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Repeated Polymerase Chain Reaction Complementary to Other Conventional Methods for Early Detection of HIV Infection in Infants Born to HIV-Infected Mothers

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The efficacy of a polymerase chain reaction (PCR) method for early detection of human immunodeficiency virus (HIV) in infants at risk for HIV infection was assessed. The PCR method was added to the routine laboratory test programme in these patients in 1988. PCR was performed in a total of 26 children at risk (age range 2 days to 58 months), including 17 infants born to HIV-infected mothers, who were followed up clinically from the time of birth for a mean period of 23 months (range 6 to 54) in a prospective study. Twelve children were PCR-positive. Eight had AIDS, ARC or symptoms suggestive of HIV infection. All these patients had at least one culture positive for HIV (6/8) and/or one positive serum p24-antigen test (5/8). One child was repeatedly PCR positive, but asymptomatic as well as virus- and antigen-negative. Three asymptomatic children with a single positive PCR result were PCR negative in subsequent tests. Fourteen children with negative PCR did not show clinical or immunological signs suggestive of HIV infection. Their cultures for HIV and antigen-p24 assays were negative. It is concluded that in addition to clinical and immunological parameters PCR is a useful technique for diagnosis of HIV infection in infants born to HIV-infected mothers. However, in case of negative HIV cultures and/or serum p24-antigen tests, single positive PCR results in asymptomatic patients must be interpreted with caution and should be confirmed by repeated tests.

Early diagnosis or exclusion of HIV infection in infants born to HIV-infected mothers remains a major obstacle in the care of these patients. The presence of transplacentally acquired maternal antibodies, the difficulty in performing viral culture, the lack of tests for HIV-specific IgM, the inability to detect serum antigen in the presence of excess serum antibody as well as the non-specific symptoms of early HIV-infection often delay the establishment of a reliable diagnosis for months. This implies a long and desperate period of waiting for the parents. On the other hand, as preliminary results of controlled clinical trials with zidovudine in early adult HIV infection are promising (1), early diagnosis of HIV infection in infants will become essential with a view to giving antiretroviral treatment prior to the manifestation of a symptomatic state.

Approximately 25–50 % of infants born to HIV-infected women will acquire HIV infection from their mothers in utero, or during labor and delivery (2–6). Intrauterine transmission was shown by culturing virus from fetal tissues or amniotic fluid early in pregnancy (7–9). Recently, Ou et al. (10) reported on the use of the polymerase chain reaction (PCR) to detect proviral sequences of HIV in peripheral blood mononuclear cells of HIV-seropositive patients. Since then this new technique has been used in adults and children with encouraging results (11–17). As this test is very sensitive and can be performed rapidly on relatively small amounts of blood, PCR might be of great value for early diagnosis of HIV infection in infants born to HIV-infected mothers. However, only one report by Rogers et al. (15) has presented repeated test results and clinical follow-up data for the study population which is crucial for the determination of the value of PCR as a diagnostic tool in infancy.

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Table 1: Characteristics of patients evaluated by PCR.

Patient no.[a]	Age at end of study (months)	Status (CDC)	Mother HIV-infected at birth	Clinical symptoms suggestive of HIV infection (age)[d]	Polymerase chain reaction (age)	Serum p24 antigen (age)	Culture for HIV (age)	HIV antibodies: latest result (age)
Group A								
1	54	P-2,C	+	+ (15m)	+ (50m)	– (cord blood) / + (50m)	+ (50m)	+ (50m)
2[b]	58	P-2,C	+	+ (?)	+ (47m)	+ (47m)	+ (47m)	+ (47m)
3[b]	53	P-2,C	+	+ (?)	+ (42m)	– (42m)	+ (42m)	+ (42m)
4	32	P-2,A	+	+ (3m)	+ (28m)	– (2d; 3m) / + (28m)	– (2d; 28m) / + (3m; 10m)	+ (28m)
5	31	P-2,A	+	+ (6m)	+ (27m)	– (6m) / + (27m)	+ (6m; 27m)	+ (27m)
6	14	P-2,A	+	+ (1m)	+ (3m)	– (3m)	+ (3m)	+ (3m)
Group B								
7	20	P-1,B (?)	+	+ (5m)	+ (3m; 6m; 12m; 18m)	– (2d; 6m; 12m) / + (3m)	– (2d; 6m; 12m; 18m)	– (18m)
8	19	P-1,B (?)	+	– (18m)	+ (7m; 12m; 18m)	– (7m; 12m; 18m)	– (7m; 12m; 18m)	+ (18m)
9	12	P-1,B (?)	+	+ (3m)	– (1d; 3m; 7m) / (+) (12m)	– (1d; 3m; 7m; 12m)	– (1d; 3m; 7m; 12m)	+ (12m)
Group C								
10	46	healthy	+	– (46m)	+ (34m) / – (46m)	– (28m; 34m; 48m)	– (3m; 34m; 46m)	– (46m)
11	27	healthy	?	– (27m)	+ (18m) / – (22m; 27m)	– (18m; 22m; 27m)	– (14m;15m;18m;22m;27m)	–/+ (27m)
12	7	P-0	+	– (7m)	+ (2d) / – (3m)	– (2d; 3m)	– (2d; 3m)	+ (3m)
Group D								
13	56	healthy	?	– (56m)	– (56m)	– (56m)	– (56m)	– (56m)
14[c]	48	healthy	?	– (48m)	– (48m)	– (48m)	ND	– (48m)
15	42	healthy	+	– (42m)	– (30m)	– (18m; 30m)	– (12m; 18m; 30m)	– (30m)
16	40	healthy	+	– (35m)	– (35m)	– (11m; 35m)	ND	– (35m)
17	35	healthy	?	– (35m)	– (35m)	– (35m)	– (35m)	– (35m)
18	34	healthy	+	– (34m)	– (12m; 16m; 22m; 29m)	– (3m;7m;12m;16m; 22m;29m)	– (1m;7m;12m;16m;22m;29m)	– (29m)
19[c]	31	healthy	?	– (31m)	– (31m)	– (31m)	ND	– (31m)
20[c]	20	healthy	?	– (20m)	– (20m)	– (20m)	ND	– (20m)
21	18	healthy	+	– (18m)	– (1d; 6m; 11m; 18m)	– (11m; 18m)	– (1d; 6m; 11m; 18m)	– (18m)
22[c]	10	P-0	+	– (10m)	– (10m)	– (10m)	ND	+ (10m)
23	9	P-0	+	– (7m)	– (1d; 3m; 6m)	– (1d; 3m; 6m)	– (1d; 3m; 6m)	+ (6m)
24	7	P-0	+	– (6m)	– (1d; 6m)	– (1d; 6m)	– (1d; 6m)	+ (6m)
25	6	P-0	+	– (6m)	– (3d; 3m; 6m)	– (3d; 3m; 6m)	– (3d; 3m; 6m)	+ (6m)
26	6	P-0	+	– (6m)	– (3d; 3m; 5m)	– (3d; 3m; 5m)	– (3d; 3m; 5m)	+ (5m)

[a] Group A: HIV-infected children. Group B: Children strongly suspected of having HIV infection (repeatedly positive PCR and/or highly suggestive symptoms). Group C: Children with one positive PCR followed by negative results without any other signs of HIV infection. Group D: Children without clinical or laboratory evidence of HIV infection.

[b] Children only seen once.

[c] Children from different hospitals whose blood was examined in our laboratory.

[d] Age at appearance of symptoms. Negative sign (–) indicates last examination without symptoms. ND: not done; d = day; m = month.

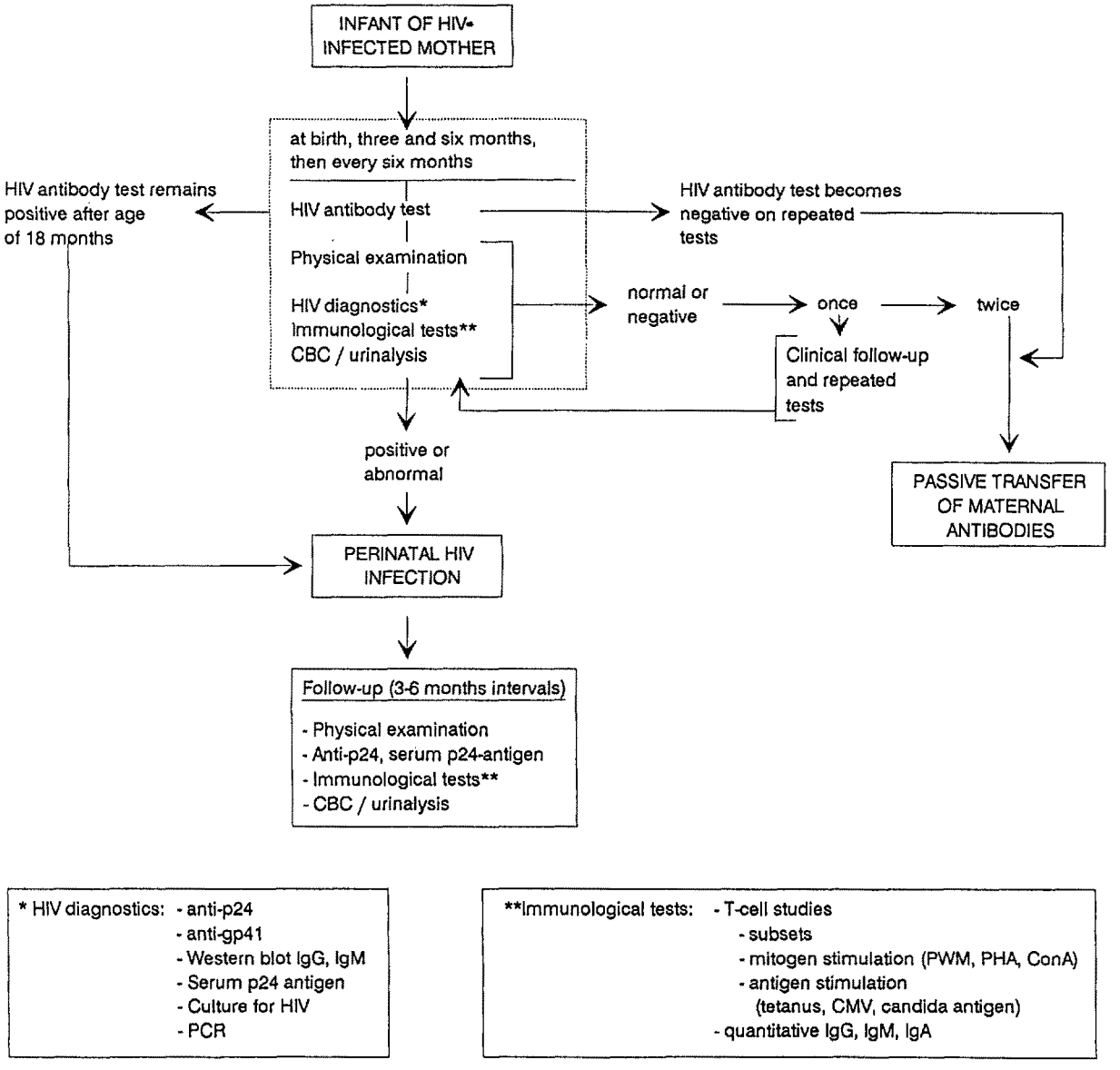

Figure 1: Follow-up of infants born to HIV-infected mothers.

To further evaluate this technique, PCR was introduced into a pre-existing follow-up programme for infants born to HIV-infected mothers. PCR was performed whenever blood samples were taken. Sequential PCR results can thus be interpreted in relation to clinical and immunological findings as well as to results of other conventional HIV tests. We present the results of the first 18 months using PCR in children in this follow-up programme (time of clinical observation 6–54 months) and in children at risk who were from outside our clinic or not part of the study.

Patients and Methods

Study Population: Twenty-six children, from 1 day to 56 months old, who were either born to HIV-infected mothers or whose mothers were known to be HIV-infected at the child's first examination, were included in this study (Table 1). Seventeen of these children (patients no. 1, 4–10, 12, 15, 16, 18, 21, 23–26) were included in a pre-existing neonatal follow-up study and had been observed from birth for a mean period of 23 months (range 6 to 54 months). Three of them (patients no. 1, 5 and 8) were entered into the study after some delay, but their follow-up data from birth were available on entering the programme. Patient no. 8 was the only one who was on breast-feeding. Fifteen of

the mothers were infected through the use of intravenous drugs, the other two through heterosexual contact with intravenous drug-using partners. Our programme comprises a physical examination at least every three months as well as periodic laboratory examinations as shown in Figure 1. The programme ends when children are classified as not being HIV-infected. This is the case when they lose maternal antibodies and when all other laboratory tests are negative or normal on two subsequent occasions. Children with proven HIV infection are enrolled in a different follow-up programme for HIV-infected children (Figure 1). The remaining nine patients were not part of this neonatal follow-up study for various reasons. Patients no. 2 and 3 were examined only once when hospitalized in our clinic for evaluation of interstitial lung disease by means of bronchoalveolar lavage. In the case of patients no. 11, 13, 14, 17, 19 and 20 it was not known if the mothers were already HIV-infected at the time of delivery of their offsprings. All these women were diagnosed to be HIV-infected some time between birth and the first examination of their children. In patients no. 14, 19, 20 and 22 blood was sent from other hospitals to our laboratory for examination.

Four different groups of patients emerged from the study depending on the HIV infection status and PCR results. Children of Group A (patients no. 1–6) were HIV-infected according to the current Centers for Disease Control (CDC) criteria (18). Group B (patients no. 7–9) consisted of children in whom, due to symptoms and/or test results, there was a strong suspicion of HIV infection. Patients of Group C (patients no. 10–12) had one positive PCR result which could not be confirmed in subsequent tests and/or other signs of HIV infection. Group D (patients no. 13–26) consisted of asymptomatic children who did not seem to be HIV-infected according to their test results. In accordance with their age, five of the patients in Group D (patients no. 22–26) had not yet lost maternal antibody and therefore still belonged to class P-0 of the CDC classification system (18) at the end of this study.

Polymerase Chain Reaction: Peripheral blood mononuclear cells of patients were separated from heparinized blood samples by density gradient centrifugation (Ficoll-Hypaque), frozen and stored in duplicates at –70 °C. DNA was isolated from these aliquots by incubating peripheral blood mononuclear cells in lysis buffer (Applied Biosystems, USA) containing 1 mg/ml proteinase K for 1 h at 60 °C. After phenol/chloroform extraction the DNA was precipitated by ethanol and redissolved in 10 mM Tris-HCl (pH 8.0)/1 mM EDTA (ethylene diamine tetraacetate).

The oligonucleotide primers used in the study were selected from two relatively conserved regions in the pol gene of HIV-1. Two pairs of 20mer primers were synthesized: 1245:5'-dCCCCACCTCAACAGATGTTG-3'. 1246: 5'-dATCAATACATGGATGATTTG-3', 1919: 5'-dTTTAGCTGACATTTATCACA-3', 1920: 5'-dGAACATGAGAAATATCACAG-3' (kindly provided by J. Jiricny, Basel). The amplified gene fragments contained HIV-1 nucleotides 2635–2734 and 3806–3913, respectively (19). The combined use of both primers lead to the detection of 24 of 25 seropositive blood samples (data not shown). The primers and oligonucleotide probes were specific for HIV-1 and did not crossreact with HIV-2 or HTLV-I under the conditions used and described below.

The amplification was carried out in a total volume of 100 µl of 1 x polymerase buffer [16.6 mM $(NH_4)_2SO_4$; 67 mM Tris-HCl, pH 8.8 at 25 °C; 6.7 mM $MgCl_2$; 1 mM dithiothreitol; 200 µg bovine serum albumine/ml] containing 1–3 µg genomic DNA, 0.8 µg of each primer and each of the dNTP (dATP, dCTP, dGTP, dTTP) at 200 µM. For the initial denaturation the samples were incubated at 95 °C for 5 min in a DNA thermal cycler (Perkin Elmer-Cetus, USA), chilled in ice, 2 U of Taq DNA polymerase were added and 100 µl mineral oil was overlaid. The denaturing procedure continued for 1 min at 95 °C and was followed by 40 programmed cycles, each consisting of 1 min denaturation at 94 °C, 2 min annealing at 51 °C and 1 min chain elongation at 72 °C.

Dot blot analysis was performed as previously described (20). Briefly, 0.5 µl aliquots of the reaction mix containing the amplified DNA were spotted onto a nylon filter (Biodyne A, Pall, Switzerland). DNA was denatured by soaking the filter in 0.5 M NaOH/1.5 M NaCl, neutralized in 0.5 M Tris-HCl pH 7.2/1.5 M NaCl and fixed by UV irradiation. The filters were then prehybridized in 5 x SSPE (750 mM NaCl; 75 mM sodium phosphate; 0.5 mM EDTA) containing 10 x Denhardt's solution (0.2 % ficoll; 0.2 % polyvinylpyrolidone; 0.2 % bovine serum albumin), 0.3 % sodium dodecyl sulphate (SDS), and 100 µl yeast tRNA/ml at 60 °C for 1 h. Hybridization was done in the same buffer containing an oligonucleotide probe specific for the amplified fragment at 60 °C overnight. The probes were 5'end-labelled with phosphorus-32 and 10^6 cpm/ml hybridization solution was used. DNA fragments amplified by primers 1245/1246 and 1920/1919 are detected by the 45mer oligonucleotides 1248: 5'-dGATCGCTCCTCTATTTTTGTTCTATGCTGCCCTATTTCTAAGTCA-3' and 1925: 5'-dTGCTACTACAGGTGGCAGGTTAAAATCACTA GCCATTGCTCTCCA-3', respectively. Filters were washed in 2.5 x SSPE containing 1.0 % SDS at 60 °C for 5 min, then in 3.0 M tetramethylammonium chloride, 50 mM Tris-HCl pH 8.0, 0.2 mM EDTA at room temperature for 10 min. Mismatched oligonucleotide probes were removed by a stringent wash with the 3.0 M tetramethylammonium chloride solution at 72 °C for 60 min. Autoradiograms were exposed for 5 h and overnight with intensifying screens at –70 °C.

DNA samples of normal blood donors were used as negative controls. Positive controls included dilutions of a DNA stock of HIV-1 infected H9 cells. The limit of detection of HIV-1 in this positive control sample was 1 pg of DNA (corresponding to a few HIV molecules). Routinely, 3–10 pg of DNA were diluted into 1 µg of DNA from normal blood donors and amplified. Samples were considered positive if one of the two primer pairs gave a clear positive signal, and indeterminate if signal intensity was low compared to the filter background. Positive samples were retested starting with DNA preparation from the second aliquot of frozen cells. Special care was taken to prevent contamination and PCR product carryover. Precautions taken were in accordance with most recommendations of Kwok and Higuchi (21), e.g. autoclaving of solutions, use of disposable pipette tips and microcentrifuge tubes for aliquoting reagents, use and frequent changing of disposable gloves, adoption of a special technique for opening vials to avoid formation of aerosols and splashes, premixing of PCR reagents and addition of DNA last; negative DNA controls were added before handling of test samples and positive controls were added last. Different working areas in one room were used for DNA extraction, pre- and postamplification procedures.

Assay for HIV-1 Antibody: HIV-1 antibodies were detected with a commercial enzyme immunoassay (Abbott, Switzer-

land). All specimens were tested with the Western blot method (Du Pont, FRG) for IgM and IgG antibodies.

Assay for p24 Antigen: HIV p24 antigen was detected in a solid-phase sandwich type enzyme immunoassay (Abbott). Positive specimens were confirmed by using a neutralising antibody (Abbott). A reduction of > 50 % of the recorded adsorbance was considered to be confirmatory.

Virus Isolation: HIV-1 cultures were performed as described elsewhere (3, 4, 14, 22, 23). Ficoll-Isopaque separated peripheral blood lymphocytes were depleted of CD8+ T cells (23) using Dynabeads M-450 (Milan Analytica, Switzerland). Of the remaining (mainly CD4+) cells, 5×10^6 to 1×10^7 were spun together with 3×10^6 peripheral blood lymphocytes from healthy donors stimulated with 0.1 % phytohaemagglutinin (PHA, Gibco, Switzerland) three days previously at 37 °C for 1 h at 2500 x *g*, and then cultured in RPMI medium (Gibco) supplemented with 10 % fetal calf serum, 20 ng/ml recombinant human IL-2 (rIL-2) and 1 mg/ml polybrene. Twice a week half of the medium was replaced by fresh medium containing rIL-2 (20 ng/ml). Aliquots of the harvested culture medium were saved for detection of p24 antigen. Once a week cultures were supplemented with fresh PHA-stimulated donor lymphocytes (3×10^6). Performing the CD8-depletion and centrifugation steps increased the sensitivity of the virus isolation procedure from around 50 % to over 90 %. Thus, in a study with serologically HIV-positive, asymptomatic pregnant women, virus isolation was successful in all of eight women employing the above protocol, in contrast to five of ten women using the conventional method without CD8-depletion and centrifugation (unpublished observation).

For some serologically negative individuals the method described by Imagawa et al. (14) was tested as well. 10^7 CD8-depleted peripheral blood lymphocytes were incubated with 0.1 % PHA, 20 ng/ml rIL-2 and 1 mg/ml polybrene for three days. The cells were then harvested and spun together with 3×10^6 PHA-stimulated peripheral blood lymphocytes from healthy donors at 37 °C for 1 h at 2500 x *g* and cultured as described above, but in the absence of PHA. No HIV-1 was isolated from eight seronegative individuals when this method and our standard protocol were tested in parallel.

All cultures were maintained for 4 to 6 weeks. Cultures were considered positive for HIV-1 if the p24 antigen level in the supernatants increased over time.

Lymphocyte Separation: Fresh blood with anticoagulant (heparin or EDTA) was diluted 1:1 with RPMI 1640 + 2 % human AB serum, separated with Ficoll-Paque, washed three times with RPMI 1640 + 2 % ABS, suspended in lymphocyte cultivation medium (RPMI 1640, Hepes, antibiotics, nonessential amino acids, pyruvate, glutamine, mercaptoethanol) and counted.

Lymphocyte Subsets: 10^6 lymphocytes separated by Ficoll-Paque were suspended in 50 µl of cold PBS + 2 % fetal calf serum and incubated with monoclonal mouse antibodies OKT3, OKT4, OKT8, OKDR in a first step and goat anti-mouse IgG in a second step, as recommended by the manufacturers (Ortho Diagnostic Systems, USA). 3 x 100 cells (manual counting) per sample were examined by fluorescence microscopy. Normal ranges were obtained from healthy donors of the same age.

Lymphocyte Stimulation Test: Stock solutions of 2 µg phytohaemagglutinin (Wellcome, UK), 4 µg pokeweed mitogen (Seromed, FRG), and 8 µg concanavalin (Pharmacia, Sweden) per ml lymphocyte culture medium were prepared. 0.1 ml of the undiluted, 1:2 and 1:4 diluted stock solution were added to 0.1 ml lymphocyte suspension (8×10^5/ml) in triplicate cultures. After 72 h at 37 °C, 1 µCi of methyl-^{3}H-thymidin (Amersham TRA 120, UK) was added, the cultures harvested after 14–24 h with a betaplate harvester (LKB Wallac, Finland) and the incorporated thymidine counted with a betaplate counter. Incorporation was judged in relation to values of healthy donors of the same age. Considering the high individual variability, values of more than 50 % of the mean values of healthy donors were considered to be normal.

CMV ad-169 glycine extracted antigen was obtained from Scripps Laboratories (USA), tetanus toxoid (partly purified) from the Swiss Serum and Vaccine Institute and candida antigen stade 2 from Institut Mérieux (France). Antigen dilutions were prepared in lymphocyte culture medium (CMV 10 and 1 EIA unit, tetanus toxoid 10–80 µg/ml, candida antigen 4–100 U/ml) and 0.1 ml added to 0.1 ml lymphocyte suspension (10^6/ml) in triplicate. Tracer was added after 120 h of incubation, and harvesting and counting done as described. The result was expressed as the stimulation index (mean cpm value of the stimulated culture divided by the mean cpm value of the control culture). A value equal to or greater than 3 was taken as a positive result.

Results

Between June 1988 and January 1990 PCR was performed in 26 children. Of these, 17 were included in a pre-existing follow-up programme for infants born to HIV-infected mothers and were observed from birth for a mean period of 23 (range 6 to 54) months. According to the study design (Figure 1) PCR was carried out more than once in 12 of these patients. The test results are summarized in Table 2.

PCR was positive in 12 (46 %) and negative in 14 (54 %) children. In five patients of Group A (patients no. 1–5) HIV infection had been proven according to the CDC criteria (18) prior to the introduction of the PCR technique. They were all PCR-positive at the first examination, and therefore the tests were not repeated. Testing of the same blood sample which was PCR positive showed that all children (all older than 18 months) were positive for antibody as well as serum-p24-antigen and/or culture postive for HIV. The serum p24 antigen assay and culture for HIV were negative in one patient each at that time. In patient no. 6 HIV infection was proven at the age of three months by a positive culture for HIV and a positive PCR. Clinical signs (generalized lymphadenopathy, hepatosplenomegaly) were first

Table 2: Summary of PCR results in 26 infants of HIV-infected mothers (patient numbers according to Table 1).

PCR positive n = 12 (46 %)	8	AIDS, ARC or symptoms suggestive of HIV infection (no. 1-7, 9)
	6	culture for HIV positive (no. 1-6)
	5	serum p24-antigen positive (no. 1, 2, 4, 5, 7)
	1	repeatedly positive by PCR, asymptomatic, no other evidence of HIV infection (no. 8)
	3	asymptomatic, PCR negative in consecutive tests (no. 10-12)
PCR negative n = 14 (54 %)		no symptoms suggestive of HIV infection (no. 13-26) no culture for HIV positive (no. 13-26) no serum p24-antigen test positive (no. 13-26)

recognized at an age of one month in this child. All patients of Group A, followed up from birth onwards by ourselves (patients no. 4–6), presented with unspecific clinical signs and symptoms when the diagnosis of HIV infection was confirmed using conventional methods in the laboratory. At that time only one patient showed slightly elevated quantitative IgG levels, whereas T-cell tests (according to Figure 1) were normal in all patients and quantitative immunoglobulin tests normal in two patients. At the following examination hypergammaglobulinemia was present in all these children, while T-cell test results became abnormal only 6 to 18 months after diagnosis. There was a very slow depletion of T-helper cells and an increase of T-suppressor cells with a T_4/T_8 ratio > 1.0 and an absolute T-helper cell count > 1.0×10^9/l for up to two years after diagnosis (patients no. 4 and 5).

Children of Group D did not seem to be HIV-infected. They were healthy, and those over 15 months of age had lost maternal antibodies. PCR, cultures for HIV and serum p24-antigen assays were negative whenever performed. All these children had normal T-cell and quantitative immunoglobulin tests as well. In accordance with their age, patients no. 22–26 still belonged to the CDC classification group P-0 (18), and therefore had still not lost maternal antibodies.

Of special interest were the patients of Groups B and C who will be discussed individually below. Children of Group B were suspected of being HIV infected due to a single positive PCR result (patient no. 9) or repeatedly positive PCR results (patients no. 7 and 8) and/or suspicious clinical symptoms (patients no. 7 and 9) in combination with minor immunologic abnormalities (patients no. 7–9). Those of Group C had a single positive PCR result but otherwise no supportive evidence of HIV infection was present (patients no. 10–12).

Group B Case Reports. Patient no. 7 was a 20-month-old girl delivered by forceps at term after a normal pregnancy. Her mother was a 26 year old primipara who was a heavy smoker and had contracted HIV infection through i.v. drug use. The patient was small for date; her birth weight was 2560 g and her length 47 cm. During the neonatal period she had slight withdrawal symptoms. At five months of age she had a first episode of diaper rash and oral thrush. One month later slight hepatomegaly was noted, and at ten months there was minimal splenomegaly as well as hyperreflexia of the lower extremities with a positive Babinsky sign. At that time the child presented with a second episode of diaper rash and oral thrush. At one year of age she had oral thrush again. At that time the splenomegaly had disappeared, but at 14 months a minor generalized lymphadenopathy, streptococcal pharyngitis and bilateral otitis media were seen. Lymphadenopathy and hepatomegaly as well as hyperreflexia of the lower extremities with a positive Babinsky sign persisted. Motor and mental development as well as head circumference, weight and length conformed to age. Values below normal range were found for IgG at 3 months and IgA at 3 and 6 months; at 18 months IgM was mildly elevated. The mitogen response was slightly diminished at 12 months, but normal at 18 months. At 3 months the patient had a single positive serum p24-antigen test. Otherwise all laboratory tests performed 4 to 5 times according to Figure 1 were normal/negative. Maternal antibodies disappeared at 18 months. The PCR was positive at 3, 6, 12 and 18 months, whereas serum p24-antigen assays (with one exception) and culture for HIV remained negative.

Patient no. 8 was a 19-month-old girl whose mother had contracted HIV infection through heterosexual contact. The child was delivered spontaneously after an uneventful 38 week gestation; her birth weight was 3150 g and length 48 cm. She was breast-fed for six months and in good health until her first visit at seven months of age. There was one episode each of diaper rash and oral thrush at 14 months gastroenteritis at 15 months and aphthous stomatitis at 16 months. No symptoms giving rise to a suspicion of HIV infection were found on physical examination. Growth,

mental and motor development were normal. Tests were performed at 7, 12 and 18 months of age. Antibodies in IgG Western blot declined without appearance of new bands but remained weakly positive for gp120/160 at 18 months. The PCR was positive three times, whereas serum p24-antigen assays and culture for HIV remained negative. Quantitative IgG was slightly increased at 7 and 12 months, but normal at 18 months, whereas IgA was normal at 7 and 12 months, but slightly decreased at 18 months. Quantitative IgM remained in the normal range for age. Aside from a slightly diminished relative proportion of helper cells at 18 months (32.7 %), T-cell subsets, the absolute T-helper cell count (2760/µl; 3383/µl; 1860/µl), and the T_4/T_8-ratio (3.2; 2.2; 2.8) were normal on each occasion. The mitogen response was normal at 7 months, but decreased at 12 and 18 months, while the antigen response remained unaffected.

Patient no. 9 was a 12-month-old boy whose 24-year-old mother contracted HIV infection through i.v. drug use. He was delivered spontaneously after a normal 40-week gravidity. His birth weight was 2700 g, length 48 cm. After birth he suffered from signs of heroin withdrawal. At 3 months he presented with a severe diaper rash. Generalized lymphadenopathy and hepatosplenomegaly were recognized at 4 months of age and persisted thereafter. At 7 months diaper rash reappeared. The child contracted bilateral otitis media three times, at 9, 11 and 12 months of age. Motor and mental development as well as growth were normal. This child was tested according to the programme outlined in Figure 1 after birth, and at 3, 7 and 12 months of age. HIV antibodies declined without appearance of new bands in the Western blot. The PCR was negative on the first day and after 3 and 7 months of life, but weakly positive after 12 months. The serum p24-antigen assay and culture for HIV were negative on every occasion. Quantitative IgG was slightly diminished after birth, and at 3 and 7 months, but normal at 12 months of age. IgM was slightly elevated at 12 months of age but otherwise quantitative IgA and IgM were in the normal range for age whenever tested. The relative proportion of T-helper cells (30.1 %; 42.2 %; 34.3 %) was slightly diminished at 3, 7 and 12 months, but the absolute numbers (2686/µl; 3450/µl; 4105/µl) as well as the T_4/T_8 ratio (4.3; 1.5; 2.0) and mitogen response remained normal. At 12 months, the in vitro antigen response was normal against tetanus toxoid, but absent against candida antigen despite recurrent diaper rashes.

Group C Case Reports. Patient no. 10 was a 46-month-old boy whose 26-year-old mother was an i.v. drug user. The child was delivered spontaneously after an uneventful full-term pregnancy (birth weight 3250 g, length 51 cm). After birth he developed signs of heroin withdrawal, *Staphylococcus aureus* sepsis, herpes simplex encephalitis, salmonella gastroenteritis, oral thrush and diaper rash. He remained in hospital for four months. He recovered remarkably after this period of severe illness, was given to a foster family; he showed normal mental and motor development and normal growth. At 22 months he had an aphthous stomatitis, otherwise he suffered no further illnesses. Minor changes in the electroencephalogram and esotropia seem to be the only sequelae. Physical examination never revealed any signs suggestive of HIV infection. Maternal antibodies disappeared at 8 months of age and the child remained antibody-negative at 16, 34 and 46 months. The serum p24-antigen was negative on 4 occasions and culture for HIV negative on 3 occasions. The T_4/T_8 ratio was 0.86 at the end of the initial period of severe illness, but thereafter the T-lymphocyte subsets, absolute T-helper cell count, T_4/T_8 ratio, mitogen and antigen responses as well as quantitative immunoglobulins were normal on at least three examinations each. The PCR was positive at the age of 34 months but this result could not be confirmed 12 months later.

Patient no. 11 was a 27-month-old girl, delivered by cesarian section at a gestational age of 33 weeks due to arrested intrauterine development. She was small for date; her birth weight was 1160 g and her length 39 cm. Her mother was found to be HIV-infected one year later, and probably contracted her HIV infection through heterosexual contact with an i.v. drug using partner. It is not known whether she was infected before, during or after pregnancy. This girl had a severe congenital malformation syndrome strongly resembling the Silver-Russell syndrome. There was a marked delay in weight and height gain, and her mental and motor development remained far behind normal. She was not able to sit by herself or to speak single words. Head circumference was normal. The girl had to be hospitalized various times due to feeding difficulties, epileptic seizures and recurrent otitis media. MRI at 14 months of age revealed minor hypoplasia of the corpus callosum but no brain atrophy or calcification of basal ganglia. There was marked muscular hypotonia. Liver, spleen and lymph nodes were always normal on palpation. Although her clinical symptoms were not suggestive of HIV infection, this girl had

an indeterminate PCR result and two single weak bands at gp120 and gp160 on the IgG Western blot at 14 months of age. One month later, she had another indeterminate PCR result and 4 months later a clearly positive PCR result, whereas on both occasions the Western blot results were negative. At 22 and 27 months of age, the PCR was negative again, but there was a single weak band at p64 on the IgG Western blot on both occasions. Four tests of T-cells and two subsequent tests of quantitative immunoglobulins revealed normal results. Six serum p24-antigen assays and five cultures for HIV were negative.

Patient no. 12 was a 7-month-old girl, the second child of a 25-year-old i.v. drug using mother. The child was born spontaneously after a normal 40-week gestation; her birth weight was 3040 g and length 49 cm. At two months of age she had transitory hepatosplenomegaly without other signs of an infectious disease, and at 3 months of age an episode of diaper rash. On her second day of life, the PCR was positive, but a confirmatory test at 3 months of age was negative. Simultaneous T-cell studies, cultures for HIV, serum p24-antigen assays and measurements of quantitative immunoglobulins were normal or negative. In accordance with her age, she was still carrying maternal HIV antibodies.

Discussion

In four (23.5 %) of 17 infants born to HIV-infected mothers and followed up prospectively from birth HIV infection was proven according to the CDC criteria (18). The disease progressed in only one of these infants to acquired immune deficiency syndrome (AIDS) (lymphoid interstitial pneumonia) at 27 months of age (patient no. 1), while the others continued to show less specific symptoms such as generalized lymphadenopathy and hepatosplenomegaly, as well as recurrent episodes of diaper rash and oral thrush. They remained in good health, the physical and immunological deterioration progressing very slowly in comparison to the observations recently published by Scott et al. (24) and Hira et al. (25). If patients of Group B, who all presented with minor immunologic abnormalities, turn out to be HIV-infected as well, the perinatal transmission rate will be 41 % (7/17). This figure corresponds to previous findings (2–6), but is significantly different from the 7.1 % recently reported by Mok et al. (26).

The PCR technique developed by Mullis et al. (27–29) can be used for the detection of HIV in infected cell lines, cells cultured from infected individuals (30) and DNA directly isolated from peripheral-blood mononuclear cells of seropositive individuals (10). It is a powerful method and its sensitivity theoretically allows the detection of one HIV DNA molecule in about 100,000 lymphocytes (13). Therefore the amplification of even the smallest amount of viral contamination can cause a false positive result. On the other hand there is the problem of false negative results. Hart et al. (31) found 1 of 21 and Ou et al. (10) 4 of 22 seropositive specimens to be negative for HIV by PCR. In the report of Rogers et al. (15) two infants who met the CDC case definition for HIV infection and one infant with a positive culture for HIV were negative on PCR. To avoid false negative results it was proposed to use more than one primer pair, and it was shown by Ou et al. (10) and Laure et al. (12) that in some cases reactive samples scored positive with only part of the combinations of primers used for the amplification assay. The problem of false positive or negative results and proposals on how to avoid them have been extensively discussed in a recent report by Clewley (32).

Our results (Table 2) are comparable to those of Edwards et al. (16) and Rogers et al. (15). All patients with HIV-infection (Group A) according to the current CDC criteria (18) had a positive PCR result, which was confirmed by either virus culture or serum p24-antigen assay or both. In addition, all the children enrolled in our follow-up programme for infants born to HIV-infected mothers at birth (patients no. 1, 4–6) had symptoms suggestive of HIV infection, but nearly always normal quantitative immunoglobulins and T-cell tests at the time we were able to confirm diagnosis in the laboratory.

According to the clinical findings, patient no. 9 (Group B) was suspected to be HIV-infected after 3 months of age. The PCR was weakly positive for the first time at 12 months of age, whereas virus culture and the serum p24-antigen assay have remained negative so far. The other two patients of Group B (patients no. 7 and 8) were believed to be HIV-infected because they had several positive PCR results on repeated tests. In these patients (22 % of the total number of patients suspected to be HIV-infected; Groups A and B) PCR detected HIV infection prior to other methods and in patient no. 8 even prior to the appearance of clinical symptoms. All patients of Group B showed minor immunological abnormalities and in addi-

tion patient no. 7 had one positive serum p24-antigen assay result at 3 months of age.

In 14 patients (Group D) with negative PCR results, culture for HIV and the serum p24-antigen assay were negative, even on repeated testing. All these children were without clinical or laboratory evidence of HIV infection. In six of them (patients no. 18, 21, 23–26) PCR was negative more than once. As five of these children (patients no. 22–26) still belonged to CDC classification group P-0 (18) according to their age (less than 15 months) at the end of the study, it is possible that one or the other will turn out to be HIV-infected despite all previously negative test results.

The patients of Group C (patients no. 10–12) are of special interest. They all had one clearly positive PCR followed by one or more negative results. In view of their age, physical examination, normal immunological findings and multiple negative cultures for HIV and serum p24-antigen assays it would be appropriate to class these children as not HIV-infected. Nevertheless, we found a few single reactive bands on IgG Western blot on four occasions in patient no. 11. In addition, she was suffering from a very complex congenital malformation syndrome which made interpretation very difficult. There was no clinical evidence that she was HIV-infected because her symptoms differed from those typical of HIV infection. Despite her neurological disorder she showed distinct developmental progress after her first visit, and MRI did not show cerebral atrophy or calcification of the basal ganglia. Her nutritional state improved, and she had no malabsorption or diarrhea. Theoretically, the number of HIV-infected peripheral blood mononuclear cells in these patients (Group C) could be too low to be detected by PCR on every occasion. For that reason neither latent HIV infection nor false positive PCR results could be ruled out with certainty in these patients. According to her age, patient no. 12 still belonged to the CDC classification group P-0 (18), and HIV infection might become evident during the next few months of life.

Our experience with sequential PCR testing disclosed some difficulties in interpreting single PCR results. Aside from consistent PCR results in groups A, B and D, three (17,6 %) (Group C) of 17 patients followed up from birth in a prospective study had a positive PCR result which has not yet been confirmed clinically or immunologically, or by repeated or different tests. Probably a very long observation time will be necessary to disclose the significance of the single positive PCR results in these patients who might have an extremely long incubation period.

Thus, at present a single positive PCR result cannot prove HIV infection with certainty in the absence of other evidence of infection. The number of such patients might be even higher if more than two pairs of primers were used simultaneously as proposed by Ou et al. (10) and Laure et al. (12). On the other hand, the presence of HIV infection cannot be ruled out by either a single or multiple negative PCRs. HIV infection can be overlooked in the case of a very low number of infected peripheral blood mononuclear cells at the time of testing, as demonstrated in patient no. 9. This can also be concluded from several previous studies (10, 15, 26).

The PCR is a very promising additional technique for early diagnosis of HIV infection in infancy, being able to detect some infections prior to the appearance of clinical symptoms, and prior to virus culture or serum p24-antigen assay. On the other hand, single positive PCR results should be interpreted with caution, and in the absence of further evidence of HIV infection, it is necessary to confirm a positive result prior to disclosure of the diagnosis to parents or introduction of a potentially toxic therapy or a preventive treatment under trial. At present a combination of different laboratory methods with clinical and immunological evaluation is essential for early recognition of HIV infection. More studies of this kind are needed to determine the value of PCR as a diagnostic tool in pediatric HIV infection. Repeated monthly or even fortnightly testing by PCR during the first few months of life and an improved technique for culture of HIV, as recently described by Ho et al. (33), could permit early detection of HIV infection in infancy prior to the appearance of symptoms.

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