A NOVEL MOLECULAR METHOD FOR HIV-1 PROVIRAL DNA DETECTION: NON-RADIOACTIVELY - REVERSED PROBE HYBRIDIZATION AND NESTED PCR

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Abstract. A novel molecular method for HIV-1 proviral DNA detection comprising two main techniques: nested PCR, amplifying a target sequence of the ENV-gene of HIV-1, and non-radioactively-reversed probe hybridization for the detection of the amplified target sequence. The dual amplification of inserted HIV-1 proviral DNA in each DNA sample to be tested was performed by nested PCR in two steps: firstly with two outer primers covering the target sequence of the ENV-gene of HIV-1; secondly with two 5′-biotinylated primers specific to the target sequence. The biotinylated PCR product could be visualized as a single band of 141bps in length on agarose gel stained with ethidium bromide. For the confirmation of the primary result, a method of reversed probe hybridization, using a nylon membrane immobilized with the oligonucleotide probe specific to the target sequence, was established. The oligonucleotide probe was given a homopolymer tail with terminal deoxyribonucleotidyl-transferase; the tail was spotted onto a nylon membrane and bound covalently by UV irradiation. Owing to its length, the tail bound to the nylon, leaving the oligonucleotide probe free to hybridize. Hybridization of the amplified target sequence to the immobilized probe was accomplished by a simple colorimetric reaction involving the enzymatic oxidation of a colorless chromogen that yielded a purple color wherever hybridization occurred.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is recognized as one of the two etiological agents of AIDS. The diagnosis of HIV-1 infection is currently based on antibody detection by immunoassay. During the first few weeks after infection, the only evidence of HIV-1 is the presence of the viral genome and, in approximately one-third of patients, the occurrence of the acute retroviral syndrome. Serological diagnosis can be made at 4 to 6 weeks by the detection of the HIV-1 antigen and, days to weeks later, by the sequential occurrence of antibodies to different HIV-1 structural proteins.

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Serological assays are presently used to screen blood and blood products for the presence of HIV-1 antibodies. Generally, serological testing is highly reliable. However, occasional false-positive results are produced by the presence of antibodies that have cross-reacted with host antigens or the antigens of related viruses. Although serological assays identify persons with prior exposure to HIV-1, they do not necessarily indicate current infection. Isolation of HIV-1 from asymptomatic seronegative persons has been reported (Salahuddin *et al*, 1984; Mayer *et al*, 1986). Conventional methods for the direct detection of the virus include co-cultivation with a susceptible cell line and the detection of viral antigens. The procedure of HIV-1 isolation is laborious, time-consuming, costly, and requires the handling of large amounts of infectious material; furthermore, viral p24 antigen in culture supernatant may not be detected for up to 4

weeks, and the direct detection of p24 antigen in patients' sera is often impossible because of either the formation of antigen-antibody complexes or the small amount of antigen present.

Since its inception, the polymerase chain reaction (Saiki *et al*, 1985; Mullis and Faloona, 1987) has been widely used to measure nucleic acid sequences. PCR is a highly sensitive and specific method for the direct detection of HIV-1. The procedure has been particularly useful in determining the infection status of individuals with indeterminate serological results (Jackson *et al*, 1990) and babies born to HIV-infected mothers (Rogers *et al*, 1989). The procedure has assisted in identifying individuals who are infected but who have not seroconverted (Farzadegan *et al*, 1988).

HIV-1 is not one simple virus: it has many subtypes and varies at a high rate even within subtypes. To minimize the likelihood of sequence heterogeniety among viral variants leading to discordant results, we designed our primers to be specific for a region that is well conserved among sequenced HIV-1 subtypes, including HIV-1 Thai E and Thai B subtypes. Our technique was developed and validated in order to produce a new, highly specific and sensitive molecular diagnostic method for the early detection of HIV-1 proviral DNA that could be routinely applied in clinical laboratories. Our novel molecular diagnostic technique for HIV-1 proviral DNA detection in human peripheral blood mononuclear cells (PBMCs) uses nested-PCR featuring two biotinylated primers specific to the ENV- gene of HIV-1 and non-radioactively- reversed probe hybridization.

MATERIALS AND METHODS

Specimen collection and PBMCs purification

2-5 millilters of whole blood were collected in an ACD tube. All samples were handled and processed as if they contained a transmissible infectious agent; the use of sharp objects and glass containers was avoided. Human PBMCs were purified from the whole blood by Ficoll-Hypaque density gradient centrifugation: the layer of PBMCs and platelets appeared as an opaque band between the Ficoll-Hypaque solution and the plasma; erythrocytes and granulocytes were at the bottom of the tube. Proviral DNA of HIV-1 was extracted from human PBMCs following the M&W method by adding a lysis buffer containing guanidine isothioicyanate (GuSCN) in the presence of silica suspension (Attatippaholkun *et al*, 1995). All the extracted DNA was eluted from the silica and kept at -20ºC until use.

DNA amplification by nested PCR

The nested PCR was performed in two-step amplification: firstly with a pair of outer primers (WAHG1 and WAHG2) and secondly with a pair of inner primers (biotinylated WATG1 (bWATG1) and biotinylated WATG2 (bWATG2); one side of each of the primers, WATG1 and WATG2 were labeled with biotin. The nucleotide sequences of all primers and probes are shown in Table 1.

PCR product analysis

The biotinylated PCR product of the HIV-1 conserved region was first analysed by agarose gel electrophoresis and stained with ethidium bromide; this primary result was then confirmed by reversed dot blot hybridization using a membrane immobilized with the WAPG probe. The hybridization was visualized non-radioactively by our locally developed reagents, which were based on a biotin-strepavidin-alkaline phosphatase system. The detection was accomplished by color staining via the enzymatic reaction: a deep purple was given off where hybridization occurred (Attatippaholkun *et al*, 1995).

RESULTS

Human PBMCs, including CD4+ T-lymphocytes and monocytes, are the major target cells for HIV-1 infection in vivo (Fausi, 1988). They could be separated from other cell types

Table 1
Nucleotide sequences of the primers and the probe used.

Primers and probe	Nucleotide sequence (5′- 3′)
Outer primers	
WAHG1	TTCCTTGGGTTCTTGGGAC
WAHG2	AGGTATCTTTCCACAGCCAG
Inner primers	
bWATG1	bGCAGCAGGAAGCACTATGG
bWATG2	bCCAGGACTCTTGCCTGGAGC
Probe	GAGCCTGTGCCTCTTCAGCTA
WAPG	CCACCG

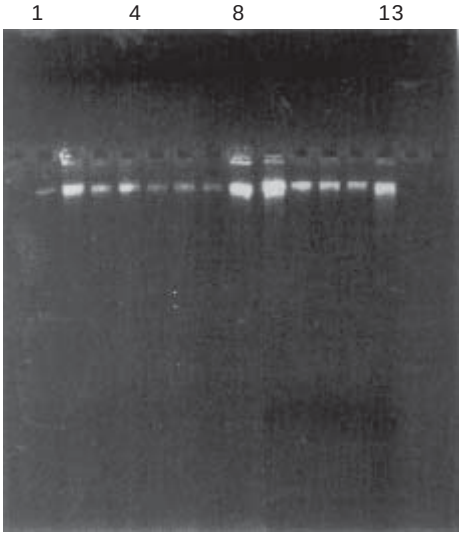

Fig 1–The DNA samples extracted from 13 randomly sampled human PBMCs were analysed by agarose gel electrophoresis stained with ethidium bromide.

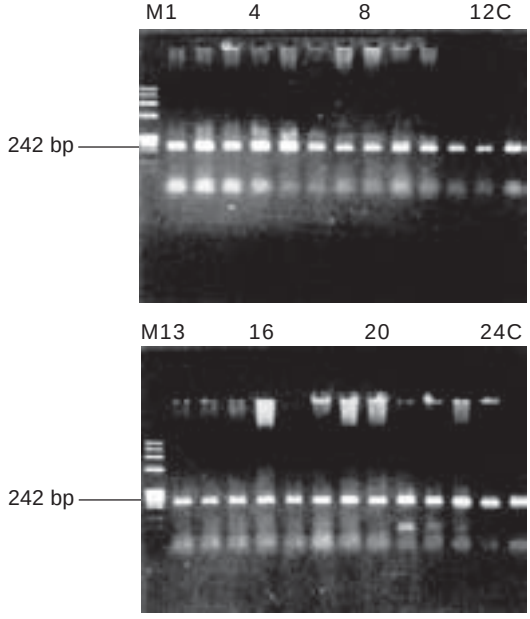

Fig 2–Qualification of human DNA samples extracted from human PBMCs by standard PCR of the 242bp HLADQ alpha locus. Twenty- four random human DNA samples were analysed.

in human peripheral blood by our modified density gradient centrifugation method (Attatippaholkun *et al*, 1995). Nucleic acids were extracted from human PBMCs by the modified method using the lysis buffer containing guanidinium isothiocyanate in the presence of silica particles (Boom *et al*, 1990). The extracted nucleic acids from 13 random samples of human PBMCs showed intact single bands of very large DNA fragments when analysed by agarose gel electrophoresis and ethidium bromide staining (Fig 1). The quality of each extracted DNA sample was determined by amplifying the 242bp DNA fragment of the HLA-DQ alpha gene (Fig 2).

Our novel molecular diagnostic method for HIV-1 proviral DNA detection comprises two main techniques: nested PCR, which amplifies a target sequence on the ENV-gene of HIV-1, and non-radioactively-reversed probe

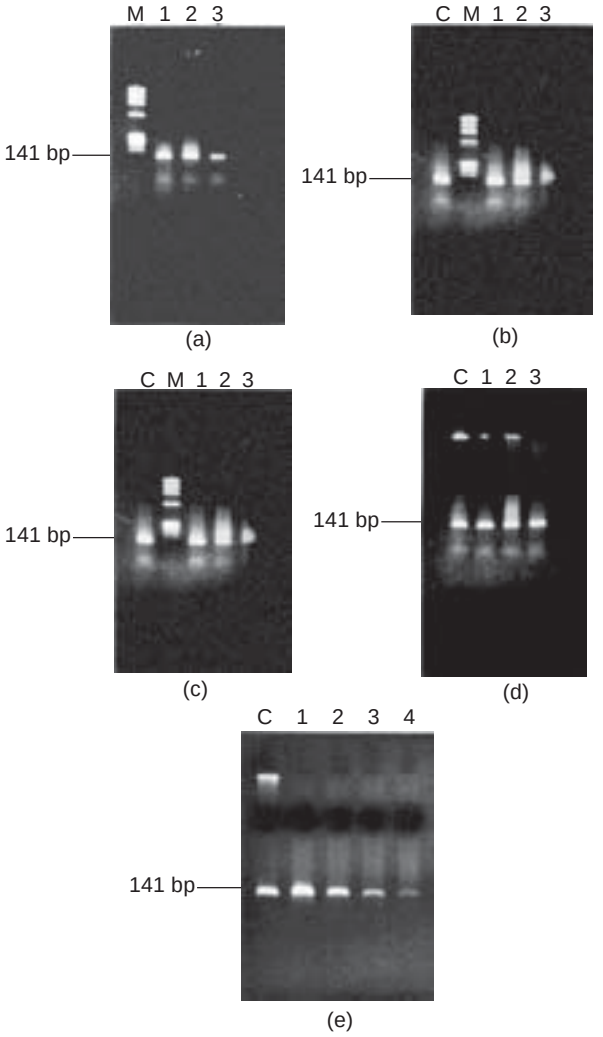

Fig 3–Optimization of nested PCR for HIV-1 ENV target. Pooled DNA of HIV-1 seropositive samples was amplified with the outer primers WAHG1 and WAHG2 followed by the inner primers (bWATG1 and bWATG2). Lane C is standard ΦX-174- Hae III DNA fragments.

(a) Annealing temperatures: 50ºC (lane 1); 55ºC (lane 2); and 60ºC (lane 3).

(b) Magnesium chloride concentrations: 2.5 mM (lane 1); 5.0 mM (lane 2); 7.5 mM (lane 3).

(c) Volumes of DNA template obtained from the first PCR: 2.5 µl (lane 1); 5.0 µl (lane 2); 7.5 µl (lane 3).

(d) Units of *Taq* DNA polymerase: 1.5 units (lane 1); 1.0 unit (lane 2); 0.67 unit (lane 3); 0.34 unit (lane 4).

(e) Cycle numbers of the second PCR: 30 cycles (lane 1); 35 cycles (lane 2); 40 cycles (lane 3); 50 cycles (lane 4).

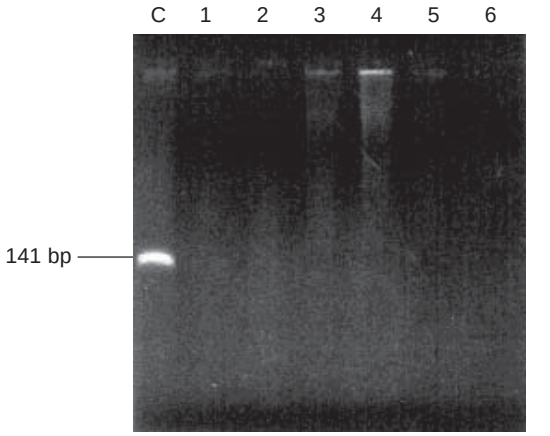

Fig 4–Specificity of the designed primers.
DNA samples from six SLE seronegative patients were amplified by nested PCR with the outer and inner primers. The PCR products were analysed by agarose gel electrophoresis and ethidium bromide staining (lanes 1-6).

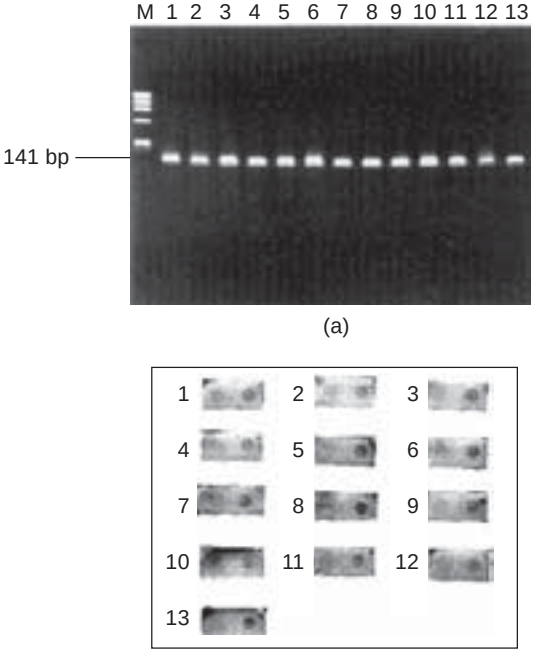

Fig 5–Within-run precision of our molecular method of HIV-1 ENV target amplification. Thirteen repeated amplifications were performed within conditions using the same reagents.

(a) Results of agarose gel electrophoresis and ethidium bromide staining.

(b) Results from reversed dot blot hybridization.

hybridization, for detection of the amplified target sequence. The inserted HIV-1 proviral DNA in each DNA sample was subjected to dual amplification: firstly with two outer primers (Table 1), which covered the target sequence of the ENV-gene of HIV-1; secondly with two 5′-biotinylated primers (Table 1) specific to the target sequence. The biotinylated PCR product could be visualized as a single band of 141bps in length on agarose gel stained with ethidium bromide (Fig 3). The optimum condition of nested PCR using the inner primers to amplify the highest yield of the PCR product was 55ºC of annealing temperature (Fig 3a), 2.5 mM $MgCl_2$ (Fig 3b), 5 µl of outer PCR product as the template (Fig 3c), 1.5 units of *Taq* DNA polymerase (Fig 3d). The nested PCR for amplifying the ENV-gene of HIV-1 with our designed outer and inner primers showed high specificity without cross-reaction for all six HIV-1 seronegative DNA samples of SLE blood patients (Fig 4). The final results, analysed by agarose gel electrophoresis as well as reversed dot blot hybridization, demonstrated the high precision of both within-run (Fig 5) and between-run assays (Fig 6). The detection sensitivity was such that it required only a single copy of HIV-1 proviral DNA: this was sufficient for the 141bp PCR product of HIV-1 to appear on the ethidium bromide stained agarose gel (Fig 7). Final validation with DNA samples of 60 HIV-1 seronegative subjects (Fig 8) and 100 HIV-1 seropositive subjects (Fig 9) was conducted and a statistical analysis allowed the comparison with the results of serological ELISA anti HIV-1 detection (Abbott Diagnostic Kit). The two-by-two comparative analysis of the results of the nested PCR and the serological ELISA indicated 100% specificity, 100% sensitivity, 100% positive predictive value, 100% negative predictive value, and 100% efficiency for the novel PCR method (Table 2).

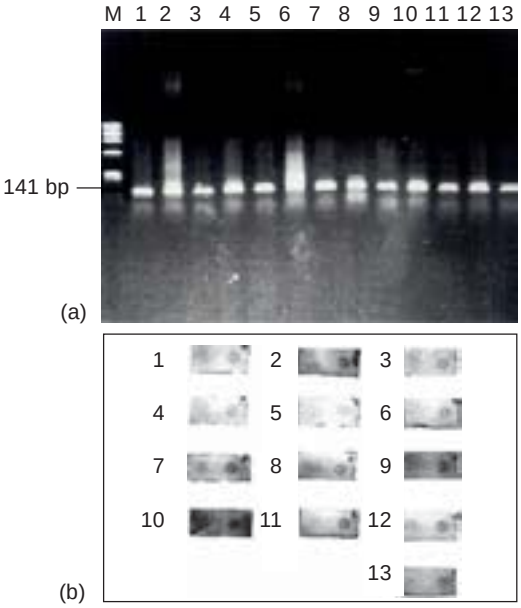

Fig 6–Between- run precision of our molecular method of HIV-1 ENV target amplification . Thirteen repeated amplifications were performed on 13 consecutive days .
(a) Results of agarose gel electrophoresis and ethidium bromide staining.
(b) Results of reversed dot blot hybridization.

Table 2
Comparison by two-by-two statistical analysis of the PCR results and the serological ELISA results of 60 HIV-1 seronegative individuals and 100 HIV-1 seropositive patients.

PCR results	ELISA results		Total
	Positive	Negative	
Positive	100	0	100
Negative	0	60	60
Total	100	60	160

Positive: either a single band (141bps in length) or one purple dot appeared on the hybridized membrane.
Negative: neither a band (of 141bps in length) nor a purple dot appeared on the hybridized membrane.
Specificity of the test = 100%; Sensitivity of the test = 100%; Positive predictive value = 100%;
Negative predictive value = 100%; Efficiency of the test = 100%

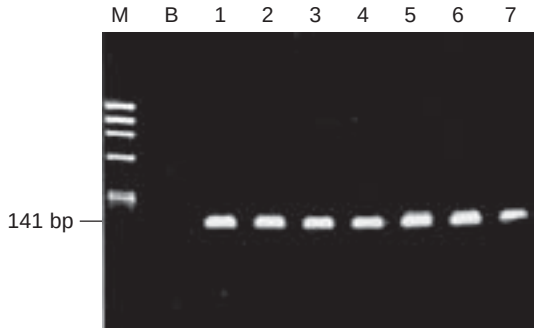

Fig 7–Detection sensitivity of our molecular method of HIV-1 ENV target amplification. Various numbers of 1, 2, 4, 6, 8,10 copies of HIV-1 positive control (Perkin Elmer Cetus) were tested (lanes 1-7). Lane C is standard Φ X-174- Hae III DNA fragments.

DISCUSSION

We have described a novel molecular diagnostic method for HIV-1 proviral DNA. Nested PCR, using two biotinylated primers specific to the conserved ENV region of HIV-1, was augmented by non-radioactively- reversed probe hybridization detection. We applied a simple, rapid, and reliable protocol for the small-scale purification of DNA from human PBMCs. The method is based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidinium isothiocyanate (Ciulla *et al*, 1988) and the nucleic acid-binding properties of silica particles (Volcani, 1981). The extracted DNA of each sample was re-

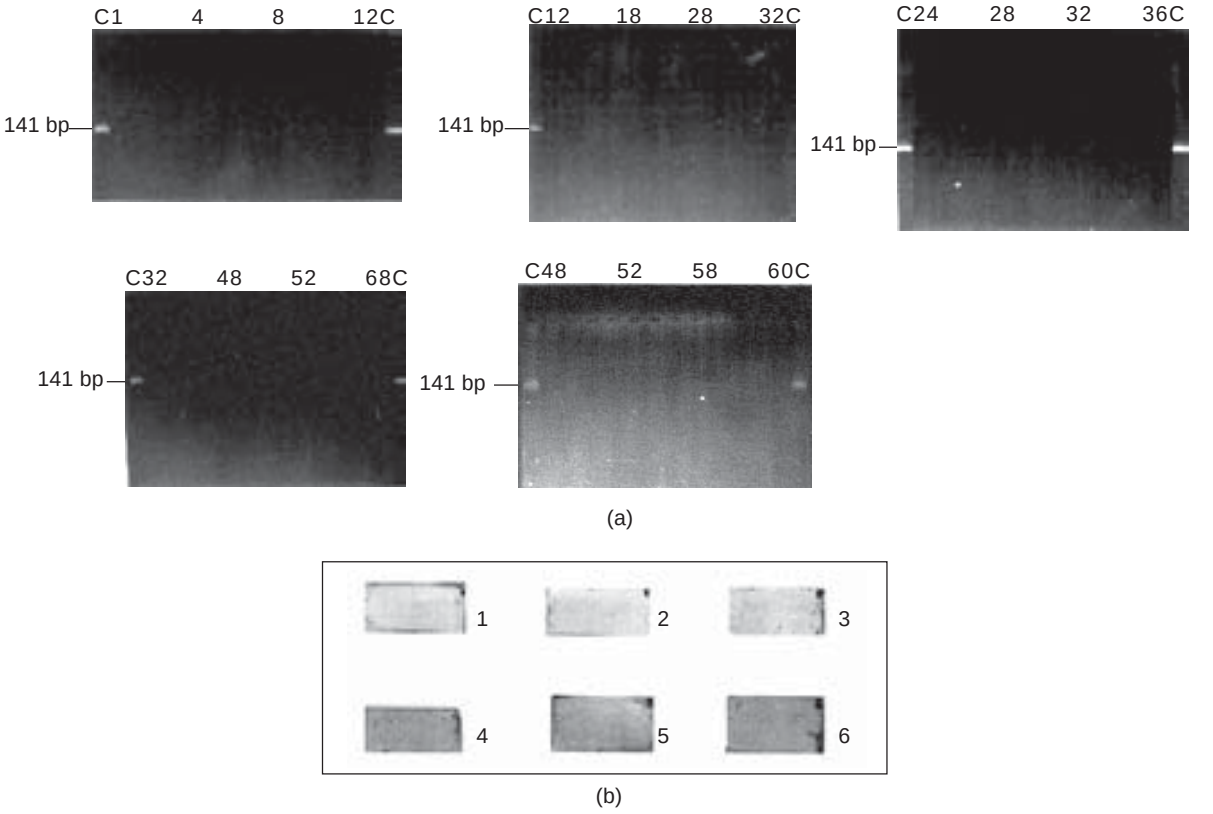

Fig 8–Analysis of 60 HIV-1 seronegative DNA samples amplified by our established molecular method for HIV-1 ENV target amplification.

(a) Results analysed by agarose gel electrophoresis and ethidium bromide staining (lanes 1-60). Lane C is standard Φ X-174-HaeIII DNA fragments.

(b) Results analysed by reversed dot blot hybridization.

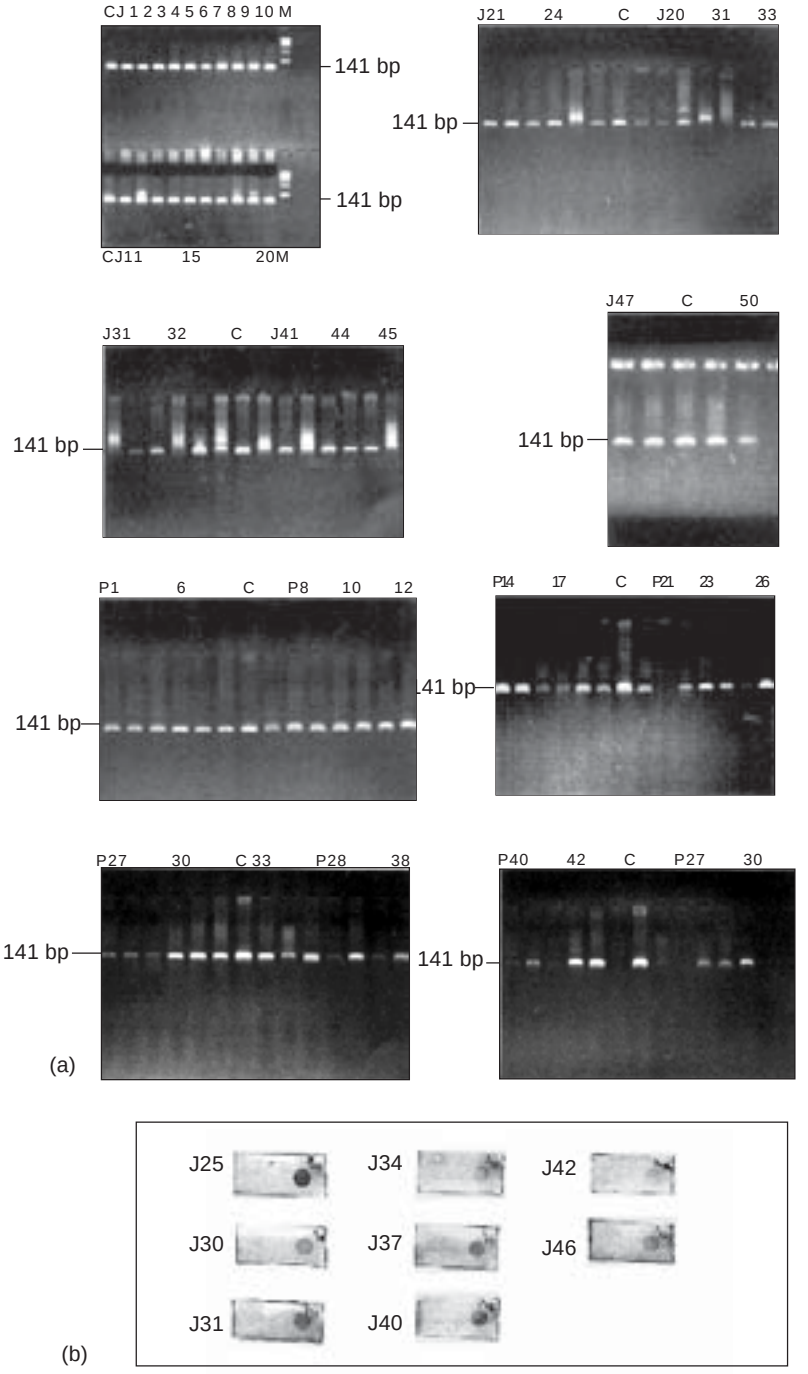

Fig 9–Analysis of 100 HIV-1 seropositive DNA samples amplified by our molecular method of HIV-1 ENV target amplification.

(a) Results analysed by agarose gel electrophoresis and ethidium bromide staining (lanes 1-100). Lane C is standard Φ X-174-HaeIII DNA fragments.

(b) Results analysed by reversed dot blot hybridization.

covered almost undegraded, gave a high yield (Fig 1), and was a good template for PCR (Fig 2). This simple protocol allowed the rapid isolation of DNA from a small quantity of human PBMCs without the need for proteinase K treatment, phenol extraction, or ethanol precipitation; it reduces the number of sample transfers and may help to reduce laboratory cross-contamination.

The low prevalence of HIV-1 proviral sequences in human PBMCs precludes the use of conventional hybridization detection techniques. PCR is an extremely powerful technique for the detection of the minute quantities of nucleic acid. Many modifications to the basic PCR process, some of which have diagnostic relevance, have been made. One such popular modification uses nested sets of PCR primers: nested PCR. We designed two sets of primers and a probe specific for the best-conserved ENV-region of HIV-1 (Table 1). The dual amplification of inserted HIV-1 proviral DNA in each DNA sample was performed by nested PCR in two steps using two outer primers and two 5′-biotinylated primers specific to the target sequence following our optimum condition (Fig 3). Nested amplification confers both advantages and disadvantages. The sensitivity of our nested amplification procedure was extremely high: a single copy of HIV-1 proviral DNA could be detected without the need for hybridization with labeled probes. Re-amplification with the inner primers served to verify the specificity of the first-round product, in much the same

way as a labeled probe would. In addition, the transfer of the reaction products of the first PCR may have diluted inhibitors that might have been present in the sample. Despite its advantages, nested amplification involving the open transfer of the amplification products from tube to tube is not recommended for routine clinical laboratory use.

The biotinylated amplification products of all tested samples were then confirmed by reversed hybridization with the WAPG probe (Table 1) that was homologous to internal sequences, *ie* the amplified region bordered by the two amplification primers (bWATG 1 and bWATG 2). The hybridization step confirmed the presence of the specific amplification products and improved detection sensitivity. Our molecular method for HIV-1 detection showed high within-run and between-run precision (Figs 5, 6); all diagnostic parameters were satisfied (100%), as shown by two- by-two analysis (Figs 8, 9) (Table 2). Our novel molecular method may provide a highly sensitive and specific technique for the direct detection of HIV-1. It has been especially useful for resolving the infection status of individuals with indetermine serological results and babies born to HIV-1 infected mothers; in addition, the method has helped to identify infected individuals who have not seroconverted.

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