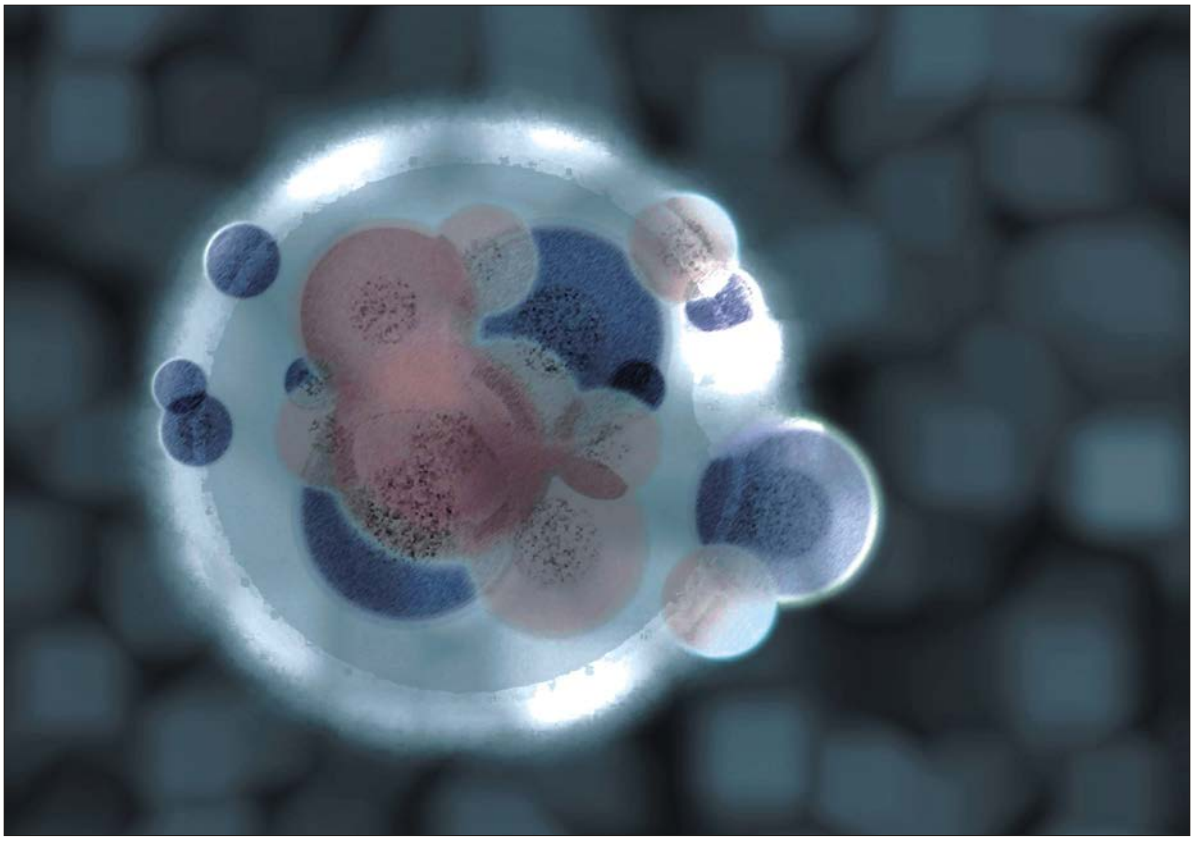

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Evaluation of a New, Highly Sensitive and Specific Primer Set for Reverse-transcriptase PCR Detection of HIV-1 Infected Patients: Comparison with Standard Primers

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Abstract: In the present study, a primer pairs in the HIV-1 POL gene were evaluated by performing RT-nested PCR and PCR and results were compared with universal GAG primers. In this study 80 HIV-1 seropositive patients from the patient cohort of the infectious disease division at Imam Khomeini Hospital in Tehran and 40 HIV-1 seronegative blood donors were evaluated by performing PCR and RT-PCR with the widely used SK primers (gag) and new designed primer set. Determination of PCR and RT-PCR sensitivity (copy number) was performed. The newly designed POL primer pairs was shown to be highly sensitive (100%) and specific (100%) for detection of HIV-1 RNA in Iranian patients. New primer designed detected HIV-1 RNA and DNA in all 80 plasma samples and 69 PBMC samples. SK38/39 could only detect HIV-1 RNA and DNA in 54 plasma samples and 37 PBMC samples, respectively. The optimum PCR profile for a specific primer pairs was defined as that which detected one copy of proviral plasmid DNA and 500 copies of viral RNA. Present results demonstrated that the use of primers designed for highly conserved regions of viral genome along with appropriate optimization of the test, leads to results with higher sensitivity. The high sensitivity of the developed RT-PCR allows its use as a qualitative screening test in patients receiving antiretroviral therapy in Iran.

Key words: New sensitive primer, HIV-1, Reverse-transcriptase PCR

INTRODUCTION

The standard diagnostic techniques for diagnosis of HIV-1 and assessment of blood and blood products are Enzyme immunoassays (EIAs) and Western Blot (WB). However, in both cases, due to a long incubation period before seroconversion, unidentified cases may occur (Newel, 2006; Sethoe *et al*., 1995). During the past several years, Nucleic Acid Testing (NAT) such as PCR has been used as a strong detection system in laboratories and blood bank organizations. PCR is recommended for diagnosis of HIV-1 proviral DNA in infants of infected mothers, monitoring HIV-1 infection prior to appearance of antiviral antibodies and in patients with indeterminate serological results. In addition, PCR can be used in laboratories without appropriate equipments and/or conditions for culturing the virus (Chamberland *et al*., 2001; Yilmaz, 2001). The use of reverse transcription PCR on viral isolates can also help in detection of various types of viruses circulating in a society (Steegen *et al*., 2006; Izopet *et al*., 1996).

One of the unique characteristics of HIV-1 is its high mutation rate that is not distributed equally around the world. This genomic heterogeneity has increased doubts with regard to the use of classical serological assays as the main techniques for diagnosis of HIV-1 (Van Binsbergen, 1996).

A key factor in determining sensitivity and specificity of PCR is selection of appropriate primers for amplification of the target sequence. It has been shown that inappropriate primers yield false negative results, which is misleading for the clinical team responsible for patient care (Engelbrecht, 1996). One of the primer sets suggested by international organizations and is commonly used in many laboratory settings is SK38/39 that is specifically designed to amplify the GAG fragment of HIV-1 (Vandamme *et al*., 1995). However, by widespread use of these primers, it has been shown in several studies that they can not be considered as optimal primers for diagnosis of predominant circulating HIV-1 in various countries, causing false negative results in a group of subjects studied (Owens *et al*., 1996).

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In this study, SK 38/39 is compared to a new set of primers designed by our group in an optimized HIV-1 PCR in a group of confirmed HIV-1 seropositive as well as HIV-1 negative control patients. Both proviral DNA in peripheral blood lymphocytes (PBLs) as well as viral RNA in plasma were evaluated.

MATERIALS AND METHODS

Patients and samples: Eighty HIV-1 seropositive patients from the patient cohort of the infectious disease division at Imam Khomeini Hospital in Tehran, Iran and 40 HIV-1 seronegative blood donors were enrolled in this study. Informed consent was obtained from all participants. All of HIV-1 positive patients and healthy blood donors were living in IRAN. Blood samples from all patients were assayed one more time for the presence of HIV-1 antibody reactivity by two standard EIA kits consisted of HIV-1/HIV-2 recombinant kit (Pasture Institute of Iran) and HIV-1 mix kit (Organon, Italy). Positive samples were then confirmed by WB analysis and those that were positive by all of the above-mentioned methods were considered as positive.

Blood was collected in EDTA-treated tubes. Plasma was stored at -80°C and PBMCs were also separated by Ficoll-hypaque gradient centrifugation. Cell pellets were prepared and stored at -80°C until use.

HIV RNA and proviral DNA extraction: Viral RNA was extracted with the QIAamp ultra sense virus kit (Qiagen, USA) following the manufacturers instructions. Purified RNA was eluted in 40 μL of the elution buffer. Proviral DNA was extracted and purified from each PBMC pellet by DNAeasy tissue kit (Roche, Germany). The DNA content of each sample was determined by spectrophotometric analysis at 260/280 nm.

Primers: The most conserved regions of HIV-1 genomes were specified via sequence multiple alignments by applying clustalx method with different reference sequences obtained from referral HIV data bank from Los Alamos Laboratory (2005). New primers were selected and designed by oligo software in our laboratory. A conserved sequence from the pol region of HIV-1 was selected as a suitable location for this primer pairs constellation. Primers amplifying cellular gene (β2 microglobulin) were included to evaluate the DNA quality of samples for the PCR assay. The primer sequences are summarized in Table 1. The universally recognized SK38/39 primers were also used in parallel experiments.

Amplification system: cDNA synthesis from HIV-1 RNA was catalyzed by the addition of 100 u recombinant AMV reverse transcriptase (Roche, Germany) in 20 μL volume that consisted of 50 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM DTT, 0.2 mM (each) dNTPs, 4 μM of specific reverse primer and 20 unit RNasin (Roche, Germany) in DEPC-treated water. The RT reaction was carried out at 42°C for 60 min and 65°C for 5 min and followed by DNA amplification.

The first-round PCR was carried out in a 25 μL reaction volume containing Taq buffer with 1.25 u Taq Polymerase, 1.5 mM $MgCl_2$, 200 nM (each) dNTPs and 500 nM (each) of the primers, 5 μL of extracted DNA or 2 μL of constructed cDNA. Optimum concentrations of $MgCl_2$ for each primer pairs was found by titration: SK38/39 (2 mM $MgCl_2$) and HIV POL (1.5 mM $MgCl_2$). Cycling conditions were summarized in Table 2. The nested round of PCR was performed under the profile b (Table 2) by applying inner primer sets with 1 μL of the first round PCR products.

The designed pol primers amplified a 183 bp fragment, the SK38/39 primers amplified a 115 bp fragment and β2 microglobulin primers amplified a 764 bp fragment (Chern and Chen, 2000).

A master cycler PCR system (eppendrof, Germany) was used for all PCR reactions. The results were checked by electrophoresis of the nested PCR products on 1.5% agarose gel and visualized with ethidium bromide under UV light. The β2 microglobulin gene was amplified in a single PCR assay for 35 cycles under the same conditions as described earlier.

Table 1: Primer sequences of HIV-1

Region	Primer code	
POL	HIVPOL 4303 outer	Forward: 5'- ATTTTAACCTGCCACCTGTAGTAGC-3'
	HIVPOL 4770 outer	Reverse : 5'- ATACTGCCATTTGTACTGCTG-3'
	HIVPOL 4502 inner	Forward: 5'- AGAAACAGGGCAGGAAACAGC -3'
	HIVPOL 4686 inner	Reverse : 5'- TCTACTACTCCTTGACTTTGGGG-3'
GAG	SK38/39 1461 outer	Forward: 5'-ACCAGGCCAGATGAGAGAACCAAG -3'
	SK38/39 1778 outer	Reverse : 5'- CAATCTGGGTTCGCATTTTGGACC -3'
	SK38/39 1544 inner	Forward: 5'- ATAATCCACCTATCCCAGTAGGAGAAAT -3'
	SK38/39 1658 inner	Reverse : 5'- TTTGGTCCTTGTCTTATGTCCAGAATGC -3'
β2 microglobulin	BU9	Forward: 5'- GGCAGGAGCCAGGGCTG-3'
	BD9	Reverse : 5'- CCCATTCTAAACTACCCTG-3'

Numbering is according to the sequence of HIV-HXB2. These primers were synthesized by (Primm, Italy)

Table 2: Profiles evaluated with different primer pairs

Primers:	SK38/39	SK38/39	HIVPOL	HIVPOL
Profiles:	a	b	a	b
Cycles	35	25	35	25
Denaturing (°C)	94	94	94	94
Time (sec)	30	30	30	30
Annealing (°C)	55	62	58	61
Time (sec)	40	40	40	40
Elongation (°C)	72	72	72	72
Time (sec)	50	50	50	50

Determination of PCR sensitivity (copy number): To standardize our PCR reactions, a plasmid control was used to confirm the number of target HIV-1 DNA molecules present. The sensitivity of amplifying HIV-1 DNA with our primer pairs was determined with this plasmid. This plasmid was constructed by insertion of our PCR amplified fragment on a TA vector (Invitrogen, Germany). This plasmid control was grown in *Escherichia coli* DH5α, the plasmid DNA was isolated using standard methods and the DNA concentration was determined using a spectrophotometer. The dilutions containing 1, 10, 100 and 1000 copies of plasmid were included in each run. Negative reagent control contained sterile water instead of template DNA.

Determination of RT-PCR sensitivity (copy number): To standardize our RT-PCR reactions, a sample of HIV-1 positive plasma quantified by the Amplicor HIV-1 monitor test was used. The stock virus concentration contained 8×10^5 RNA copies p mL^{-1}. Prior to the RNA purification procedure, the HIV-1 stock was diluted in HIV-1 negative plasma to achieve several scalar dilutions (from 5×10^5 to 5 copies p mL^{-1}). The aliquots were kept in -70°C freezer until used.

RESULTS

Eighty seropositive and 40 seronegative individuals were evaluated by performing PCR and RT-PCR with the widely used SK primers (gag) and new designed primer sets. Both primer sets evaluated showed a 100% specificity since no amplification was observed when 40 seronegative individuals were tested. In PCR and RT-PCR assays, significantly lower primer sensitivity was observed with the SK38/39 primers, 46 and 67%, respectively (Table 3 and 4). The pol region specific primers demonstrated a high sensitivity in amplifying HIV-1 RNA from specimens (Fig. 1). The overall sensitivity of the nested primers, using the pol was 100% in RT-PCR and 86% in PCR, which are significantly higher than the overall sensitivity obtained with SK38/39 primers tested here ($p<0.001$) (Fig. 2).

To test the sensitivity of the PCR with pol primers, a 10-fold dilution of plasmid was subjected to the PCR. PCR sensitivity of one copy proviral genome could be detected with pol primer set (Fig. 3).

Table 3: Cumulated performance of the primers on the PCR assay performed on PBMCs

Primers	Tested positives on 80 true positives N (% sensitivity)	Tested negatives on 40 true negatives N (% specificity)	Tested false positive on 40 true negatives	Tested false negative on 80 true positives, N (%)
GAG SK38/39	37 (46%)	40 (100%)	0	43(54%)
POL	69 (86%)	40 (100%)	0	21(31%)

Table 4: Cumulated performance of the primers on the RT-PCR assay performed on plasma

Primers	Tested positives on 80 true positives N (% sensitivity)	Tested negatives on 40 true negatives N (% specificity)	Tested false positive on 40 true negatives	Tested false negative on 80 true positives, N (%)
GAG SK38/39	54 (67%)	40 (100%)	0	26 (33%)
POL	80 (100%)	40 (100%)	0	0

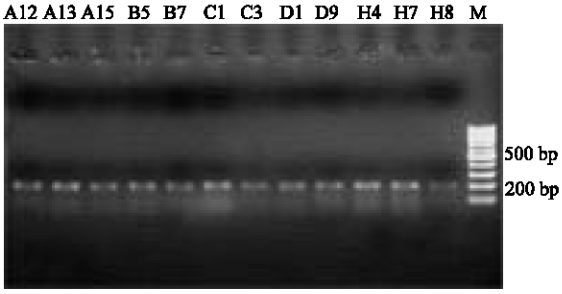

Fig. 1: Composite to show a set of RT-PCR results using HIV POL primer pairs. A 3 μL sample from each patient (as indicated above each lane) was run on 1.5% agarose gels and stained with ethidium bromide. The fragment sizes in base pairs are 183. M = 100 bp ladder of Fermentas

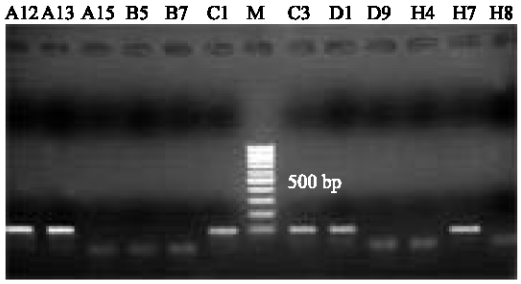

Fig. 2: Composite to show a set of RT-PCR results using SK38/39 primer pairs. A 3 μL sample from each patient (as indicated above each lane) was run on 1.5% agarose gels and stained with ethidium bromide. The fragment sizes in base pairs are 115. M =100 bp ladder of fermentas

To determine the sensitivity of the RT-PCR in plasma, ten fold serial dilutions of one sample, from an untreated HIV-1 infected person was performed. RT-PCR sensitivity

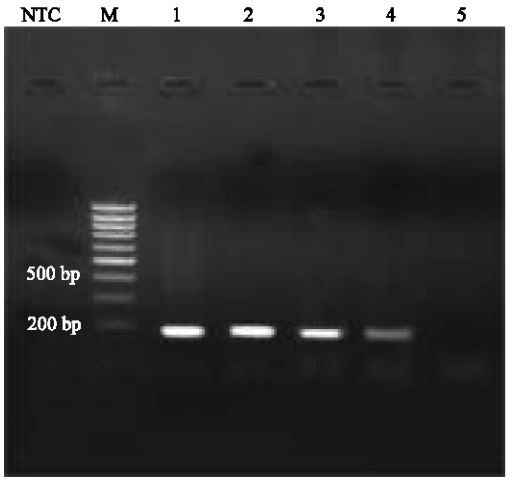

Fig. 3: The sensitivity of amplifying HIV-1 DNA with POL primer pairs. NTC = Non template control. M = 100 bp ladder of fermentas. Lanes 1-5: POL fragment (183 bp) of a 10-fold dilution of the plasmid control; 1000, 100, 10, 1 and 0 copy, respectively

of 500 copies viral RNA could be detected using the pol primer set.

DISCUSSION

Due to length of time for appearance of HIV-1 antibodies, PCR has been increasingly used as an attractive alternative for diagnosis of the virus as well as monitoring therapeutic approaches in HIV-1 positive individuals. On the other hand, the high mutation rate of HIV-1 and presence of various genotypes and subtypes in various regions of the world has to be considered in designing of any nucleic acid testing (McDonald and Burnett, 2005 and Steegen, 2006). Previous unpublished results of our laboratory showed that PCR techniques using SK38/39 primer set were unable to amplify a limited number of Iranian HIV-1 isolates.

The goal of this study was to develop and evaluate a PCR system for detection of viral DNA and RNA in HIV infected patients using pol region specific primers. The results demonstrated positive RT-PCR results for viral RNA in 80 seropositive specimens. While only 54 specimens had positive results for viral proviral DNA. None of the control seronegative specimens were positive by PCR, indicating a specificity of 100% for the new test.

Nested PCR protocols potentially increase carry-over contamination problems in the laboratory, which can contribute to an increased false positive rate. This was not the case in our laboratory because extensive precautions were taken. Besides the physical separation of pre-nesting and post-PCR activities, we used filter tips, made stock solutions and used disposables. Especially in the working where the nesting procedure was performed, contamination of the surfaces was eliminated by working area using UV irradiation overnight and washing bench surfaces every week with bleach.

Many scientists have proven that in order to have an appropriate clinical test for diagnosis of HIV-1 infection, several primers should be used in parallel (Vandamme, 1995 and Owens, 1996). Present results demonstrated that the use of primers designed for highly conserved regions of viral genome along with appropriate optimization of the test, leads to results with higher sensitivity. Using these primers, it was demonstrated that one copy of a plasmid containing proviral DNA could be detected by electrophoresis. As expected, the dilution of 1:10 copies of this plasmid was negative and there was no false positive result. The genetic variability between different HIV-1 viruses is high and may affect the detection of virus by PCR and RT-PCR. The low reactivity rate with the SK primers on Iranian strains is probably due to sequence diversity. Mismatches in the 5' terminal region of the sequence to be amplified, can significantly reduce the yield of PCR products and cause false negative results. High numbers of mismatches could also increase the rate of non-specific products (McClure *et al.*, 2000). When sequence of SK38/39 of HIV gag genes of various isolates as judged from the most recent Los Alamos consensus HIV-1 sequence were assessed, 4-5 mismatches were observed in the 5' terminal region. We did not detect any mismatches in the 3' terminal region. Finally, the present study illustrates the urgent need to generate more sequence data on HIV-1 isolates from Iranian origin and to make data more rapidly available to Iranian sequence data bank, which will enable design of more sensitive primer sets.

Using HAART in limited numbers of clinical centers for managing HIV and AIDS in Iran has increased the demands for accurate monitoring of patients undergoing retroviral therapies. Using accurate and correct monitoring techniques could control an HIV-1 epidemic in developing countries. Development and use of laboratory techniques for monitoring of antiviral drug side effects and their success and failure is necessary.

In this report, a diagnosis technique for HIV-1 RNA with high sensitivity is described. On the other hand, the high sensitivity of the developed RT-PCR with the detection limit of less than 500 copies per mL, allows its use as a qualitative screening test in patients receiving antiretroviral therapy in resource limited countries.

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