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A simple competitive RT-PCR assay for quantitation of HIV-1 subtype B and non-B RNA in plasma

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Abstract

An easy, inexpensive competitive RT-PCR assay for HIV-1 RNA quantitation was constructed. A 138-bp sequence in the HIV-1 *gag* p24 region was selected as the target and co-amplified with competitor RNA containing an internal 44-bp deletion. Quantitation of serial dilutions of control RNA samples prepared from the LAI isolate demonstrated a good linearity ($R^2 = 0.991$) within the range between 10 and 250 copies/sample. The detection limit of the assay was determined to be 3.8 copies/sample by Probit analysis and corresponded to 110 copies/ml in plasma. The intra-assay CV value was 9.1%, and the inter-assay value was 25.9%. Both were comparable to those obtained with commercially available HIV-1 RNA quantitation kits. The correlation efficient for the results obtained in 47 plasma samples from HIV-1-infected individuals (subtype A in 1, subtype B in 25, subtype C in 4, subtype F in 1, and CRF01_AE in 16) with the competitive RT-PCR and Cobas Amplicor HIV-1 Monitor test v1.5 was 0.956 for subtype B and 0.947 for subtype non-B. The assay devised is a good alternative for monitoring antiretroviral therapy in resource-poor countries.

Keywords: HIV-1; Monitoring; Competitive RT-PCR; Viral load

1. Introduction

The pandemic of human immunodeficiency virus type 1 (HIV-1) infection has been the greatest threat to human health worldwide since its emergence two decades ago, and today about 40 million people are living with HIV/AIDS (Simon et al., 2006). As has been the pattern with respect to many other infectious diseases, the regions of the world experiencing the most significant spread of HIV-1 infection are poor. Ninety five percent of individuals infected with HIV-1 are living in developing countries with few resources, such as the countries of sub-Saharan Africa and Southeast Asia. According to the World Health Organization (WHO), nearly 6 million HIV/AIDS patients are in urgent need of antiretroviral therapy (ART), yet only 5% have access to the necessary drugs, and the remaining 95% are hopelessly waiting to die (WHO, 2004). To address this problem, WHO has launched the so-called "3 by 5" initiative (Jong-wook, 2003), which sought to treat 3 million people by the end of the year 2005, a goal made more likely by agreements enabling the use of generic antiretroviral drugs. The project appears to be operating effectively, and more patients than ever are being treated with antiretrovirals. Ironically, the introduction of antiretrovirals to countries with few resources has raised another issue: how best to monitor patients' response to treatment. In most developing countries where the "3 by 5" initiative has been implemented, it has been difficult to routinely monitor patients' CD4-positive T-cell counts or, what is worse, their plasma viral RNA concentrations (Russell, 2004). The plasma viral load is an important surrogate marker that is used to make prognoses, evaluate response to treatment, and estimate the probability of emergence of drug resistance (Kalish et al., 1999; Kempf et al., 1998; Leriche-Guerin et al., 1997; McDermott et al., 2005; Piliero, 2003). Three types of viral load quantitation kits are available commercially: the Cobas Amplicor HIV-1 Monitor test (Roche Diagnostics, Branchburg, NJ) (DiDomenico et al., 1996), the Quantiplex HIV-1 RNA Assay (Bayer, Mannheim,

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Germany) (Collins et al., 1997; Pachl et al., 1995), and the NucliSens EasyQ HIV-1 assay (bioMérieux, Boxtel, The Netherlands) (Kievits et al., 1991; de Baar et al., 1999; Yao et al., 2005). All three commercial kits have high sensitivity and reproducibility, and have been strictly validated. One significant drawback, however, is their high price. The cost per sample between 50 and 80 US dollars is too expensive to use for routine monitoring in resource-poor countries.

Competitive reverse transcription (RT)-PCR was the first method to quantitate HIV-1 RNA in plasma and used to show that viral load in plasma can be used as an important surrogate marker to assess the disease progression and the efficacy of ART (Piatak et al., 1993). Competitive RT-PCR involves co-amplification of a competitive RNA template that uses the same primers as for the target cDNA but that can be distinguished from the target after amplification by introducing a deletion, insertion, or a new restriction enzyme site. In conventional competitive RT-PCR, the amplified products of the target sequence and competitor are quantitated on agarose gel electrophoretograms, which is easy and inexpensive to perform, but has never been developed as a commercial assay. Several in-house competitive RT-PCR methods have been reported (Ernest et al., 2001; Johanson et al., 2001), but they have not been fully evaluated for quantitation of HIV-1 subtype non-B RNA in plasma samples. This paper describe a simplified, less expensive protocol for plasma viral load quantitation that is also applicable to HIV-1 subtype non-B.

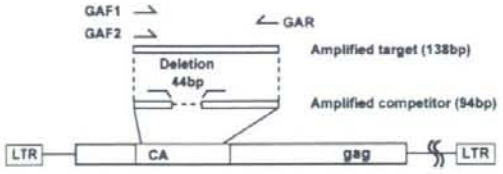
2. Materials and methods

2.1. Subjects

Blood samples were obtained from 47 HIV-1 seropositive individuals (subtype A in 1, subtype B in 25, subtype C in 4, subtype F in 1, and CRF01_AE in 16). The subtype was determined by phylogenetic analysis of sequences in RT and PR regions in *pol* and the *env* V3 region (Kato et al., 2003). The patients provided written informed consent to the study, and the study was approved by the local ethics committee.

2.2. Preparation of competitor RNA and control HIV-1 RNA

A template DNA consisting of an HIV-1 *gag* sequence (positions 1372–1509) with a 44-bp deletion (1418–1461) (Fig. 1) and a T7 promoter (TAATACGACTCACTATAGGGAGA) at the 5′ end was constructed from HIV-1 DNA clone NL4-3 by the recombinant PCR technique (Higuchi, 1990). Competitor RNA was synthesized from the template DNA with an *in vitro* transcription system and T7 RNA polymerase (Promega, Madison, WI). QIAamp UltraSens Virus Kit (QIAGEN, Hilden, Germany) was used to extract HIV-1 LAI RNA from the culture supernatant of peripheral blood mononuclear cells infected with the LAI isolate, and the extracted LAI RNA was stored at −20 °C until used as an external control for HIV-1 quantitation. The exact HIV-1 RNA concentration of the LAI RNA solution was determined by spectrophotometry and Poisson distribution analysis of the positive scores of nested RT-PCR at the endpoint dilution (Kato et al., 2006).



Primer	Sequence	Position
GAF1	5'-CAAGCAGCCATGCAAATGTTAA-3'	1372-1393
GAF2	5'-CAGGCAGCAATGCAAATGTTAA-3'	1372-1393
GAR	5'-AGTAGTTCCTGCTATGTCACTTCCC-3'	1509-1485

Fig. 1. Construction of the competitive RT-PCR assay: target genome and primer design. An HIV-1 *gag* p24 region was selected as the target for the competitive RT-PCR. Both the target and the competitor RNA molecules were co-amplified with a primer mixture of GAR, GAF1, and GAF2. The amplicons of the target and the competitor were distinguished by a 44-bp deletion. The primer sequences of GAR, GAF1, and GAF2 are shown. The nucleotide numbers are based on the HIV-1 NL4-3 sequence (GenBank accession no. M19921).

2.3. Competitive RT-PCR assay for viral RNA quantitation

Primer binding sites were selected in the most conserved sequences in the p24 region of the *gag* gene among all subtypes and CRFs of HIV-1 (Fig. 1) according to the HIV sequence database (Los Alamos database— http://hiv-web.lanl.gov/content/hiv-db/mainpage.html). Two oligonucleotides that differ in nucleotide at positions 1374 and 1380, GAF1 and GAF2, were used together as upstream primers to minimize mismatches with HIV-1 sequences reported in the database (Fig. 1). The size of amplicons of the target RNA and competitor RNA was expected to be 138 and 94 bp, respectively.

RNA was extracted from 140 μl of patient plasma with a QIAamp Viral RNA kit (QIAGEN) with elution in 80 μl. A 20 μl volume of 1, 1/10, and 1/100 dilutions of the eluate were used for subsequent competitive RT-PCR. Three different copy numbers of LAI RNA (10, 50, and 250) were assayed in duplicates as external controls.

RT-PCR was carried out in a 50 μl solution containing primers (0.2 μM GAR, 0.1 μM GAF1, and 0.1 μM GAF2), competitor RNA equivalent to 50 copies, and 1 × SuperScript III RT/Platinum *Taq* Mix (Invitrogen, Carlsbad, CA) with a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal profile of RT-PCR was: 10 min at 50 °C for RT; 2 min at 94 °C for initial denaturation; 5 s at 94 °C, 10 s at 48 °C, and 15 s at 72 °C for 5 cycles; 5 s at 94 °C, 10 s at 62 °C, and 15 s at 72 °C for 35 cycles; and 1 min at 72 °C for extended elongation. Introduction of the elongation step at 48 °C was intended to minimize the effect of mismatches between primers and naturally occurring HIV-1 variants.

The plasma samples were also assayed with COBAS Amplicor HIV-1 Monitor Test v1.5 (Roche Diagnostics).

2.4. Electrophoresis and evaluation

A 20 μl sample of PCR products was electrophoresed in a 2% agarose gel at 160 V for 40 min. The gel image was photographed with a Polaroid camera under UV illumination and

scanned with a CanoScan FB-636U scanner (Canon, Tokyo, Japan). The band intensity of targets and competitors was evaluated with the NIH Image program (http://rsb.info.nih.gov/nih-image/Default.html). A calibration curve was obtained from the ratios between the bands of the external controls and competitors with MS Excel software (Microsoft, Seattle, WA). The copy numbers of the samples were determined by fitting their ratios to the calibration curve.

3. Results

3.1. Competitive RT-PCR system

Five different copies (10, 25, 50, 125, and 250) of control HIV-1 LAI RNA were amplified together with a fixed dose of competitor RNA, and the ratios between the band intensity of target RNA and the band intensity of competitor RNA were plotted against the input copy numbers of target RNA (Fig. 2A). Good linearity was obtained, with a high correlation coefficient of 0.995.

A photograph of a typical electrophoretogram of competitive RT-PCR for RNA samples from plasma of HIV-1-infected individuals is shown in Fig. 2B. DNA fragments of two different sizes were observed: a 138-bp fragment amplified from target HIV-1 RNA and a 94-bp fragment amplified from competitor RNA. In practical runs, a calibration curve was obtained from co-amplification of each of three different numbers of copies (250, 50, and 10) of LAI RNA with competitor RNA. Fig. 2B shows that ratios of target and competitor RNA bands for patients 1 and 2 were calculated as 2.66 and 4.10, respectively, and applying them to a calibration curve revealed HIV-1 RNA copy numbers per milliliter of 4500 and 6600, respectively.

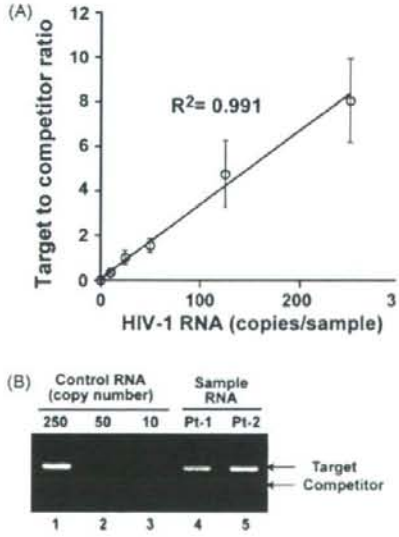


Fig. 2. Linearity and a typical agarose electrophoretogram of the results of competitive RT-PCR. (A) Linear relationship between target/competitor band ratios and initial HIV-1 RNA copy numbers. Each measurement was performed in five replicates. Means and standard deviations are indicated by circles and bars, respectively. (B) Lanes 1–3 are control LAI RNA at levels of 250, 50, and 10 copies/sample, respectively. Lanes 4 and 5 are two clinical samples (Pt-1 and Pt-2). Target/competitor band ratios of the clinical samples (2.66 and 4.10) were translated into copy numbers (160 and 240 copies per sample) using a calibration curve obtained from lanes 1–3.

Table 1
Detection limit of competitive RT-PCR by limiting dilution assay

HIV-1 RNA (copies/sample)	Target amplification	
	+	−
20	10	0
10	10	0
5	7	3
2.5	2	8

Control LAI RNA samples of two-fold serial dilutions were assayed in 10 replicates, and the detection limit was calculated by Probit analysis.

3.2. Detection limit and reproducibility of the competitive RT-PCR

The lower detection limit of the assay was assessed by testing multiple replicates of dilutions of the HIV-1 LAI RNA (subtype B) that had been quantitated by using the Poisson distribution analysis. Dilutions equal to 560, 280, 140, and 70 copies/ml were tested in 10 replicates at each dilution (Table 1). A Probit analysis showed that the 50% detection limit was 110 copies/ml. Since samples of HIV-1 subtypes A, B, C, and F and CRF01_AE exhibited equivalent efficiency of amplification and detection as described below (Fig. 3), the detection limit for subtype non-B viruses was concluded to be at the same level. The dynamic range of 110–700,000 copies/ml was obtained by testing three serial dilutions (1, 1/10, and 1/100) of the RNA sample.

The reproducibility of the competitive RT-PCR assay was assessed by assaying five seronegative plasma samples spiked

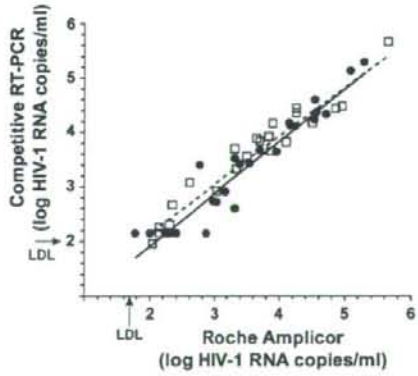


Fig. 3. Comparison between the results of competitive RT-PCR and Cobas Amplicor HIV-1 Monitor test v1.5. Plasma HIV-1 RNA levels of 47 clinical specimens containing subtype B (solid circle) and non-B (open square) are plotted. The lower detection limit (LDL) by competitive RT-PCR and Amplicor was 110 and 50 copies/ml, respectively. The correlation coefficient was 0.956 for subtype B and 0.947 for non-B. Simple regression lines for subtype B (dotted line) and non-B (solid line) are shown.

Table 2
Reproducibility of competitive RT-PCR

Test[a]	Sample[b] (copies)					
	A	B	C	D	E	Mean ± SD
1	48.5[b]	39.4	37.2	36.8	30.9	37.2 ± 6.4
2	47.6	35.8	37.9	34.8	27.4	35.8 ± 7.3
3	41.5	47.8	40.2	36.2	33.2	40.2 ± 5.6

[a] Viral RNA extraction and competitive RT-PCR reaction were performed in triplicate in three independent experiments.
[b] Fifty copies of LAI RNA were added to the plasma from a healthy human subject in each sample tube.

with 50 copies of HIV-1 LAI RNA in triplicate (Table 2). The inter-assay and intra-assay CV values were 25.9% and 9.1%, respectively, and comparable to those obtained with commercially available quantitation kits (16.2–87.5%) (Lin et al., 1998; Muyldermans et al., 2000).

3.3. Quantitation of clinical samples

The HIV-1 RNA concentration of 47 seropositive plasma samples of subtypes A, B, C, and F and CRF01_AE was analyzed with both Cobas Amplicor HIV-1 Monitor test v1.5 and the competitive RT-PCR assay (Fig. 3). The results showed good agreement between the two assays: the correlation coefficient for subtype B and subtype non-B was 0.956 and 0.947, respectively; and the ratio between the two measurements was 0.97 ± 0.10 (mean ± S.D.) for the subtype B samples and 1.01 ± 0.07 for the subtype non-B samples. Although the number of samples in this study was limited, these data suggest that the competitive RT-PCR allows quantitation of both subtype non-B and subtype B of the viral load.

4. Discussion

In order to establish a quantitation method for HIV-1 RNA in plasma that could be used as a routine clinical test in resource-poor countries, an attempt was made to develop an alternative to the commercial kits. The assay described above is based on a competitive RT-PCR technique and is characterized by several improvements over similar methods reported previously. First, it enabled quantitation of HIV-1 RNA in 47 plasma samples containing different HIV-1 subtypes (A, B, AE, C, F) belonging to group M. The viral loads measured with the present assay were consistent with those determined with Cobas Amplicor HIV-1 Monitor, and the ratio was 0.97 ± 0.10 (mean ± S.D.) for the subtype B samples and 1.01 ± 0.07 for the subtype non-B samples. Two competitive RT-PCR assays have been previously reported to detect or quantitate non-subtype B viruses (Emery et al., 2000; Ernest et al., 2001), but since both assays tested primary isolates containing extremely high titers instead of viruses in patients' plasma, the inhibitory effect of plasma components on RT or PCR was not examined. The target of PCR in the present study was selected as the most conserved sequence in the *gag* gene among all subtypes and CRFs of HIV-1 group M (HIV Sequence Database, Los Alamos). Furthermore, to mitigate the influence of sequence variations among subtypes on viral RNA quantitation, a mixture of two upstream primers was employed that differ at two nucleotide positions to minimize mismatches between primers and HIV-1 RNA variants, and 5 cycles with a low annealing temperature of 48 °C were introduced during the early phase of PCR.

The present assay is simple and easy to perform, and the entire procedure, from RNA extraction to analysis of electrophoresis images, takes only 4 h. The use of hot-start DNA polymerase (Platinum *Taq*) enables preparation of PCR samples at room temperature and combination of RT and PCR into one step. Because of the shortness of the amplicon (138 bp), brief reaction times were adequate for RT (10 min) and DNA elongation (15 s each) during PCR. High sensitivity for HIV-1 RNA detection was also achieved. A Probit analysis showed a 50% detection limit of 110 copies of HIV-1 RNA per ml in plasma, which is comparable with the results reported in previous studies (Piatak et al., 1993; Venturi et al., 2000), although a smaller sample volume (140 μl) was used than in those studies (2.8 and 1.8 ml, respectively).

Several commercial HIV-1 quantitation assays, such as Amplicor (Roche Diagnostics), Nuclisens (bioMérieu), and Quantiplex (Bayer), are being used as routine tests to monitor patients being treated with antiretroviral drugs in developed countries. However, they are expensive and require the use of special instruments that are often unaffordable in developing countries. The present method costs only about 10 dollars per sample and does not require any special instruments except a microcentrifuge, gel electrophoresis system, and ultraviolet transilluminator, and the simplicity of the method means that no special training program is needed for clinical technologists. These features should be advantageous in regard to adoption of the assay in resource-poor countries.

One common problem of competitive RT-PCR is the narrow dynamic range. In the current assay, 1-, 1/10-, and 1/100-fold dilutions of plasma RNA samples were subjected to RT-PCR separately to cover a quantitation range from 110 to 700,000 copies/ml. Because an adequate dilution ratio cannot be predicted for each plasma sample, a fixed amount of competitor RNA (50 copies) is added to a reaction mixture rather than a plasma sample. Thus, this protocol does not correct measurements of viral loads in terms of RNA extraction efficiency, although the recovery of RNA by QIAamp Viral RNA kits (QIAGEN) was found to be almost constant at 77% (Table 2).

In conclusion, the competitive RT-PCR assay described in this study is a good candidate for an HIV-1 viral load assay for use in monitoring ART in resource-poor settings.

Acknowledgments

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GENETICS

The development of novel quantification assay for mitochondrial DNA heteroplasmy aimed at preimplantation genetic diagnosis of Leigh encephalopathy

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Abstract *Purpose*: To perform preimplantation genetic diagnosis (PGD) of Leigh encephalopathy, we developed a rapid and reliable quantification assay for the percentage of T8993G mtDNA mutation and analyzed various specimens.

Methods: We prepared the standard curve by measuring serial proportion of 8993T/G cloned plasmid DNA using real-time PCR, and measured (1) mutant DNA (known proportions by PCR-RFLP), (2) single lymphocytes from 46% mutant carrier, (3) 123 blastomeres from 20 abnormal embryos.

Results: (1) These were within −5~+6% error range, (2) mean 44.3% (11–70%), (3) Five embryos harbored T8993G mutation (4–22%). Embryos from same person indicated different degrees of heteroplasmy, and blastomeres from same embryo demonstrated limited dispersion of heteroplasmy (2–11%).

Conclusions: (1) This method provides rapid and reliable PGD for Leigh encephalopathy. (2) The variable heteroplasmy with somatic mitosis was suggested. (3) T8993G mutation was existed in undeveloped embryo, and the bottleneck theory was supported. The limited heteroplasmy dispersion of blastomeres from same embryo also supported reliability of PGD for T8993G mutation.

Keywords Mitochondrial DNA · Heteroplasmy · Real-time PCR · Preimplantation genetic diagnosis · Leigh encephalopathy

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Introduction

The mitochondrial (mt) DNA is a circular double-stranded DNA molecule with 16.6 kilobases [1]. It presents in several copies in each mitochondrion. Human individual cells contain 100,000 to 200,000 copies of mtDNA [2]. The mtDNA has been recognized that it is maternally inherited and nearly all of the mtDNA is identical in normal individuals. Mutation of mtDNA is seriously concerned with mitochondrial disease. Heteroplasmy (the presence of both normal and mutant mtDNA at different levels within the same cell) accounts for the phenotypic variability of mitochondrial disease [3, 4]. From human maternal carrier of mitochondrial disease to pedigrees, rapid shifts in mtDNA mutant frequency have been observed. To explain this phenomenon, it has been hypothesized that the number of mtDNA within any one oocyte is reduced to as few as five or less during oogenesis, especially in primordial germ cell [5–9]. This is termed "bottleneck theory." Thus oocytes develop extremely different heteroplasmy ratios of mtDNA mutation (a state termed "mutant load").

To date, there have not been attempts generally for preimplantation genetic diagnosis (PGD) of mitochondrial disease

caused by mtDNA mutation. This is due to three possible main reasons: (I) the mutant load measured in the blastomere will not be the same as the mutant load in the other fetal tissues, (II) the mutant load measured in the blastomere will change during embryogenesis or after birth, (III) the correlation between the mutant load and the disease severity is not obviously understood.

In the majority of mtDNA disorders, these features of mtDNA are fulfilled [10, 11] and prenatal diagnosis or PGD is difficult to perform. However in families with mutations at the mtDNA T8993G point mutation, the mutant load is usually similar in different tissues, indicating that there is no tissue-specific selection of age-related variation, and there is also a strong correlation between the mutant load and the disease severity [12, 13]. Thus PGD may be feasible for this mutation.

The T8993G point mutation in the ATPase6 gene was first described in an adult with neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP) [14], and is also a common cause of Leigh syndrome [15, 16]. The clinical severity is strongly associated with mutant load. The patient with Leigh syndrome has very high mutation load, typically >90% mutation of mtDNA, and has severe clinical symptoms. NARP is related with intermediate mutant load of 60 to 80%, while mutant load less than 60% are commonly not associated with clinical symptoms [17, 18].

The only way to perform PGD for mitochondrial disease is analysis of the heteroplasmy ratio in a single blastomere of an early stage embryo (4–8 cells stage). Although the mutation ratio used to be quantified by PCR-RFLP and this method has high sensitivity with low standard deviation [19], it requires many procedures; PCR amplification, digestion with restriction enzyme, and electrophoresis in a genetic analyser. Consequently more simple procedure is desirable for safety and reliable PGD. In the present study, we developed a simple and precision quantification assay of the T8993G mtDNA point mutation using real-time single PCR for PGD of Leigh encephalopathy and analyzed various types of cells and DNAs about this mutation to verify this assay. We also analyzed the segregation patterns of mtDNA in preimplantation development by measuring heteroplasmy ratio of human blastomeres.

Materials and methods

Plasmid DNA and specimens preparation

DNA for the wild-type (8993T) and mutant (8993G) target sequences was generated from cloned plasmid DNA containing pCR® 2.1-TOPO® vector (Invitrogen, USA) and PCR products of primers mtF8838 and mtR9139, and to be sized about 4200bp. The copy numbers of the wild-type and mutant DNA sequences were calculated based on the size and molecular weight of the plasmid DNA. These were mixed to each mutant load, and diluted into 0.04 pg/μl (equivalent to mtDNA from a single blastomere) by TE buffer with 5 ng/μl salmon DNA (Sigma-Aldrich, USA) to avoid adsorption of small DNA to real-time PCR wells.

Four different heteroplasmy types of specimens (lymphocyte cell line or whole blood) from female carriers and patients affected by Leigh encephalopathy with T8993G mutation and whole blood samples from normal woman and MOLT4 cell line as controls were obtained. Four specimens have been analyzed to have 28%, 46%, 62%, 98% of T8993G mutant load by PCR-RFLP respectively. The PCR restriction analysis was undertaken as previously described [20]. MOLT4 cell line was established from lymphocyte of human acute lymphoblastic leukemia and considered to have no T8993G mutation. DNA was extracted from these six types of specimens by using SepaGene (Sanko Junyaku, Japan) and diluted into 10 pg/μl, amounts that equal mtDNA from a single blastomere (10^4 copies). When a fresh or cultured single lymphocyte with 46% mutation was analyzed, cells were handled in a clean bench with a mouth-controlled fine heat-polished glass micropipette in drops of PBS using an inverted microscope.

Twenty human embryos confirmed to contain three polar bodies (3PN) were donated to this research by thirteen unrelated normal couples. Embryos were considered to be abnormal inadequate embryo to transfer at 24 h following insemination and cultured up to 2–12 cleavage stage, and placed in an equilibrated medium (Sydney IVF Embryo Biopsy Medium: Cook, USA). Then Embryos were irradiated by a non-contact 1.48 μm diode laser system (OCTAX Laser Shot: MTG, Germany) for the piercing of the zona pellucida. Two or three short pulses (2.9 ms) were applied, and the biopsy pipette was inserted in the hole and a blastomere was removed by aspiration. Under an inverted microscope the blastomeres were rinsed in a drop of PBS, and transferred into individual PCR tubes containing 3 μl of cell lysis solution (0.2% sarcosyl + TE buffer with 10 mM EDTA). Whole blastomeres per embryo were examined.

All specimens were collected after receiving informed consent. The research procedure was approved by the Research Ethics Committee of Keio University School of Medicine and the Japan Society of Obstetrics and Gynecology.

Real-time PCR quantification of mtDNA heteroplasmy ratio

Real-time PCR primers and two fluorescent probes (TaqMan®MGB probe; Applied Biosystems, USA) corresponding to normal (8993T) and mutation (8993G)

were prepared. The forward primer (5′-CGAAACCAT CAGCCTACTCATTCAA-3′) spanned nt 8958 to nt 8982. The reverse primer (5′-CCTGCAGTAATGTTAG CGGTTAGG-3′) spanned nt 9026 to nt 9003, and total length of the amplified product was 69bp. The probe for normal (8993T; CCAATAGCCC[T]GGCCGT) had "VIC" fluorescent and the probe for mutation (8993G; AATAGCCC[G]GGCCGT) had "FAM" fluorescent. The source of the probes was obtained from Applied Biosystems. To confirm PCR product, PCR amplified DNA was sequenced using ABI PRISM 310 genetic analyzer.

Twentyfive μl PCR mixture was set up with final concentrations: 12.5 μl TaqMan Universal PCR Master Mix, No AmpErase UNG (2X), 0.625 μl 40X Assay Mix, 8.875 μl (6.875 μl) distilled water, 0.5 μl × 2 TaqMan MGB Probe, 1.0 μl × 2 PCR primer, 1.0 μl (3.0 μl) specimen. The reactions were performed as follows: initial denaturation at 50°C for 2 min and 95°C for 10 min, and 45 cycles at 92°C for 15 sec (denaturation), 60°C for 1 min (annealing and extension). The allelic discrimination assay using real-time PCR (ABI PRISM 7000) was used to measure each fluorescent, and proportion of these values (mutation [FAM]/normal [VIC] + mutation [FAM]) was calculated and analyzed for the following studies.

Preparing the standard curve

(1) Two types of plasmid DNA (8993T and 8993G) were mixed and prepared to each mutation ratios (0, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%). The concentration of plasmid DNA was adjusted into 0.04 pg/μl as stated above. Measurements were performed in ten times, and the average value was applied for standard curves. (2) DNA extracted from 98% mutation carrier cell line and normal woman were mixed to each mutation ratios (0, 1, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, 98%). The concentration of extracted DNA was adjusted into 10 pg as aforesaid. Measurements were performed in ten times.

Analysis of various specimens

(1) 28% mutation DNA (n = 20), (2) 46% mutation DNA (n = 20), (3) 62% mutation DNA (n = 20), (4) 98% mutation DNA (n = 20), (5) Single lymphocytes of fresh blood and established cell line derived from the same 46% mutation carrier (n = 40/40 respectively), (6) Single lymphocytes derived from MOLT4 cell line (n = 20), (7) Distilled water as negative control (n = 20), (8) 123 individual blastomeres from 20 cleavaged embryos as a simulation of the actual PGD.

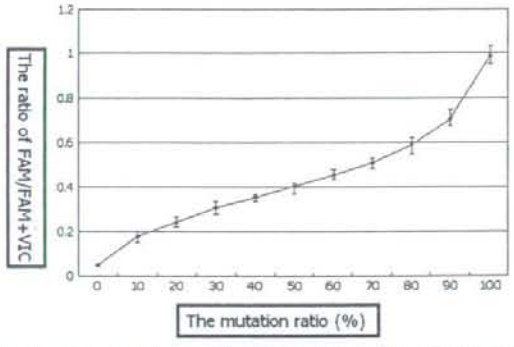

Fig. 1 The standard curve of mutation ratio analysis; two types of plasmid DNA (8993T/G)

Measured values were calculated and applied to standard curve of plasmid DNA, then converted to mutation percentage.

Results

Linear standard curves were obtained from the proportion of VIC and FAM fluorescence (Figs. 1 and 2). Values of correlation coefficient of VIC for normal sequence and of FAM for mutating sequence were 0.9697 and 0.9745 respectively. Standard curves from carrier DNA and plasmid DNA demonstrated an approximate curve, but carrier DNA showed slightly low values (0.0165~0.0475; 0%~−8% [mean −3.6%]). A standard deviation of each curve was demonstrated in very low level (≤0.05).

The sequencing result of PCR product showed identical arrangement with mtDNA nt 8958 to nt 9026 (Fig. 3).

The standard curve obtained from plasmid DNA was applied to measure mutant load of various specimens. Four different types of heteroplasmic DNA were consistent with standard curve within −5~+6% error range (Fig. 4).

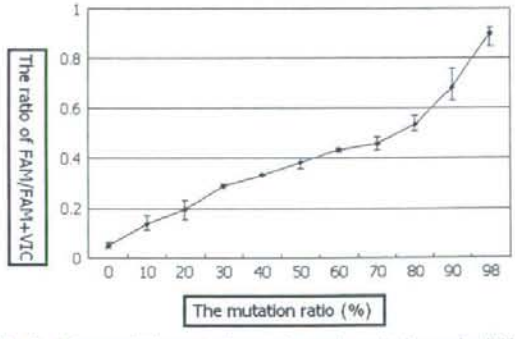

Fig. 2 The standard curve of mutation ratio analysis; carrier DNA (98% mutation)

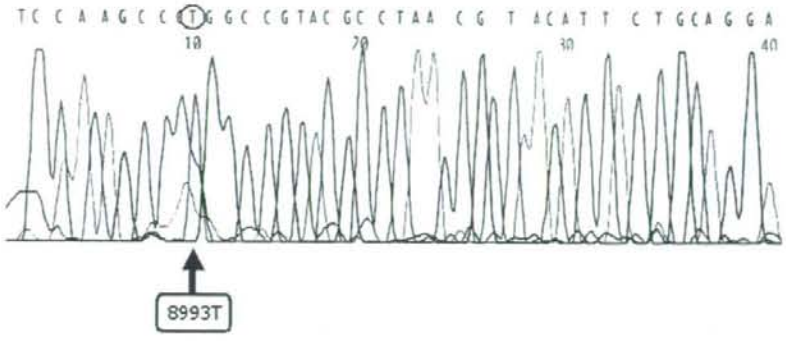

Fig. 3 The sequencing result of PCR product

Actual measurement data was as follows; (1) 28%DNA: upper extreme 34%/upper quartile 31%/lower quartile 29%/lower extreme 26%, (2) 46%DNA: 49/48/47/45%, (3) 62%DNA: 68/68/63/60%, (4) 98%DNA: 97/97/94/93%.

In the analysis of fresh blood single lymphocytes from 46% mutation carrier, a scattering was observed. An average mutant load of 40 lymphocytes was 44.3% with 11–70% wide range among individual cells (upper extreme 70%, upper quartile 52%, lower quartile 38%, lower extreme 11%), and this mean value was close to the result of PCR-RFLP (46%) (Fig. 4). While 80% lymphocytes (32/40) from fresh blood indicated 46% ± 10% range of mutant load, only 45% lymphocytes (18/40) from established cell line showed same range of mutant load (Fig. 5). Mutant load of MOLT4 lymphocytes and negative controls were both 0% (Figs. 4 and 6). While MOLT4 lymphocytes showed only normal fluorescent (VIC), negative controls indicated neither fluorescent.

T8993G mutation was detected in five embryos (25%; 5/20) with different degrees of heteroplasmy (4~22%; embryo6,7,11,16,17) (Fig. 6). Blastomeres derived from same embryo indicated limited dispersion of heteroplasmy within 2 to 11% range (embryo6; 4~15%, embryo7; 9~20%, embryo11; 11~22%, embryo16; 10~12%, embyo17; 18~21%). Embryos derived from same person indicated different degrees of heteroplasmy (embryo 5,6,7 [0~8%, 4~15%,9~20%]/embryo 16,17 [10~12%,18~21%]).

Discussion

A linearity of standard curve was secured with very low standard deviation and actual measurement data of heteroplasmic DNA was calculated within low error range. However in different DNA concentration, measurements showed a little change (data not shown). This standard curve was prepared under a condition of 10^4 mtDNA copies per blastomere, but several percentage differences were observed under 10^3 or 10^5 mtDNA concentration.

Studying single lymphocytes of the 46% carrier indicated wide range variation of mutant load (11~70%[fresh]/0~79%[cultured]). This data suggested the possibilities of the variable heteroplasmy at the cellular level by a mtDNA random distribution with somatic mitosis. Previously it has been pointed out that the mutant load of established cell

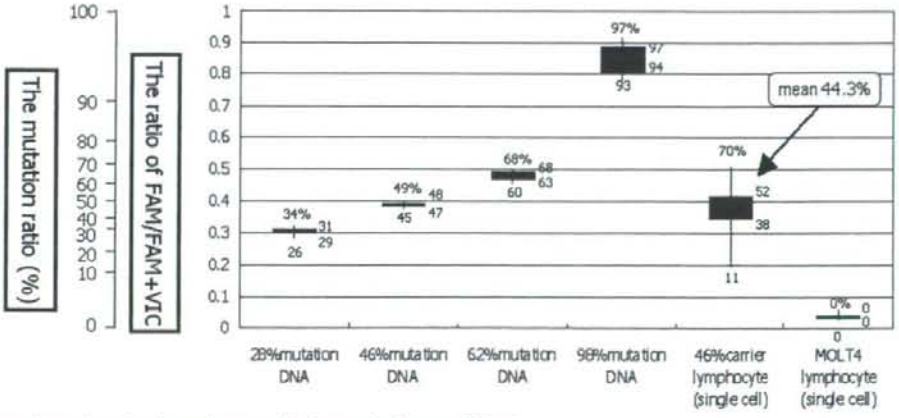

Fig. 4 The mutation ration of each specimens applied to standard curve of Fig. 1

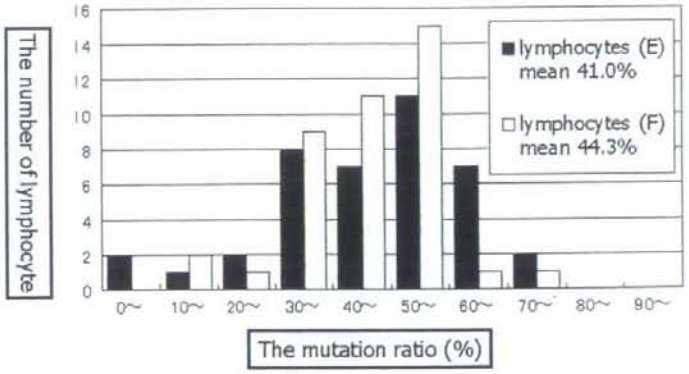

Fig. 5 The distribution of single lymphocytes from 46% mutation carrier fresh blood (F) and established cell line (E)

lines might be changed as a result of cell selection during cell culture [21], therefore we expected the result to be different distribution between fresh and cultured lymphocytes. However, significant changes were not seen between them.

The analysis of blastomeres proved an existence of T8993G mtDNA mutation in 3PN embryo from women thought to have no mtDNA mutation. To the best of our knowledge, few existences have been reported so far, although in abnormal cleavage embryo. Mutant loads among embryos from same person were different (embryo 5,6,7 [0~8%,4~15%,9~20%]/embryo 16,17 [10~12%, 18~21%]). These differences were measured more than measurement error of present real-time PCR method. Therefore it suggested an existence of bottleneck theory during oogenesis. The ratio of the heteroplasmy was demonstrated to be a limited dispersion in the individual blastomeres of the same embryo. This result means that the mutant load of single blastomere is practically equal to that of the same embryo. These data are consistent with results from previous studies in mouse embryos [22] and human embryos [19], detecting the distribution of mtDNA polymorphisms in blastomeres. It also supports reliability of PGD for T8993G mutation.

In actual PGD of Leigh encephalopathy, considering various factors (measurement error, difference of first mtDNA amount, random distribution with somatic mitosis etc.), the cut-off value should be established to subtract 10~15% from mutation ratio of carrier mother. However it is considered that the avoiding offspring of more severe mutant load will be not difficult, because previous data shows a much skewed segregation of the T8993G mutation in gametes of T8993G carriers. Most oocytes inherited either a very low or a very high proportion of mutant mtDNA [23].

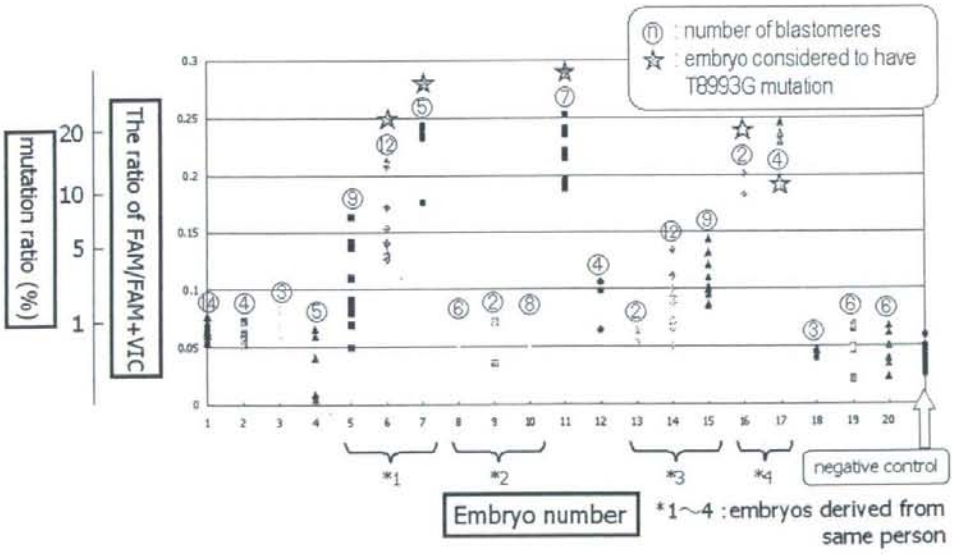

Fig. 6 The variation of mutation ratio in blastomeres derived from normal women (3PN embryo → cleavage embryo)

There is a possibility of nuclear transplantation for mtDNA mutation carriers. However many problems exist in nuclear transplantation, principally on safety and the DNA originality of the individual human. At the moment, PGD by measuring mutant load is the only way which can provide an opinion to carrier mother and family for avoiding the transmission of high mutant mtDNA.

Conclusions

These results demonstrate that it is technically and logically possible to perform PGD for T8993G Leigh encephalopathy by measuring the mtDNA heteroplasmy ratio (even small percentages) by allelic discrimination assay using real-time single PCR from small amounts of DNA. This method requires only few hours to obtain results of mutant load, and needs only few procedures, therefore it is expected to avoid misdiagnosis by manipulations and to perform the rapid and accurate PGD.

The research of single lymphocytes and blastomeres suggested the variable heteroplasmy with somatic mitosis, the existence of T8993G mutation in abnormal embryo and the bottleneck theory during oogenesis

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GENETICS

Well-devised quantification analysis for duplication mutation of Duchenne muscular dystrophy aimed at preimplantation genetic diagnosis

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Abstract *Purpose:* Preimplantation genetic diagnosis (PGD) has been performed for deletion and point mutation type of Duchenne muscular dystrophy (DMD). Our aim was to develop a PGD technique, not yet established, to directly detect duplication mutation instead of substitute diagnosis similar to gender determination.

Methods: Our method is based on comparative quantification using conventional duplex PCR, real-time PCR and gender determination. We evaluated this method in single lymphocytes from a duplication type of DMD patient and a normal male.

Results: There was a significant difference in the mean values of the ratios (the mutation locus/a normal reference): mean value ± SE was 1.84 ± 0.15 in the duplication patient, and 1.00 ± 0.09 in the normal male ($p < 0.001$).

Conclusion: It is suggested that our comparative quantification method could be a new option in PGD for carriers with duplication mutation who wish to have an unaffected son.

Keywords DMD · A single cell · Duplication · Real-time PCR · Quantification

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Introduction

Duchenne muscular dystrophy (DMD) (NIM #310200) is a lethal X-linked recessive disorder affecting 1 in 3,500 male births [1]. Few efficient treatments have been developed so far for DMD, and genetic treatment research continues to be performed [2, 3]. Genetic counseling and molecular diagnosis that allow the identification of carriers are therefore the only solutions offered to DMD families. DMD is caused by mutations in the dystrophin gene located at Xp21.2, which encodes protein of the membrane cytoskeleton in skeletal muscle (dystrophin) [4]. The dystrophin gene is the largest encoding gene found in nature to date, measuring 2.4 Mb and containing 79 exons that encode 14 kb mRNA, and probably accounts for the high frequency and high proportion of de-novo mutations [5]. Two-thirds of DMD mutations are deletions, with the remaining mutations consisting of large duplications and point mutations [6].

Preimplantation genetic diagnosis (PGD) is an evolving technique that provides a practical alternative to prenatal diagnosis and termination of pregnancy for couples who are at substantial risk of transmitting a serious genetic disorder to their offspring [7]. Samples for genetic testing are obtained from oocytes or cleaving embryos after in vitro fertilization (IVF) [7]. Only embryos that are shown to be free of the genetic disorders are made available for replacement in the uterus, in the hope of establishing a pregnancy [7]. In the mid-1980s, the advent of polymerase chain reaction (PCR) provided a far superior method for genetic testing, making it possible to carry out a diagnostic test on highly concentrated and relatively pure amplified PCR fragments that spanned the appropriate genetic mutation [8]. Substantial groundwork for the clinical application of PGD to various conditions was

undertaken in the late 1980s. The first pregnancies that resulted from the transfer of embryos that had been genotyped as female were reported in 1990 [9], and the first live birth was reported following PGD for cystic fibrosis in 1992 [10]. However, the majority of PGD for heterozygous female patients have so far consisted of the positive selection of female embryos [11]. The main disadvantage of this approach is that half of the discarded male embryos are unaffected. Accordingly, allelic diagnosis by direct detection of a mutant gene has also been performed in the course of PGD [12].

PGD for DMD has also been performed by gender determination when no specific PCR test was available, but recently techniques have been developed that can be used for direct detection of mutation. The first specific PGD for DMD was performed for a family carrier of a deletion encompassing exon 17 of the dystrophin gene, through the study of this exon in single blastomeres [12]. There are several reports that describe a single cell multiplex PCR protocol allowing the analysis of dystrophin gene combined with gender determination [11, 13, 14]. More recently, Girardet et al. [15] clinically applied this approach to PGD for a family displaying a large deletion extending from exon 21 to exon 34 of the dystrophin gene. Deletions in the dystrophin gene that account for approximately 60% of mutations responsible for DMD are diagnosed by single cell multiplex PCR. The remaining cases are due to point mutation (25–30%) [16] or duplications (5–10%) [5, 17], but there have been no reports of PGD for DMD to the best of our knowledge. PGD for point mutation was performed for familial amyloidotic polyneuropathy (FAP) by nested PCR and single-stranded conformational polymorphism (SSCP) analysis [18], and for β-thalassaemia using sequencing of single cell PCR products [19]. Meanwhile, only a few reports exist concerning PGD for duplications [20, 21], of which the detection is commonly possible using DNA probes located in the duplicated region (FISH or Southern blotting method). Pregnancy after PGD for Charcot-Marie-Tooth disease type 1A caused by a duplication of a 1.5 Mb region was first reported in 1998, in which mutations were detected by fluorescent PCR with a polymorphic $(CA)_n$ marker localized within the duplication [22]. In cases where polymorphic $(CA)_n$ markers are localized out of the duplication, however, diagnosis has to rely on linkage analysis that does not involve direct detection of mutations.

The ethical committee of the Japanese Society of Obstetrics and Gynecology (JSOG) has been carefully considering the clinical enforcement of PGD, especially for DMD within the context of Japanese history and society. From an ethical aspect, normal embryos should not be discarded on the basis of gender due to the fact that embryos are considered as precious form of life. From a technical aspect, there is a need not only for high diagnostic accuracy but also, as a consequence, for a specific method to clarify gene mutation.

Table 1 Normal reference in comparative quantification: the ratio between the duplicated exon and the normal reference

Normal reference	A normal male XY (1)*	A DMD patient *X*Y (2)*	A normal female XX (2)*	A DMD carrier *X*X (3)*
Autosome	0.5(1/2)	1(2/2)	1(2/2)	1.5(3/2)
X chromosome	1(1/1)	2(2/1)	1(2/2)	1.5(3/2)
Y chromosome	1(1/1)	2(2/1)	Y(–)	Y(–)

*Number of duplicated exon copy.

X: the X chromosome with the duplicated exon.

In previous deliberations of the JSOG, the substitute diagnosis including gender determination for X-linked genetic disorders has been dismissed. In addition, each PGD case requires JSOG approval. It will be necessary to overcome the formidable hurdle that this decision represents, even for duplication mutation for PGD. We aimed to develop a PGD technique for direct detection of duplication mutation.

Quantitative real-time PCR has evolved over the last few years as a valuable tool in molecular diagnosis. We examined whether a single cell specimen can be quantified by using real-time PCR, and also tested a comparative quantification method: conventional multiplex PCR followed by quantitative real-time PCR using the unspecific dsDNA dye SYBR green 1. At the same time, we used gender determination to simplify discrimination of the normal, the carrier, and the affected, because comparison within the dystrophin gene on X chromosome is more complicated than comparison between the mutant locus on X chromosome and a normal reference on Y chromosome (Table 1). This approach for DMD patients with a large duplication in the dystrophin gene was technically improved in this study to the clinically available level of PGD for the carrier of duplication mutation. This study has obtained the approval of the institutional ethical committee of Keio University School of Medicine.

Materials and methods

Sampling of single lymphocytes

Venous blood samples from a normal male, and a DMD patient who was 27 years old and was proved to have mutation of exon 8–9 duplication by Southern blot analysis, were collected into heparinized tubes. Blood from the DMD patient was drawn from the internal carotid vein after informed consent was obtained at their bedside because their limb muscles and peripheral vessels were atrophic and made it difficult to collect blood samples. Blood was then diluted 2-fold with Dulbecco's phosphate-buffered saline (PBS) (Gibco BRL, France), layered over Lymphoprep (NYCOMED PHARMA

AS, Norway) and centrifuged at 1600 rpm for 10 min. The interface containing the mononuclear cell fraction was collected and cells were washed three times with PBS. The cells were then resuspended in 1 ml Roswell Park Memorial Institute (RPMI1640) medium (Invitron, France) containing 20% fetal calf serum (FCS) and 10% dimethylsulphoxide (DMSO)(Sigma) and frozen at −80°C until further processing. At the same time, Epstein-Barr-transformed lymphoblast cell lines were established to avoid the repeated drawing of blood. For isolation of single lymphocytes, an aliquot of the cell suspension was thawed and 1 μl was added to 4 ml of 90% RPMI1640 supplemented with 10% FCS in a culture plate. Individual lymphocytes were deposited in 4 μl of lysis solution 1 (0.2% sarcosyl, TE containing 10 mM EDTA) [23], with a mouth-controlled glass microcapillary under an inverted microscope. The cells were then denatured at 65°C for 10 min and kept frozen at −20°C until the PCR procedure.

Single cell multiplex PCR

A multiplex PCR strategy was designed so that the ratio between the test exon (mutation locus) and a normal reference could be compared. We chose exon 8 of the dystrophin gene as the test exon and the SRY gene of the Y chromosome as the normal reference (both are single copy genes). The primers of exon 8 have been previously described [24, 25] on the Leiden Muscular Dystrophy pages (http://www.dmd.nl/), and the primers of the SRY gene have been previously described [26] (Table 2). The first PCR was performed by a duplex PCR of exon 8 and the SRY gene for preparation of the next quantification and gender determination procedure.

The first round PCR reaction contained 4 μl of lysis solution 1, 6 μl of lysis solution 2 (50% glycosyl, 10 mM Tris-Hcl, pH 8.0, 1 mM EDTA, pH8.0, 10 ng/μl poly A, 10 mM ascorbic acid), 5 μl of 25.0 mM $MgCl_2$ solution, 5 μl of 8 mM dNTP mix, 2.5 μl (10 pM) each of the two pairs of primers, 2.5 U AmpliTaq Gold and water in a total volume of 50 μl. PCR was carried out on a GeneAmp 9700 thermocycler (Applied Biosystems, USA) using the following program: 10 min denaturing at 95°C, then 25 cycles of 30 s at 95°C, 30 s at 60°C, 30 s at 72°C reactions, followed by an extension phase at 72°C for 7 min.

Table 2 Primer sequences

Name	Sequence	Size
Exon 8 of tne dystrophin gene		
Outer forward	GGCCTCATTCTCATGTTCTAATTAG	
Outer reverse	GTCCTTTACACACTTTACCTGTTGAG	360
Inner forward	CACTCTTCCAAGTTTTGCCTCAA	
Inner reverse	GCAACATTTCCACTTCCTGGAT	63
The SRY gene		
Outer forward	GTTGTCCAGTTGCACTTCGCTGCA	
Outer reverse	CAGTGTGAAACGGGAGAAAACAGT	351
Inner forward	AACGCATTCATCGTGTGGTC	
Inner reverse	GCATTTTCCACTGGTATCCCA	109

Real-time quantitative PCR

One microliter of the first round PCR products was then aliquoted into two separate tubes for each of the individual second round real-time PCR. Primers were designed according to the manufacturers' recommendations with the Primer Express software version 2.0 (Applied Biosystems, USA) within the first PCR products (Table 2). All primers were conformed so as not to form primer dimer and hair-pin structure.

The PCR reaction mix consisted of SYBR Green PCR Master Mix (Applied Biosystems, USA) (25 μl), 900 nM (4.5 μl) forward primer, and 900 nM (4.5 μl) reverse primer. One microliter of the first round PCR products was transferred to this PCR reaction mix, and individual real-time PCR was carried out using the ABI Prism™ 7000 sequence detection system and 96-well MicroAmp™ optical plates (Applied Biosystems, USA). The pre-run thermal cycling conditions were 10 min at 95°C, the thermal cycling conditions were 40 cycles at 95°C for 15s and 65°C for 1 min.

Comparative Ct method, as previously described by Livak [27], detected relative gene expression, or, in our case, the relative gene number. The fractional cycle number (Ct) at which the amount of amplified target reaches a fixed threshold is directly related to the amount of starting target. The ratio of the amount of the first PCR products is determined by measuring their Ct values. The Ct value was analyzed using the Sequence Detection System (SDS version 1.1, Applied Biosystems, USA). The following formula can be used to show the gene copy number: $2^{-(\Delta\Delta Ct)}$, where $\Delta\Delta Ct = \Delta Ct$ (the patient male) $- \Delta Ct$ (a normal male), and $\Delta Ct = Ct$ (the test exon) $- Ct$ (a normal reference). The ratio between the DMD patient and the normal male was determined by calculating the expression $2^{-(\Delta\Delta Ct)}$. This formula was based on the assumption that the rate of Ct change versus the rate of target DNA copy change was identical for both genes, and also that doubling of the target gene copy number resulted in a 1-cycle decrease in the measured Ct. Subsequently, the ratios between the test exon (the mutation locus) and the normal reference were calculated: the ratios of 1.0, 1.5, and 2.0 theoretically indicate a normal male/female, a carrier, and a patient with duplication, respectively. The mean values and distribution of the patient male and the normal male were statistically analyzed with Student's t-tests.

Results

Duplex PCR from single cells

Conventional duplex PCR from single lymphocytes was performed with two pairs of primers: one pair for the mutation locus (exon 8), and the other for the normal reference locus and gender determination (SRY gene). A single lymphocyte is taken to contain 4–10 pg of DNA and is considered to be insufficient for quantification in real-time PCR alone. Single lymphocyte must first be amplified by conventional PCR before quantification, and the product amount should be within the dynamic range of the next real-time PCR (1–1000 ng). First of all, we optimized the PCR condition in the number of cycles. Because plateau of amplified DNA product exists in the latter phase of PCR amplification cycles, the first PCR must be completed in the exponential phase before it reaches plateau. As a preliminary experiment, we examined the amplification curve in real-time PCR using single lymphocytes. Due to instability in amplification curve from a single lymphocyte alone, we analyzed 10 lymphocytes together in one tube. It revealed that the PCR reaction was characterized by an exponential increase in the amount of the PCR product below 35 cycles (data not shown). Too much amplification in the first PCR resulted in PCR plateau, and too little resulted in sample insufficiency in the next real-time PCR. The product amount was about 100–500 ng after 25 cycle amplification in multiplex PCR of exon 8 and the SRY gene.

Real-time quantitative PCR

After the first round PCR optimization, we examined real-time PCR as nested PCR and quantification system to detect duplication mutation from the amplified products of single lymphocytes. Three detection methods were tested: fluorescently labeled specific hybridization probes (TaqMan probe) (Applied Biosystems, USA), the fluorogenic primers (LUX Primer) (Invitrogen, USA), and the unspecific dsDNA-dye SYBR green (Applied Biosystems, USA). Discrimination between the patient male and the control male was unclear from single cells with both the TaqMan probe and the LUX Primer, but relatively clear with the SYBR green dye. Therefore, we used the unspecific dsDNA-dye SYBR green that was simple to design and cost-effective. The first round PCR products were amplified with a pair of primers for the test exon (exon 8), and then for the normal reference and gender determination (SRY gene) in separate reactions (Fig. 1(a), (b)).

Figure 2 shows the mean values of exon 8/SRY gene ratio in the DMD duplication patient and the normal male. Mean value ± SE of the ratio was 1.85 ± 0.15 in the duplication patient (29 mesurements) and 1.00 ± 0.09 in the normal male (29 mesurements) respectively, and it was significantly different (p-value of <0.001). The distributions of the DMD patient and the normal male are shown in Fig. 3; the range of 25–75% was differentiated, but overlap was observed. Two overlapping distributions of results are transected by a cut-off line. The region beneath the distribution of results for the DMD patient that lies above the cut-off line corresponds to the test's true positive (i.e. sensitivity); the region that lies below the cut-off line corresponds to the false negative. For the distribution of results for the normal male, these two regions correspond to the false positive and the true negative (i.e. specificity). For the two overlapping distributions, moving the cut-off line affects the sensitivity and specificity, but in opposite directions. Sensitivity and specificity cannot both be improved by changing the cut-off line, but rather only by changing the distributions such that less overlap occurs (i.e. by improving the discrimination level of the test itself). We examined diagnosis accuracy and efficiency assuming PGD by calculating the false negative rate (leading to a misdiagnosis) and transferable male embryo rate (below cut-off line) on the assumption that the cut-off line was set at 1.75, 1.20, 0.88, and 0.70 of the distributions (corresponding to upper 10, 25, 50, and 75% of the control male) (Table 3). The false negative rate was too high to be performed clinically with single cells, but was decreased by analyzing single cells in duplicate and only using those which gave identical results (Table 4). In theory, half of male embryos are normal, and all female embryos are unaffected (normal or carrier). When the cut-off line is set at 0.88, 12.2% of male embryos (24.4% of healthy male) can be transferred. Although all male embryos were discarded in PGD by previously performed gender determination, about half of normal male embryos can be rescued by this method.

Amplification rate

A total of 200 single lymphocytes including preliminary tests were analyzed using this conventional duplex PCR followed by separate nested real-time PCR with co-amplification of exon 8 and one of the following loci (exon 12, 45, and SRY

Table 3 Calculated false negative rate and transferable male embryo rate based on our results–1 blastomere diagnosis

Cut-off line	False negative rate	Transferable male embryo rate
1.75 (upper 10%)	35.7% (15/42)	72.4% (21/29)
1.20 (upper 25%)	24.1% (7/29)	50.0% (14.5/29)
0.88 (upper 50%)	17.6% (3/17)	29.3% (8.5/29)
0.70 (upper 75%)	12.5% (1/8)	14.8% (4/29)

The false negative rate is that of affected male embryos identified as normal, which leads to a dangerous misdiagnosis in PGD. The transferable embryo rate is the proportion of male embryos identified as normal. In theory, 50% of male embryos are normal, while 100% of female embryos are unaffected (normal or carrier).

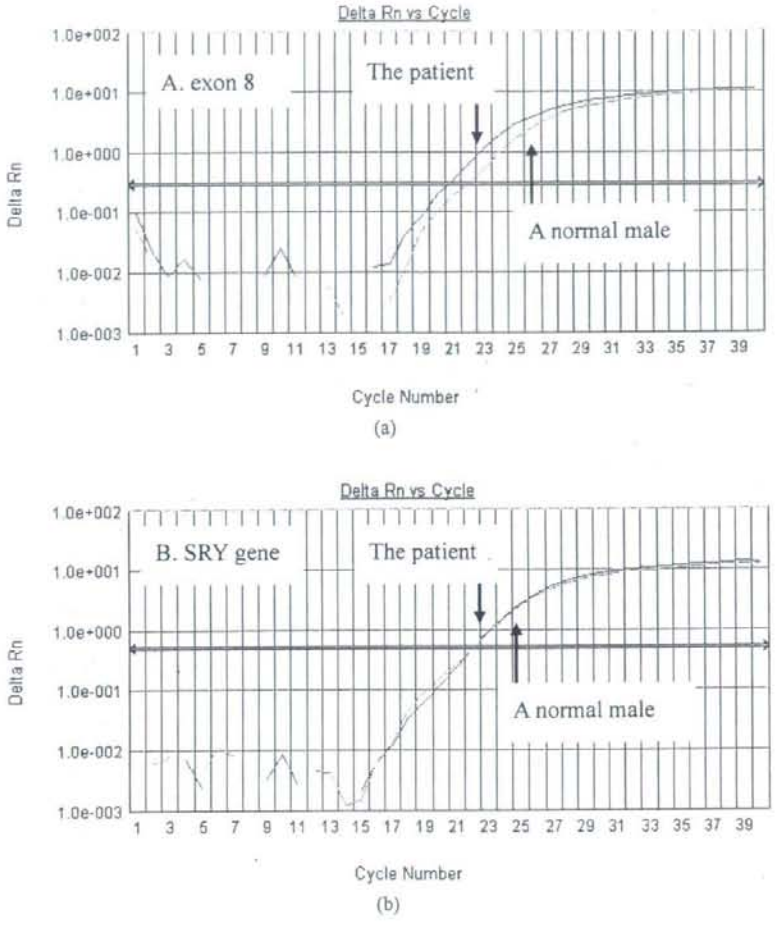

Fig. 1 AB: Amplification curve of exon 8 (a) and SRY gene (b) from the same first round duplex PCR product. Amplification curve of exon 8 in the DMD patient shifted to the left due to the extra exon (the duplicated exon), resulting in smaller Ct value.

gene). The efficiency of the individual loci (exon 8, 12, 45, and SRY gene) was 188/200 (94.0%), 60/60 (100%), 61/70 (87.1%), and 67/70 (95.7%) respectively. The overall amplification rate was 376/400 (94.0%) and the failure rate was 6.0%.

Table 4 Calculated false negative rate and transferable male embryo rate based on the results–2 blastomere diagnosis

Cut-off line	False negative rate	Transferable male embryo rate
1.75 (upper 10%)	23.6% (225/954)	56.7% (477/841)
1.20 (upper 25%)	9.2% (49/533)	31.6% (266/841)
0.88 (upper 50%)	4.4% (9/205)	12.2% (103/841)
0.70 (upper 75%)	2.0% (1/50)	3.0% (25/841)

The false negative rate was too high to allow PGD to be performed clinically with single cells, but was reduced by analyzing single cells in duplicate and only using those which gave identical results. However, the transferable male embryo rate was reduced simultaneously (12.2% of the transferable male embryos is equal to 24.4% of the healthy male embryos).

Discussion

The aim of this study was to develop PGD technique for direct detection of DMD duplication by quantification method, which is a common diagnostic method in duplication mutation. Although many duplication mutations differing in their localization and extent have been described so far in DMD patients (the Leiden muscular dystrophy pages), a versatile and effective detecting method for PGD has not been developed and mutation diagnosis at PGD is not available for about 10% of DMD patients with duplication mutation. In recent years, techniques for the detection of duplications have multiplied (reviewed in Armour et al. [28]), and very few protocols have been published proposing quantitative real-time

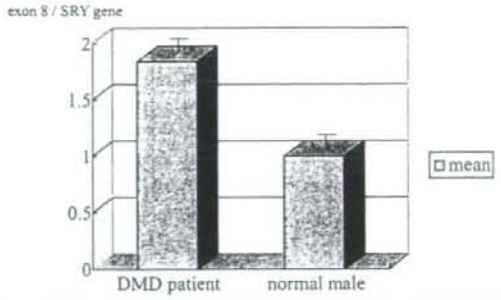

Fig. 2 Mean values of the ratio between the test exon and the normal reference. The ratios between exon 8 and SRY gene were calculated from the difference in Ct values. Mean ± SE was 1.84 ± 0.15 in single lymphocytes of the DMD patient (29 measurements), and 1.00 ± 0.09 in single lymphocytes of the normal male (29 measurements). Their mean values were significantly different (p-value of < 0.001).

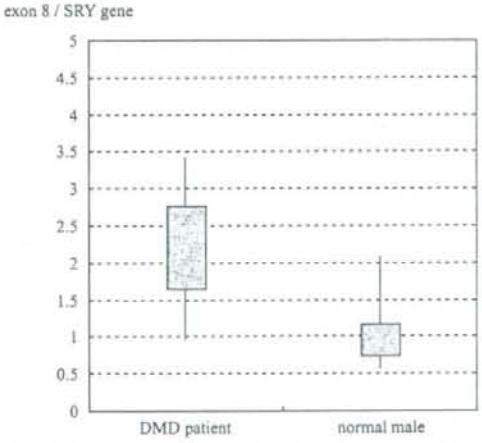

Fig. 3 Distributions of the ratio between the test exon and the normal reference. Box plot of the ratios obtained for a DMD patient and a normal male (29 measurements, respectively); box: 25th–75th percentile; error bars: 10th–90th percentile. Two overlapping distributions of results are transected by a cut-off line.

PCR for measuring copy number changes in patients with duplications spanning one or more exons [29]. Conventional PCR strategies only analyze final PCR products without visualizing the kinetics of the reaction and are not adequate for quantification due to their inability to indicate the exponential phase. In spite of this demerit of conventional PCR, we have succeeded in quantification analysis of single cells by coordinating the cycle numbers of nested conventional PCR and using capillary electrophoresis (ABI Prism™ 310 GeneScan Analysis: Applied Biosystems, USA) (data not shown). This approach allowed duplication mutation in the dystrophin gene to be distinguished from normal status, but the optimized course was complicated and time-consuming. Unlike endpoint quantitative PCR, real-time PCR is suitable for quantification because the quantity is defined by a point threshold cycle (Ct) when PCR amplification is still in the exponential phase [30].

We presented a method of conventional duplex PCR followed by a rapid real-time PCR using SYBR-green dye for monitoring product formation. Discrimination of single cells between the DMD patient and the normal male was achieved under the optimum conditions by quantification and subsequent comparison of test exons (exon 8 of dystrophin gene) and a normal reference (SRY gene). Real-time PCR is suitable for quantification because of its robustness, rapidness, and high reproducibility. Its rapid analysis ability is essential for time-constraints related to PGD. Due to a large dynamic range of quantification (1–1000 ng) and a very small amount of starting material in real-time PCR, an unknown sample can be easily quantified. DNA from a single cell is insufficient (4–10 pg) to be analyzed and must be amplified to the dynamic range prior to quantification. Single cells can be amplified by conventional PCR, of which the cycle number should be adjusted in the exponential phase so that the initial dose rate of two samples could be maintained. In addition, at insufficient cycles the amount of the PCR products did not reach the detection range for the following real-time PCR analysis. Therefore, determining an appropriate number of cycles is important. Secondly, making or selecting primers is important at the first round duplex PCR, in which the Tm value of the primers and product sizes should be adjusted. Decreasing the Tm difference increases the efficiency of the duplex PCR. The closer the two product sizes, the more similar the two product amounts are, and this makes the following quantification easier. Furthermore, proper detection method of the mutation in real-time PCR is essential for precise quantification. Because exons of the dystrophin gene are short (about 100 bp), the selection of primers and probes is restricted and difficult to make. Although fluorescence-labeled TaqMan-probes or LUX-primers are a little complicated and expensive to synthesize, SYBR-green dye is cost-effective and reliable. In addition, Joncourt et al. [29] have described that fluorescence signals of the hybridization probes decreased over time even when probes were stored as stock solutions at a constant temperature of −20°C and the stability of the probes was difficult to control. We also experienced instability of quantification analysis using fluorescence-labeled TaqMan-probes and LUX-primers. Compared with other gene dosage techniques, quantification with SYBR-green dye has many advantages in terms of low costs, less labor, and short preparation time.

Comparative quantification using a normal reference is necessary due to various DNA quantities in single cells (4–10 pg). The number of duplicated exon copy is 1, 2, 2, and 3 in a normal male, a duplication patient, a normal female, and a carrier female respectively. When the normal reference is a gene on the X chromosome, the expected ratio

between exons located in the mutation region and the normal reference is 1:2:1:1.5 in a normal male, a duplication patient, a normal female, and a carrier female respectively (Table 1). In a preliminary experiment, we examined exon 8/45 ratio on the dystrophin gene. The results showed significantly different mean values, but also showed overlapping distributions. False negative results which lead to a misdiagnosis arose due to two overlapping distributions. It is important to detect affected male embryos in PGD, and discrimination is easier (discrimination of 1:2) by using the SRY gene on the Y chromosome as the normal reference (Table 1). Consequently, comparison of mutation locus (exon 8) and Y chromosome-related single copy gene (SRY gene) can provide quantification and gender determination simultaneously. All female embryos without the SRY gene and male embryos with the SRY gene in which the ratio of exon 8/SRY gene is below the cut-off line can be transferred. As performing PGD, two blastomeres can be examined from one embryo at the same time, providing a double check for the results. It was reported that pregnancy and implantation rates did not appear to be significantly affected in spite of the biopsy of the two cells from embryos with 7 or more cells [31]. When the results of both blastomeres are identically normal, there is a high probability that the embryo will also be normal. Female embryos are all unaffected and can be transferred. Male embryos could be diagnosed using this method with a high degree of accuracy by examining the two blastomeres respectively, although the number of transferable male embryos decreases (Tables 3 and 4). Transferable embryos identified as unaffected should be selected in descending order of embryo quality. A low cut-off line reduces the false negative rate, but it also reduces the transferable embryo rate. The cut-off line should be decided in advance with the client's opinion after informed consents.

The main reason for the overlapping distributions is the limitation of detection ability with this method. The distribution is wider in patient's lymphocytes: some of the patient's lymphocytes were categorized as normal contrary to expectations. These results may be due to PCR amplification failure in duplicated exons in the same way as the phenomenon that results in the failure to amplify one allele of a heterozygous locus, commonly called allele drop-out, of which the rate ranges from 0 to 12.9% with the average rate taken to be 7.5% [31]. Amplification failure, which is a dangerous risk in PCR from single cells, can lead to a misdiagnosis, especially in gender determination. If the SRY in an affected male embryo doesn't amplify, the embryo will be look as a female embryo and transferred. With our method, the amplification failure rate of the SRY gene was found to be 4.3%. Therefore, the possibility of misdiagnosis regarding gender determination would be very low by analyzing two single cells respectively.

In conclusion, we performed DNA quantification from single cells of a normal male and a DMD patient, and there was a significant difference in the mean values between the two groups. Duplication mutation differs from type to type in localization and extent, and its primers and probes must be carefully matched in each case. The simplicity of this protocol will allow its easy implementation in diagnostic laboratories. Linkage analysis through repetitive micro satellite measurement may be a good method in PGD in terms of accuracy. However, as it is a substitute method, it doesn't meet the ethical committee's requirements. The location detection and sequencing of the junctional locus are very difficult in DMD duplication mutation due to the enormous size of the dystrophin gene. In addition, unaffected male embryos discarded by mere gender determination can be successfully transferred by our quantification method. In this respect, this method ideally meets the JSOG committee's requirements which stipulate that the indication of PGD should be strictly restricted and that clarifying gene mutation is a must. The problem lies with the distribution overlap, and the fact that false negative results can not be avoided, although the rate can be reduced to 5% or lower. This problem can be dealt with through informed consent based on adequate explanation and subsequently, prenatal diagnosis. We propose that this comparative quantification method, with gender determination using two blastomeres, can be a choice for DMD carriers who wish to have unaffected sons, or live in strictly restricted countries for PGD, due to the fact that there is no other PGD technique of direct detection of duplication mutation currently available.

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ヒト免疫不全ウイルス（ＨＩＶ）感染症：東京都における検査と解析

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Human immunodeficiency virus (HIV) infection : Diagnosis and Analysis in Tokyo

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Yasuko YOSHIDA * and Kazuyoshi YANO **

Keywords : 後天性免疫不全症候群 Acquired Immunodeficiency Syndrome:AIDS), ヒト免疫不全ウイルス Human immunodeficiency virus:HIV, サブタイプ Subtype, HIV検査 HIV Diagnosis, 薬剤耐性 Drug Resistance, 解析 Analysis, 東京 Tokyo

1．はじめに

1981年6月5日付のMortality and Morbidty Weekly Report（MMWR）に5例のニューモシスチス・カリニの患者報告がなされた[1]ことを契機に，米国疾病管理センター(CDC:Centers for Disease Control)が，この疾患の調査を開始した（表1）．その後全米各地から，日和見感染症等を伴う重症の免疫不全患者が相次いで報告されたことから，CDCはこの原因不明の免疫不全症を，後天性免疫不全症候群(Acquired Immunodeficiency Syndrome : AIDS)[2]と命名した．AIDSの病原体は，1983年にフランスのパスツール研究所のMontagnierら[3]がLAV（Lymphadenopathy-associated virus）というウイルスが原因であることを最初に報告した．その後，1984年にGalloら[4]によりHTLV-Ⅲ（Human T-lymphotropic virus type Ⅲ），さらにLevyら[5]によりARV（AIDS-associated retrovirus）として発見されたが，3つのウイルスはすべて同一であることが判明し，1986年の国際命名委員会でウイルスの名称はヒト免疫不全ウイルス(Human immunodeficiency virus:HIV)[6]に統一された．さらに，西アフリカに限局して発生していた同様の疾患から，遺伝子学的に異なるHIVが，Montagnierらにより発見され[7]，先に発見されたHIV-1と区別されHIV-2と命名された．HIVは，輸血，非加熱血液製剤，男性同性間感染，異性間性的接触，薬物静注あるいは母子感染等の感染経路で，全世界へと広がっていった．

表1．エイズ年表（1981年－1987年）

1981年	CDCは5人のカリニ肺炎患者をMMWRにて報告
1982年	CDCがAIDSという名称を採用
1983年	AIDSの原因ウイルス分離がMontagnierらによりScience誌に報告
1984年	エイズ調査検討委員会（後のエイズサーベイランス委員会）が設置
1985年	日本のエイズ患者第一号
1986年	国際命名委員会にて，ウイルスの名称がHIVに統一 MontagnierらによりHIV-2発見
1987年	日本国内初の女性患者を確認，発表，エイズパニック 全国の保健所で検査受付開始

2．我が国におけるHIV/AIDS監視体制

1984年9月にエイズ調査検討委員会（後のエイズサーベイランス委員会）が設置され，全国的なエイズ患者の調査が開始された．1985年に日本のエイズ患者第一号が報告され[8]，1987年には神戸で不特定多数の男性と性交渉を持った女性のエイズ患者がマスコミで報道されたことが[9]，大きな社会問題となった．同年2月の厚生省通知[10]により，全国の保健所でエイズ相談窓口が開設され，東京都では東京都立衛生研究所（現健康安全研究センター）ウイルス研究科がHIV検査を担当することとなった。

1989年からは「後天性免疫不全症候群の予防に関する法律」（エイズ予防法）に基づき[11]，1999年3月まで実施された。同年4月からは，「感染症の予防および感染症の患者に対する医療に関する法律」（感染症法）の施行に伴い[12]，後天性免疫不全症候群は第四類感染症，2004年には感染症法改正により第五類感染症全数把握疾患として位置づけられた。

現在，エイズ発生動向調査は感染症法に基づく感染症発生動向調査の一部として整備され，エイズ動向委員会は3

表2．HIV感染症の診断

・下記の抗HIV抗体スクリーニング検査が陽性で，確認検査が陽性の場合 1) ELISA法，2) ＰＡ法，3) IC法，4) その他
・確認検査 1) Western Blot法，2) IFA法 3) HIV病原検査（抗原検査，ウイルス分離，PCR法）
・周産期に母親が感染していたと考えられる18ヶ月未満の児の場合 1) HIV病原検査が陽性の場合 2) CD4陽性Tリンパ球数の減少，CD4/CD8比の減少

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