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Application of polymerase chain reaction (PCR) to the microscopically identified cells on the slides: evaluation of specificity and sensitivity of single cell PCR.

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Abstract

The sensitivity and specificity of single cell polymerase chain reaction (PCR) were studied. Its high sensitivity enabled detection of a single-copy gene, such as human T-lymphotropic virus type I genome in paraffin sections. The rate of obtaining positive signals with this method was affected by the number of copies of the gene in the target cell. Specificity was satisfactory if the procedure was properly and carefully followed. Since the single cell PCR is a time-consuming method which requires skill and experience to pick up the target cells accurately, the applicability of this method is limited. It works best when it is used to analyze a single or a few copy genes in histologically identified cells.

KEYWORDS: polymerase chain reaction, human T-lymphotropic virus type I, paraffin section, single cell, single copy gene

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Application of Polymerase Chain Reaction (PCR) to the Microscopically Identified Cells on the Slides: Evaluation of Specificity and Sensitivity of Single Cell PCR

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The sensitivity and specificity of single cell polymerase chain reaction (PCR) were studied. Its high sensitivity enabled detection of a single-copy gene, such as human T- lymphotropic virus type I genome in paraffin sections. The rate of obtaining positive signals with this method was affected by the number of copies of the gene in the target cell. Specificity was satisfactory if the procedure was properly and carefully followed. Since the single cell PCR is a time-consuming method which requires skill and experience to pick up the target cells accurately, the applicability of this method is limited. It works best when it is used to analyze a single or a few copy genes in histologically identified cells.

Key words: polymerase chain reaction, human T-lymphotropic virus type I, paraffin section, single cell, single copy gene

The polymerase chain reaction (PCR) technique is a powerful molecular biological method with a wide range of applications in the field of genetics and in clinical diagnosis. Since it was reported that fragmented DNA in paraffin-embedded sections was available for PCR amplification (1, 2), PCR has become a simple and essential technique in molecular pathology. PCR has enabled us to obtain much information from a large number of paraffin blocks in pathological laboratories, which had previously been used only for light microscopical study. As a result, many cases have now become available for retrospective study. Detection of viral genomes and genetic mutation was investigated with paraffin blocks; for example, Epstein-Barr virus (EBV) in Hodgkin's disease (2-4) and Sjögren's disease (5), papilloma virus in the uterine cervix (6) and the p53 tumor suppressor gene mutation in various cancers (7).

With improved sensitivity and the wide applicability of PCR, it is desired to connect this high sensitivity with histological features of the lesions. For this purpose, *in situ* gene amplification was proposed by several researchers (8-10), but, there has been no report describing the successful detection of single copy genes in paraffin-embedded tissue sections. Now, it is the only practical way to pick up the cells on sections and to amplify their DNA.

In this article, we describe the applicability of single-cell PCR for the detection of one or a few copies of gene such as the human T- lymphotropic virus type I (HTLV-I) genome.

Materials and Methods

Materials. Cell lines used for analysis included MT-2 (an HTLVI-immortalized human T cell line), TALL-1 (a human acute T-cell leukemia cell line, HTLV-I non-infected), HeLa (a human cervical adenocarcinoma cell line) and U251-MG (a human glioblastoma cell line). MT-2 and TALL-1 cells were autosmeared on glass slides by centrifugation at 1,200 rpm for 5 min. HeLa cells were cultured in a two-chambered slide (Nunc, Inc., Lab-Tek®, IL, USA) for 3 days and were further incubated with MT-2 cells (1.0×10^6 cells) for a few min. The slides were centrifuged at 1,500 rpm for 5 min to stick MT-2 cells to the glass surface. These cells were stained with Giemsa solution. U251-MG and 10,000 rad-irradiated MT-2 cells were co-cultured for 3 days, and then MT-2 cells were washed out every day for 7 days. After

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fixation in acetone, co-cultured U251-MG cells were immunostained with anti-HTLV-I gag protein (p19) monoclonal antibody (Sera-Lab, United Kingdom) by the avidin-biotin-peroxidase complex method. The cells were counterstained with hematoxylin. $p19^-$ U251-MG cells, $p19^+$ U251-MG cells and $p19^+$ giant polykaryocytes were microscopically identified and subjected to the analysis. Smears of peripheral blood cells from a patient with adult T-cell leukemia (ATL) were also analyzed. Red blood cells (RBC) and flower-like cells of ATL, atypical T lymphocytes diagnostic of ATL, were identified in May-Grünwald-Giemsa-stained specimens. Tissues resected from the stomach and parotid gland of ATL patients, which showed leukemic cell infiltration, were fixed in 4 % paraformaldehyde solution and embedded in paraffin. Six-μm-thick paraffin sections were prepared by standard methods and stained with hematoxylin or immunostained with OPD4 reacting with $CD4^+$ T cells in paraffin sections (11). ATL cells, RBC and normal glandular epithelial cells were subjected to PCR.

Single-cell PCR. Single-cell PCR was done according to the method previously described with minor modification (12). Target cells on the mounted specimens were observed microscopically and photographed. The precise locations of the cells were recorded by a micro-scanner (Sapporo Brewery Co, Tokyo, Japan), which made it easy to localize the target cells. After demounting, they were picked up using a micromanipulator (Eppendorf 5170) without touching the adjacent cells. The picked cells were then placed on a coverglass and sucked up by a micropipette, observing microscopically whether the target cells had been successfully sucked up or not. These cells were transferred to 600-μl PCR tubes. The portion of the specimen from which the target cell was picked up was photographed. The photographs before and after this procedure were compared to exclude the possibility of contamination. The transferred cells were digested with 1 mg/ml Proteinase K at 37°C for 2h in 30μl of digestion buffer; 10mM Tris-HCl (pH7.6), 0.45 % NP-40 and 0.45 % Tween-20. Proteinase K was inactivated by boiling at 97°C for 10min. All the solutions were subjected to 60 cycles of gene amplification. PCR was performed in 100μl of PCR mixture with *Taq* DNA polymerase (50mM KCl, 10mM Tris-HCl pH 8.3, 2.5mM $MgCl_2$, 0.02 % porcine gelatin, 200μM dNTPs, 100pmol primers) or *Tth* DNA polymerase (16.6mM $(NH_4)_2SO_4$, 6.7mM $MgCl_2$, 10mM 2-mercaptoethanol, 200μM dNTPs, 100pmol primers). For detection of HTLV-I, PCR primer pairs SK43, 44 and SK54, 55 were used (13); these primer pairs can amplify a part of the HTLV-I *pol* and *tax* sequences, respectively. The β-globin gene was analyzed using the primer described previously (14). Amplified DNA fragments were analyzed with ethidium bromide staining of the gel and Southern blot hybridization with internal oligonucleotide probes. Some samples were analyzed with liquid hybridization as previously reported (13).

Results and Discussion

Figs. 1a and 1b are the photographs taken before and after picking up an HTLV-I $p19^+$ giant polykaryocyte, respectively, which appeared in the U251-MG culture co-cultivated with irradiated MT-2 cells. ATL cells that were successfully picked up from the salivary gland are shown in Fig. 2a-c. These pictures clearly show that the target cells were successfully removed from the specimens without picking up other cells. This procedure requires substantial training to pick up the target cells. Taking photographs of the specimens before and after picking up the target cells helped to prevent picking up unrelated cells.

HTLV-I genomic and β-2 globin DNA sequences were amplified from one to five specific cells. Some representative results for HTLV-I are shown in Figs. 3 and 4. HTLV-I specific sequences were detected in

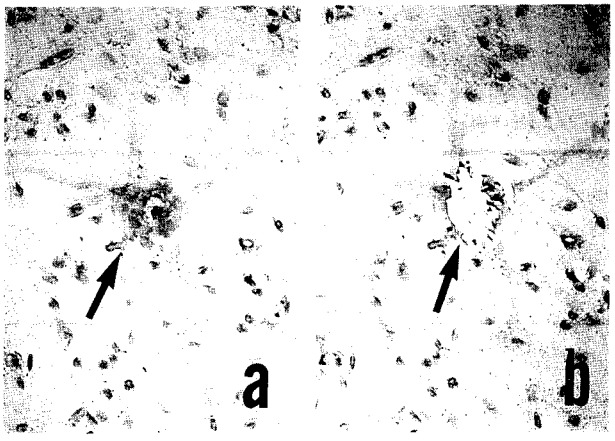


Fig. 1 Picking up a $p19^+$ polykaryocyte in U251-MG culture co-cultivated with irradiated MT-2. **a**, before picking up. **b**, after picking up. An arrow shows a polykaryocyte immunohistochemically positive for p19.

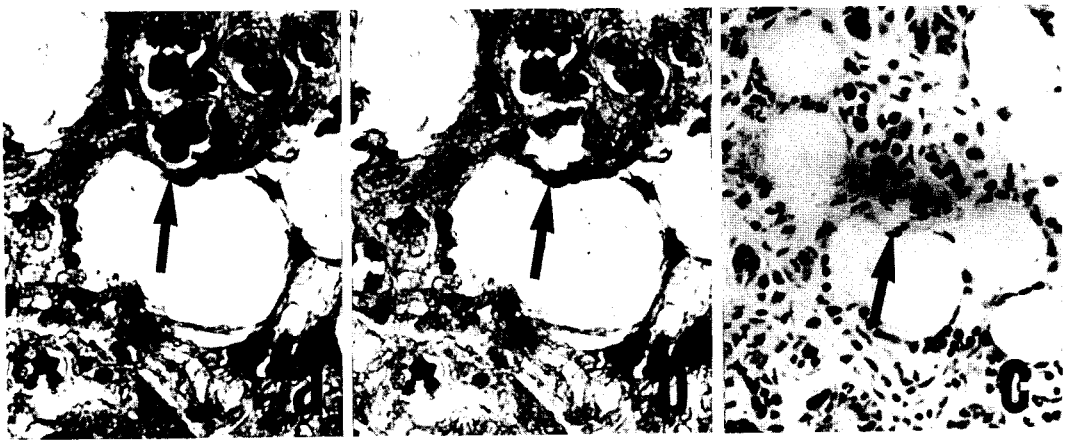


Fig. 2 Picking up adult T-cell leukemia (ATL) cells in the parotid gland. **a**, before picking up. **b** and **c**, after picking up; **a** and **b** were photographed without a coverglass.

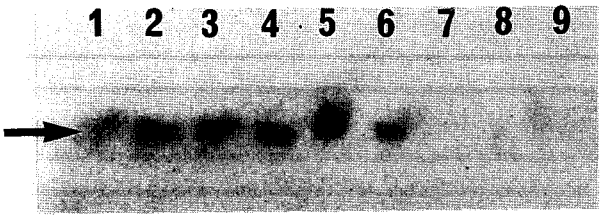
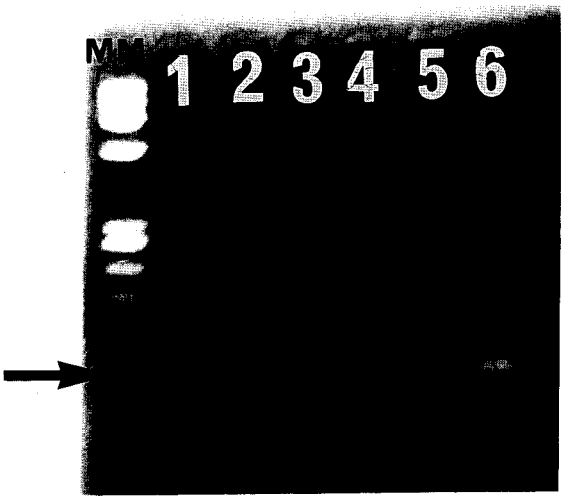


Fig. 3 Liquid hybridization of polymerase chain reaction (PCR) products amplified with SK43 and SK44. Lanes 1–4, MT-2; lanes 5, 6, p19$^+$ U251-MG polykaryocyte; lane 7, p19$^-$ U251-MG; lane 8, HeLa; lane 9, TALL-1. Four to five cells were applied to PCR. SK45 was used as a probe. Arrow indicates positive signals.



amplified DNA from the flower-like cells in the peripheral blood smears of an ATL patient, ATL cells in the paraffin-embedded specimen of ATL-affected organs, MT-2 cells, and p19$^+$ mononuclear and multinucleated U251-MG cells. In contrast, such sequences were detected neither in the HTLV-I untreated TALL-1 cells, nor in HeLa cells intermixed with MT-2 cells, p19$^-$ cells in U251-MG culture co-cultivated with irradiated MT-2 cells or in RBCs of ATL patients. This indicated that contamination with HTLV-I-infected cells did not occur during this procedure.

As for the sensitivity of this method, single copy genes such as the HTLV-I genome in ATL cells and β-globin genome were detected in 24 of 41 trials (59 %), in which 4–5 cells were applied (Table 1). Even in the case of ATL cells in paraffin sections, HTLV-I-specific DNA was successfully amplified in 13 of 25 trials (52 %). When 4 to 5 MT-2 cells, which have multiple copies of HTLV-I genome (15), were examined, sensitivity was 6 of 6 (100 %). In this study, DNA could be amplified from the cells in paraffin sections. Fixation and embedding procedures may not reduce sensitivity if a short

Fig. 4 Single-cell PCR of ATL cells in the paraffin-embedded specimens. MM shows the molecular marker ($\Phi\Psi$174/*Hae* III digest). Arrowed band is 118 bp. Lanes 1 and 2, ATL cells; lane 3, parotid gland epithelium; lane 4, RBCs in the lesion; lane 5, distilled water; lane 6, MT-2 (Positive control). DNA from these cells was amplified with SK54 and SK55. After electrophoresed in 4 % NuSieve gel, PCR products were visualized with 1 μg/ml of ethidium bromide.
PCR, ATL: See Figs 2, 3.

Table I Summary of single-cell polymerase chain reaction

Target DNA	Target cell	No. cells examined	Positive rate[a]
β-globin gene	MT-2, U251-MG, HeLa and TALL-1	4-5	5/8
HTLV-I	MT-2 cells	1	3/8
	MT-2 cells	4-5	8/8
	p19 positive U251-MG polykaryocyte	1	3/3
	Smeared ATL cells	4-5	3/5
	ATL cells in paraffin section	4-5	13/25
	HTLV-I uninfected cells[b]	4-5	0/25

a: Number of experiments showing positive signal/Number of total experiments
b: Red blood cells, epithelial cells of stomach and parotid gland, normal lymphocytes in parotid gland, and smooth muscles of stomach were taken from ATL patients. HeLa, TALL-1, and p 19-negative U251-MG cells were also examined.
HTLV-I: human T- lymphotropic virus type I; ATL: adult T-cell leukemia.

target sequence is amplified. We showed that the HTLV-I genome in ATL cells could be amplified from the paraffin sections in two cases. Single-cell PCR is the only way to detect the HTLV-I genome in specific cells in tissues, because genes of HTLV-I are not expressed in ATL cells *in vivo*.

As for specificity, no contaminated signals were detected in any series of experiments, so specificity was 100 %. Contamination occurs relatively easily with PCR, but it is avoidable. The critical step at which contamination usually occurs is extraction of the DNA and preparation of the PCR mixture. Avoiding these steps may have contributed to the high specificity for single-cell PCR. Of course, it is essential to pick up the target cell precisely.

The sensitivity of single-cell PCR is influenced by the copy number of the target sequence in cells and the size of the nucleus. The HTLV-I sequence in MT-2, which had multiple copies of HTLV-I genome, was more easily amplified than that in ATL cells which had a single copy of HTLV-I genome. Since a sliced large nucleus contains only a small fraction of whole genome, it is difficult to amplify a single-copy gene from it. Length of the target sequence may affect the sensitivity although we have no precise data about the effect of target length on sensitivity. The target sequences used in this experiment were as follows; 119 bp for HTLV-I *pol*, 158 bp for HTLV-I *tax* (13), and 118 bp for β-2 globin (14). As we could amplify the 287 bp LYDMA (EBV) sequence from paraffin embedded sections (unpublished data), it appears that target sequences shorter than 300 bp in length can be amplified from paraffin sections. Most DNA, however, in routinely processed paraffin blocks, is fragmented into length less than 500 bp length (16). Therefore, long target sequences, may be difficult to prime successfully.

In situ hybridization is used to localize the viral genome and gene expression in the specimen. Compared with single-cell PCR, however, this method is less sensitive for unexpressed genes. *In situ* PCR recently developed has improved the sensitivity of *in situ* hybridization, but this method has not been applied to the detection of single copy genes in paraffin-embedded tissue sections. This is because the sensitivity is insufficient for detecting a single copy gene, as suggested by Komminoth *et al.* who showed that the efficiency of gene amplification estimated to be about 50-fold after 30 cycles in suspended cells was significantly lower in cytospin preparations and was especially low in tissue sections (10, 17). Single-cell PCR was best applied to histologically characteristic target cells. For example, *in situ* hybridization for EBERs of EBV can easily detect EBV-infected small lymphocytes (18), but single-cell PCR is useless in such cases. The advantage of single-cell PCR is that it can detect unexpressed genes and that products by single-cell PCR can be used for further analyses. PCR primer pairs, which detect the difference of the target sequence, can reveal the divergency of the target cells. In contrast, the greatest difficulty associated with single-cell PCR is that of picking up the specified cells precisely. This method is a time-consuming procedure that requires skill and care. It is not applicable to large-scale analysis.

Taking these points into consideration, single-cell PCR may be useful when: a) histologically identified,

specific cells need to be analyzed, b) extremely high sensitivity is required, and c) diversity of target sequence can be analyzed.

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