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Identification of HTLV-I Sequence in Cord Blood Mononuclear Cells of Neonates Born to HTLV-I Antigen/Antibody-positive Mothers by Polymerase Chain Reaction

Shigeru Saito, Kazuhiko Furuki, Yoshiya Ando, Takuo Tanigawa, Kazuhiko Kakimoto, Ikuko Moriyama and Motohiko Ichijo

Department of Obstetrics and Gynecology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634

We developed a polymerase chain reaction (PCR) method which has high sensitivity and simple technique in order to investigate the presence or absence of human T lymphotropic virus type I (HTLV-I) provirus in cord blood mononuclear cells of neonates born to HTLV-I carrier mothers. Out of 40, three subjects were found to contain the HTLV-I provirus genome. These three subjects remained HTLV-I sequence-positive in follow-up study. On the other hand, when examined by a conventional technique for detection of HTLV-I-associated antigen on peripheral mononuclear cells, all 40 neonates were HTLV-I-associated antigen-negative. These results suggest that PCR is more sensitive than the conventional antigen detection method and is useful in early detection of HTLV-I infection in neonates born to HTLV-I carriers.

Key words: HTLV-I — Cord blood — Polymerase chain reaction — Intrauterine infection

Human T lymphotropic virus type I (HTLV-I) is an etiological agent of adult T cell leukemia (ATL)[1-3] and HTLV-I-associated myelopathy[4]/tropical spastic paraparesis[5] (HAM/TSP). HTLV-I is known to be vertically transmitted from mothers to children.[6,7] It has been reported that HTLV-I is mainly transmitted from mother to child via breast milk on the basis of the conventional antigen detection method.[8,9] However, HTLV-I infection has also been reported in children who had not been fed with breast milk, suggesting the possibility of intrauterine and transvaginal HTLV-I infection.[9,10] We recently attempted to detect HTLV-I DNA in cord blood mononuclear cells of babies born to HTLV-I-carrying mothers using the polymerase chain reaction (PCR) method,[11,12] which is more sensitive and simpler than the conventional antigen screening method.

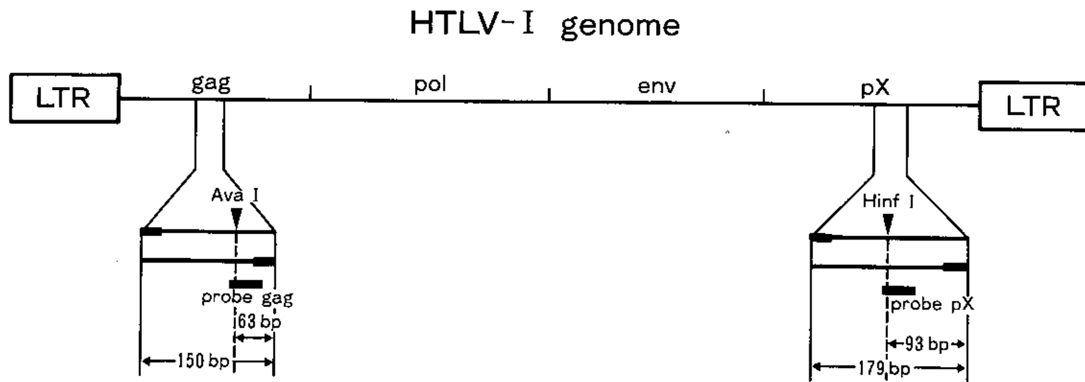
SUBJECTS AND METHODS

HTLV-I antibody titer was determined by an ELISA[13] (Eitest ATL kit, Eisai, Tokyo) method or a particle agglutination[14] (Serodia ATL kit, Fujirebio, Tokyo) method.

HTLV-I-associated antigen was screened by the method described elsewhere.[7] In brief, peripheral mononuclear cells were incubated for 4 weeks in RPMI 1640 medium containing 20% lectin-free TCGF. HTLV-I-associated antigen on lymphocytes was detected by the indirect immunofluorescence method using Gin14[15] (anti *gag*, p19) and F10[16] (anti *env*, gp21). Samples were considered to be positive when more than 0.1% of cells were positive for fluorescence under microscopy.

The PCR test was performed as described.[12] In brief, after separation of peripheral mononuclear cells by the Ficoll-Hypaque method, genomic DNA was extracted from them by the reported method.[17] Primer oligonucleotide and probe oligonucleotide corresponding to HTLV-I *gag* and *pX* were synthesized using an automatic DNA synthesizer (Applied Biosystems). Sequences of synthetic oligonucleotide primers and probes are shown in Fig. 1. In the presence of HTLV-I provirus, *gag*-encoding 150 bp DNA and *pX*-encoding 179 bp DNA were amplified by this PCR system. A 100 μl aliquot of solution containing 1 μg of DNA (derived from peripheral mononuclear cells), 50 mM KCl, 10 mM Tris (pH 8.4), 2.5 mM $MgCl_2$, 1 μM primer (primer gag, gag′ or pX, pX′), 200 μM dNTP, 200 μg/ml gelatin and 2.5 U of Taq polymerase (Stratagene, USA) was incubated for 1 min at 92°C, 3 min at 42°C and 2 min at 72°C. The same procedure was repeated 20 times. Then, after adding 2.5 U of Taq polymerase, the above procedure was further repeated 20 times, and 10 μl of the amplified DNA solution was applied to 6% polyacrylamide gel. After electrophoresis for 2 h at 100 V, alkaline blotting was performed using Hybond-N membrane (Amersham). Ultraviolet irradiation (3 min) and prehybridization were performed, followed by hybridization using 5′-endolabeled probe gag or pX. In HTLV-I sequence-positive cases, PCR was repeated to confirm that a positive result was reproducible.

In 40 babies born to 40 HTLV-I carrier mothers who were HTLV-I antibody-positive and HTLV-I *gag, env* antigen-positive, we applied the PCR method to detect *gag* and *pX* sequences of HTLV-I. In these infants, breast feeding had been replaced with bottle feeding or feeding frozen-and-thawed breast milk to prevent vertical infection of HTLV-I.



Sequences of synthetic oligonucleoticle primers and probes

Primer or Probe	Sequence	Length of amplified product (bp)
primer gag	GACCTCCAACACCTCCTGCAGTACCTTT	
primer gag'	ATTGTTGGCTTGGACACGGAGGGGACC	150
probe gag	GAAACCCGAGGTATTACAGGTTATAACCCA	(Ava I 63)
primer pX	ATGCGCAAATACTCCCCCTTCCGAAATGG	
primer pX'	AGGAGGGGCCAGGTGATGGGGGGGGAAAG	179
probe pX	CCCGGACTCCGGCCCCAAAACCTGTACACC	(Hinf I 93)

Fig. 1. Sequences of synthetic oligonucleotide primers and probe for detection of HTLV-I *gag* and *pX* sequence using the PCR method. The size of the target sequence of *gag* is 150 bp and that of *pX* is 179 bp. The hybridized *gag* band is confirmed by the change in the size of the band to 63 bp from 150 bp after digestion with *Ava* I, while the *pX* band shifts to 93 bp from 179 bp after digestion with *Hinf* I.

RESULTS

Fig. 2 shows the results of the assay using the PCR method. MT-1 cells[18] and MT-2 cells, which are known to be infected with HTLV-I, showed an amplified *gag*-specific 150 bp band alone (Fig. 2, lanes 1 and 2) and an amplified *pX*-specific 179 bp band (Fig. 3, lanes 1 and 2). No *gag*- or *pX*-specific band was detected in HTLV-I-noninfected cases (Fig. 2, lanes 4 and 5) or in another 20 HTLV-I-noninfected cases (data not shown). The specificity of the hybridized *gag* and *pX* bands was demonstrated by the change in the size of the band to 63 bp from 150 bp after *Ava* I digestion (Fig. 2, lane 3) and to 93 bp from 179 bp after *Hinf* I digestion (Fig. 3, lane 3). To study the sensitivity of PCR, genomic DNA from

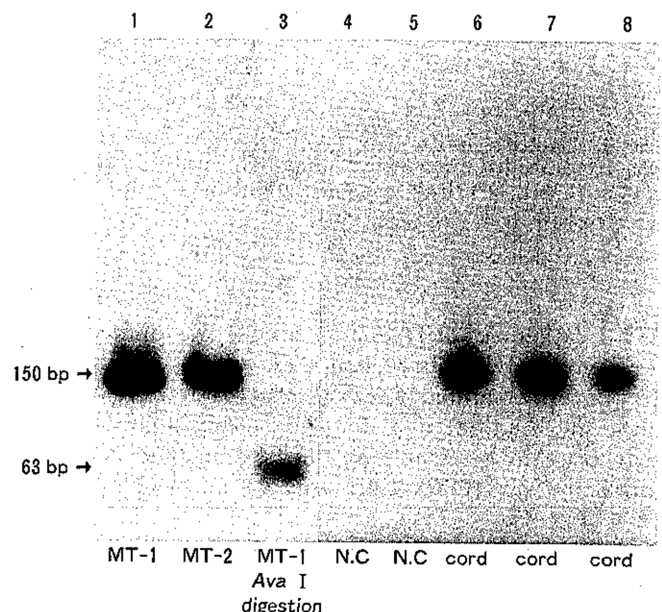


Fig. 2. Southern blot assay of the PCR-amplified HTLV-I *gag* site. Lanes 1 and 2 are MT-1 and MT-2 cells, respectively. Lane 3 is MT-1 treated with *Ava* I. Lanes 6, 7 and 8 are case numbers 3, 19 and 23 in Table I, respectively. Lanes 4 and 5 are HTLV-I-noninfected cases.

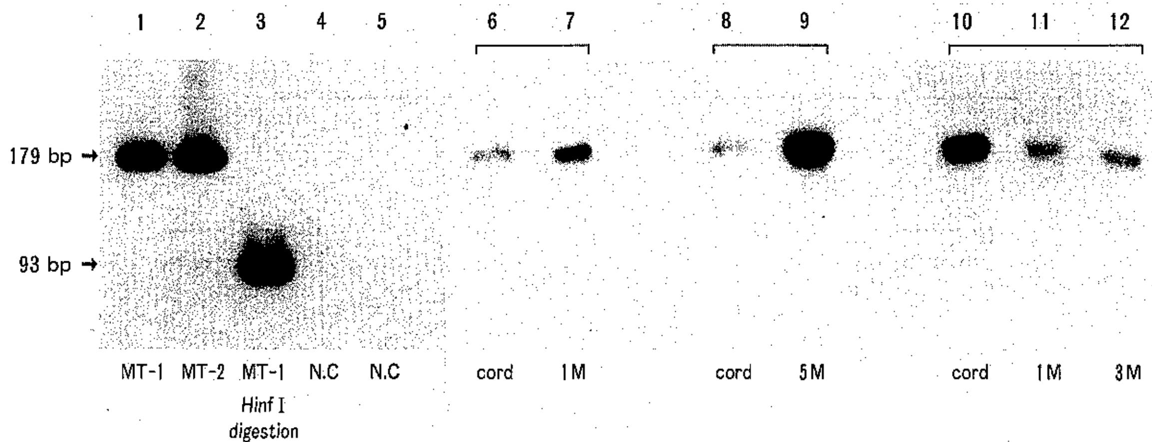


Fig. 3. Southern blot assay of the PCR-amplified HTLV-I *pX* site. Lane 1 is MT-1 cells and Lane 2 is MT-2 cells. Lane 3 is MT-1 treated with *Hinf* I. Lanes 6 and 7 are case number 3 in Table I. Lanes 8 and 9 are case number 19 in Table I. Lanes 10, 11 and 12 are case number 23 in Table I. Lanes 4 and 5 are HTLV-I-noninfected cases.

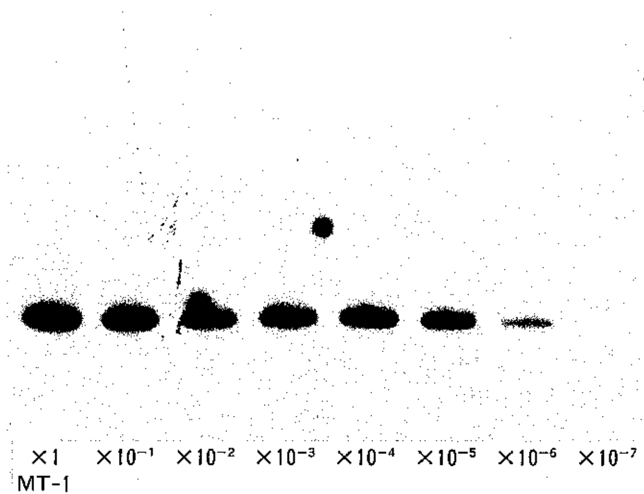


Fig. 4. The sensitivity of the HTLV-I PCR. The sensitivity was demonstrated by serially diluting MT-1 cells (10 copies of HTLV-I per cell) with genomic DNA of a non-HTLV-I-infected case.

MT-1 (10 copies of HTLV-I per cell) was diluted $1{:}10^n$ by genomic DNA from non-HTLV-I-infected subjects. In this examination, a *pX*-specific band was observed until the dilution of $1{:}10^6$. This means that HTLV-I sequence was detectable by the PCR method if one or more copies of HTLV-I provirus were present in 1×10^5 cells (Fig. 4).

Although HTLV-I-associated antibody was detected in cord serum from all 40 neonates born to HTLV-I carriers, none of them was HTLV-I-associated antigen-positive when examined by the conventional antigen screening method (Table I). However, HTLV-I *gag* and *pX* sequence were found in 3 children by the PCR method (Fig. 2, lanes 6–8; Fig. 3, lanes 6–12). When 21 subjects with negative HTLV-I sequences by the PCR method were followed up and re-examined for the presence of HTLV-I provirus, HTLV-I DNA remained negative in all of them (Table I). On the other hand, all of the infants in whose umbilical blood HTLV-I sequence was detected by the PCR method remained HTLV-I sequence-positive in follow-up (Table I, Fig. 3, lanes 6–12).

DISCUSSION

Human immunodeficiency virus (HIV), which is a retrovirus like HTLV-I, has been found to be transplacentally transmitted, and the incidence of transplacental or transvaginal infection with HIV has been studied.[19–21] Regarding intrauterine HTLV-I infection, we can find no report other than a single case report by Komuro *et al.*,[10] who used a conventional HTLV-I antigen detection method. In our previous study using the conventional antigen screening method, HTLV-I-associated antigen was absent in umbilical blood specimens of all 62 neonates born to HTLV-I carriers, although vertical HTLV-I transmission occurred in 2–3% of these children even when they were not fed with breast milk.[9] This previous finding suggests the possibility of transplacental or transvaginal infection with HTLV-I. In the present study of cord blood of 40 neonates born to HTLV-I antigen/antibody-positive mothers, HTLV-I genome was detected in 3 neonates (7.5%), although

Table I. Detection of HTLV-I Provirus in Cord Blood Mononuclear Cells by PCR

Case No.	Weeks of gestation	Sex	Weight (g)	Cord blood			Follow-up
				PA-Ab[a]	IF-Ag[b]	PCR	PCR
1	38	♂	2960	(+)	(−)	(−)	
2	37	♂	3526	(+)	(−)	(−)	
3	40	♂	2902	(+)	(−)	(+)	1M (+)
4	40	♀	3304	(+)	(−)	(−)	
5	38	♀	3004	(+)	(−)	(−)	1M (−) 3M (−)
6	40	♂	4254	(+)	(−)	(−)	
7	38	♀	3500	(+)	(−)	(−)	7M (−)
8	39	♀	3118	(+)	(−)	(−)	1M (−)
9	38	♂	2806	(+)	(−)	(−)	1M (−)
10	34	♂	2320	(+)	(−)	(−)	
11	38	♂	3048	(+)	(−)	(−)	
12	39	♀	2760	(+)	(−)	(−)	1M (−) 6M (−)
13	38	♀	2730	(+)	(−)	(−)	
14	38	♀	2766	(+)	(−)	(−)	1M (−)
15	41	♂	3226	(+)	(−)	(−)	1M (−)
16	41	♀	3240	(+)	(−)	(−)	1M (−)
17	40	♂	2736	(+)	(−)	(−)	
18	35	♂	2185	(+)	(−)	(−)	
19[c]	41	♀	3646	(+)	(−)	(+)	5M (+)
20	40	♂	2978	(+)	(−)	(−)	
21	40	♀	3510	(+)	(−)	(−)	
22	40	♀	3646	(+)	(−)	(−)	1M (−) 3M (−)
23	40	♀	3224	(+)	(−)	(+)	1M (+) 3M (+)
24	35	♂	2558	(+)	(−)	(−)	
25	40	♂	3560	(+)	(−)	(−)	1M (−) 3M (−)
26	40	♀	3676	(+)	(−)	(−)	1M (−) 3M (−)
27	41	♀	3740	(+)	(−)	(−)	3M (−)
28	40	♀	3306	(+)	(−)	(−)	
29	39	♀	2582	(+)	(−)	(−)	
30	39	♂	3724	(+)	(−)	(−)	1M (−)
31	38	♀	3804	(+)	(−)	(−)	1M (−)
32	40	♀	3202	(+)	(−)	(−)	
33	39	♀	3436	(+)	(−)	(−)	1M (−)
34	40	♀	3394	(+)	(−)	(−)	
35	40	♂	3592	(+)	(−)	(−)	1M (−)
36	39	♂	3408	(+)	(−)	(−)	1M (−)
37	41	♀	3682	(+)	(−)	(−)	3M (−)
38	40	♀	3208	(+)	(−)	(−)	3M (−)
39	39	♂	2900	(+)	(−)	(−)	3M (−)
40	37	♀	3192	(+)	(−)	(−)	1M (−)

a) HTLV-I-associated antibody detected by the particle agglutination method.
b) HTLV-I-associated antigen detected by the immunofluorescence method.
c) Rh-incompatible pregnancy and indirect Coombs test positive.

these three positive cases were not disclosed by the conventional antigen screening method. This result suggests that: (1) the PCR method is more sensitive than conventional antigen screening to detect the presence of HTLV-I provirus, and (2) even when HTLV-I provirus is detected by PCR, the HTLV-I antigen may be expressed in too small a number of blood cells or expressed at too low a level to be detected by the conventional antigen screening method.

When the 3 children were followed up, all of them remained HTLV-I genome-positive by the PCR method although HTLV-I-associated antigen was negative by the

conventional antigen detection method. We can therefore rule out the possibility of maternal lymphocyte contamination in the neonatal cord blood during collection or the possibility that umbilical blood specimens were contaminated by a very small amount of HTLV-I during the PCR procedure. In case 19, the baby, whose blood type was Rh-positive, was born to an Rh-negative mother, and the indirect Coombs test was positive after the 24th week of pregnancy, suggesting transplacental HTLV-I infection. In cases 3, 19 and 23, which terminated in spontaneous deliveries, the details for weeks of gestation, birth weight and sex were 40 weeks, 2902 g, male; 41 weeks, 3646 g, female; and 40 weeks 3224 g, female respectively. Unlike the HIV-infected children, none of these three neonates was premature or showed intrauterine growth retardation, indicating that HIV-infected children clinically differ from HTLV-I-infected children.

These cases of intrauterine or transvaginal HTLV-I infection must be carefully followed up, and it will be interesting to see when they become positive to the conventional antigen screening method. Although ATL occurs in adults, HAM/TSP has been reported to have occurred also in children.[4] Careful monitoring is therefore important. We have previously detected, using the PCR method, 4 HTLV-I-infected cases (8.0%) who were fed with bottled or frozen-and-thawed milk.[22] Interestingly, this vertical transmission rate of HTLV-I is similar to that in cord blood.

In conclusion, intrauterine or transvaginal infection of HTLV-I does occur, and is not uncommon.

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