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ORIGINAL ARTICLE

Design and Development of a Multiplex Real-Time PCR Assay for Detection of HIV-1 and HCV Using Molecular Beacons

Mahdi Paryan · Samira Mohammadi-Yeganeh · Siamak Mirab Samiee · Houri Rezvan

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Abstract At least 10 million individuals worldwide are co-infected with immunodeficiency virus type 1 (HIV-1) and hepatitis C virus (HCV). These two viruses are transmitted most primarily by exposure to infected blood or blood products. Various nucleic acid assays have been developed for diagnostics and therapeutic monitoring of infections. In the present study, a multiplex real-time PCR assay for simultaneous detection of HCV and HIV-1 using molecular beacons were designed and validated. A well-conserved region in the HIV-1 *pol* gene and 5′NCR of HCV genome were used for primers and molecular beacon design. The analysis of scalar concentrations of the samples indicated that this multiplex procedure detects at least 1,000 copies/ml of HIV-1 and 100 copies/ml of HCV with linear reference curve ($R^2 > 0.94$). The results demonstrate that a specificity of 100 % and sensitivity of 96 % can be achieved. The analytical sensitivity study with BLAST software demonstrated that the primers do not attach to any other sequences except for that of HIV-1 or HCV. The primers and molecular beacon probes only detected HIV-1 and all major variants of HCV. This assay may represent an alternative rapid and relatively inexpensive screening method for detection of HIV-1/HCV co-infection especially in blood screening.

Keywords HIV-1 · HCV · Multiplex real-time PCR · Molecular beacon

M. Paryan · S. Mohammadi-Yeganeh
Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

M. Paryan (✉) · S. Mohammadi-Yeganeh
Department of Molecular Biology and Genetic Engineering, Stem Cell Technology Research Center, No. 9, Saadat abad ave, P.O. Box 19977-75555, Tehran, Iran
e-mail: mparyan@gmail.com

M. Paryan · S. Mohammadi-Yeganeh · S. Mirab Samiee · H. Rezvan
Day General Hospital Laboratory, Tehran, Iran

S. Mirab Samiee
Food and Drug Laboratory Research Center, Ministry of Health and Medical Education, Tehran, Iran

Introduction

In the past decade, molecular techniques and particularly PCR as a rapid and sensitive method has revolutionalized detection of a variety of infectious viruses [1, 2]. Typically NAATs[1] require a significant investment in equipment, training and infrastructures. In this respect, World Health Organization (WHO) has recommended that diagnostic devices should be "ASSURED": affordable, sensitive, specific, user-friendly, rapid and robust, equipment free, and deliverable to end users, especially in case of developing countries. Application of these molecular techniques as diagnostic tools has become increasingly important [3–7].

Multiplex assays in which two or more viruses with common transmission routes can be detected are also becoming important in particular areas, for example triplex assays in which nucleic acid of blood borne viruses HBV, HCV and HIV are detected in one assay have proven to be most useful in cutting time of screening tests and shortening of window period of these viruses, thereby improving blood safety [8–12]. Similarly, multiplex assays in which two viruses of similar transmission route that can affect treatment of co-infected patients is also becoming a

[1] Nucleic acid amplification techniques.

useful tool [13]. For example Hepatitis C virus (HCV) infection has emerged as an important co-infection in the clinical and immunological development and treatment of HIV infection. Co-infected individuals may have an altered response to anti-retroviral treatment (ART) and are at increased risk of ART-related hepatotoxicity [14–17].

The present study was therefore designed for development of a multiplex real-time PCR assay for simultaneous detection of HCV and HIV using molecular beacons. Molecular beacons, first demonstrated by Tyagi and Kramer, are new optical tools that can be used to detect the presence of a specific oligonucleotide sequence in a mixture of targets [18]. As shown in Fig. 1, these DNA oligonucleotides contain a 5′ fluorophore, a 3′ quenching group, and 4–6 complementary bases on the 3′ and 5′ "stem" ends. When hybridized with a complementary sequence, the hairpin structure becomes linearized distancing the fluorophore and quencher resulting in fluorescence signal (Fig. 1). The hybridization signal depends on their molecular structure. Therefore, for detecting each pathogen, a separate molecular beacon with appropriate conformation in unhybridized, linearized and hybridized conformations should be designed [18–21]. This probe may provide an alternative method for detection of HIV-1 and HCV viruses with a simpler approach.

Materials and Methods

Probe and Primer Design

We designed and validated two sets of primers and molecular beacon probes on the most conserved region of *pol* gene in HIV-1 virus and 5′ non coding region (5′NCR) in HCV virus by aligning sequences in nucleotide database of NCBI (www.ncbi.nlm.nih.gov/nucleotide) containing full genomes of HIV-1 and HCV. All the alignments were carried out using Mega4 software. *Pol* gene in HIV-1 genome and 5′NCR of HCV genome were selected due to better sequence conservation in comparison with other genes.

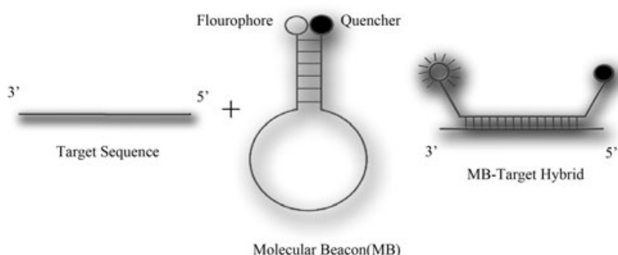

Fig. 1 Schematic mechanism of molecular beacon

The design of primers and probes was carried out using Beacon Designer 7 Software (Palo Alto, CA). The primer set chosen for HIV-1 virus amplified a 179 bp fragment in the HIV-1 *pol* gene. The primer sequences were 5′-GTACAGTGCAGGGGAAAG-3′ (forward) and 5′-CCAGAGTAGTTTTGCTGGTC-3′ (reverse). HCV primers were 5′-CATGGCGTTAGTAYGAGTG-3′ (forward) and 5′-CTATCAGGCAGTACCACAAG-3′ (reverse). The primer set selected for HCV virus amplified a 241-base pair fragment in the 5′NCR of HCV. To allow distinction between the fluorescence signal of HIV-1 and HCV, sequence-specific probes with two different reporter dyes were used.

The specific probe for HIV-1 was 5′-CCCGTGGTTTATTACAGGG ACAGCAGAACGGG-3′, labeled with the fluorescent reporter dye Tet (tetrachloro-6-carboxyfluorescein) at the 5′ end and the quencher dye BHQ-1 (Black Hole Quencher-1) at the 3′ end. The probe specific for the HCV was 5′-CCGATCAGCCATAGTGGTCTGCGGAAGAT CGG-3′ labeled with the fluorescent reporter dye FAM (6-carboxyfluorescein) at the 5′ end and the quencher dye BHQ-1 at the 3′ end. Stem sequences are indicated with underline.

Sample Preparation

Whole blood samples were collected from 70 adult individuals (All the received clinical specimens were untagged and re-labeled by numbers as sample identifier) including 30 HIV-1/HCV co-infected patients (group A), 10 HIV-1 (group B) and 10 HCV (group C) infected patients. These samples were previously screened by serological experiments and quantified by Artus HCV RG RT-PCR and Artus HIV-1 RG RT-PCR Kits (Qiagen, Hilden, Germany). In order to assess the specificity of the assay, 20 HIV-1 and HCV seronegative patients (group D) were tested. Twenty samples were manually prepared by spiking normal plasma with HIV-1 and HCV (group E).

Blood samples were collected in EDTA-containing tubes and were centrifuged at 2,500 rpm for 20 min and stored at −80 °C. Nucleic acids were extracted by QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany), eluted using 50 μl nuclease-free water, and stored at −80 °C until use.

Reverse Transcription Reaction

Following RNA extraction, the samples were reverse transcribed using expand reverse transcriptase (Roche Diagnostics GmbH Mannheim, Germany). 5 μl extracted RNA was added to 2 μl HCVR and 2 μl HIV-1R (2 mM), 2 μl dNTP (10 mM) and 1 μl distilled water and incubated at 65 °C for 10 min. The tubes were held in ice and 4 μl 5× RT-Buffer (Roche), 2 μl DTT (10 mM), 0.5 μl RNase

inhibitor (20 U), 0.5 µl distilled water and 1 µl expand reverse transcriptase (50 U) was added. The cDNA synthesis was carried out at 43 °C for 60 min and then at 95 °C for 2 min for inactivation of the enzyme. The cDNA was stored at −20 °C until use.

HIV-1 and HCV Standards

Limit of detection of this method was calculated using, PCR products cloned in T/A cloning vector. In vitro transcription was performed in a final volume of 50 µl using the recombinant plasmid and T7 RNA Polymerase (Fermentas, Germany); in presence of 2 mM NTP mix; 10 µl transcription buffer; 50 U RNase inhibitor and 30 U T7 RNA Polymxerase in a 50 µl reaction. After transcription 5 U (2 U per 1 µg of DNA used) of RNAse free DNAse (Fermentas, Germany) was added for 30 min at 37 °C to degrade the template DNA. The transcribed RNA was purified by Trizol extraction. The integrity of RNA was checked on a 2 % formaldehyde agarose gel by electrophoresis and quantitated by measuring the optical density (OD) at 260 nm. The diluted RNA was tested for DNA contamination by PCR. Before extraction, the in vitro transcribed RNAs were added to HIV-1 and HCV negative plasma samples and mixed to achieve HIV-1 or HCV scalar dilutions of 10^2–10^6 copies/ml.

Multiplex Real-Time RT-PCR Using Molecular Beacon

HIV-1/HCV multiplex real-time PCR assay was performed in 25 µl PCR mixture volume composed of 10 µl 2× Quantifast Multiplex Probe PCR Master Mix (Qiagen), 0.4 µM HIV-1 and HCV oligonucleotide primers, 0.2 µM HIV-1 and HCV probes and 5 µl cDNA. Amplification was performed in the following conditions: activation step at 95 °C for 5 min and 45 cycles of three thermal amplification steps : 94 °C for 10 s, 56 °C for 30 s and 72 °C for 30 s. Single fluorescence detection was performed in each cycle at 56 °C to reveal the positive samples. Amplification, data acquisition and analysis were performed on Rotor-Gene 3000 (Corbett Research, Australia) using Rotor-Gene 6.1 software. All samples were run in duplicate.

Results

Probe and Primer Design

Genotype independent detection of viruses such as HIV-1 and HCV relies on finding the most conserved region of viruses by using a complete databank of aligned sequences for genome of both viruses. Comparison between HCV sequences of several genotypes and also between HIV-1 sequences of three genotypes and several subtypes are represented in (Figs. 2, 3), respectively. Two sets of primers and molecular beacon probes were designed and validated.

Multiplex Real-Time RT-PCR Optimization

A Multiplex real-time PCR was evaluated for simultaneous detection of HIV-1 and HCV viruses in plasma samples. HIV-1 and HCV specific oligonucleotides pairs which were able to detect all significant HIV-1 subtypes and HCV genotypes were selected (Fig. 4). HIV-1 primers and probe specific oligonucleotides capable of recognizing a well-conserved region in the HIV-1 *pol* gene amplifying a 179 bp fragment were used. This oligonucleotide pair is known to be effective in the amplification of major HIV-1 subtypes and has been successfully employed for real-time RT-PCR technique. On the other hand, the HCV specific oligonucleotides were designed to amplify a 241 bp fragment within the 5′NCR of HCV genome. These primers and probes comply with the well-conserved sequences among different HCVs. The oligonucleotide pairs selected were checked by BLAST analysis. These sequences did not show any relevant homology with other viral or human sequences while revealing all representative HIV-1 subtypes and HCV genotypes.

Sensitivity and Specificity of the Assay

The assay was evaluated on transcribed RNA dilutions of HIV-1 or/and HCV. Invitro-transcribed RNA scalar dilutions were added to uninfected plasma samples (Table 1). After extraction and real-time RT-PCR, the assay sensitivity limit, determined as the dilution in which 100 % of samples were revealed by our technique, was 1,000 copies/ml for HIV-1 and 100 copies/ml for HCV. The Ct values showed linear reference curves for both viruses ($R^2 > 0.94$). The analytical sensitivity of Multiplex PCR could be affected by high concentration of each target sequence, a serial dilution of each viral standard were tested against a high copy number/ml (10^6) standard of the other one. As shown in the (Table 2) high viral load of one target sequence does not influence the analytical sensitivity of the other virus with low concentration. In order to determine the specificity, first a BLAST was carried out using NCBI Nucleotide BLAST software (http://blast.ncbi.nlm.nih.gov/). It was demonstrated that the primers do not attach to any other sequences except for that of HIV-1 or HCV. Then, some blood transmitted viruses such as HBV, HTLV-1, TTV, B19, HSV-1, HSV-2, HHV-6, HHV-8, HCMV and EBV were evaluated

Fig. 2 Conserved genomic sequences were applied for HCV primers and probe design

Geno type	Accession Number	Forward Primer Region	Reverse Primer Region	Probe Region
1a	AF290978	CATGGCGTTAGTAYGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
1a	AF009606	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
1b	AB016785	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
1b	D89815	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
1c	E08443	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
1c	D14853	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
2a	AF169003	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
2a	AF169004	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
2b	AB030907	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
2b	AF238486	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
2c	D50409	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
2k	AB031663	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
3a	AF046866	CATGGCGTTAGTACGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
3a	D28917	CATGGCGTTAGTACGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
3b	E10840	CATGGCGTTAGTACGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
3b	D49374	CATGGCGTTAGTACGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
3k	D63821	CATGGCGTTAGTACGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
4a	Y11604	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCGCAAG	AGCCATAGTGGTCTTCGGAA
5a	Y13184	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
6a	AY859526	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
6a	Y12083	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
6b	D84262	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTAGTCTGCGGAA
6d	D84263	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
6g	D63822	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
6h	D84265	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTGGTCTGCGGAA
6k	D84264	CATGGCGTTAGTATGAGTG	CTATCAGGCAGTACCACAAG	AGCCATAGTAGTCTGCGGAA

by the designed primers and probes. Only HIV-1 and HCV (and no other irrelevant genomes) were detected.

Analysis of Patients' Plasma by Multiplex Real-Time RT-PCR Using Molecular Beacon

These HIV-1/HCV positive samples were previously screened by serological experiments and quantified by Artus HCV RG RT-PCR and Artus HIV-1 RG RT-PCR Kits (Qiagen, Hilden, Germany). Samples quantified between 75 IU/ml and 6,700 IU/ml were chosen. Multiplex real-time RT-PCR using molecular beacon on co-infected patients, we used group A and E. 48 out of 50 positive samples were positive for both viruses and two samples were positive only for HCV. Storage conditions of the extracted sample and repeated freeze and thawing may have caused the negative results. We did not have access to the patients for resampling. Moreover, all samples in group B (HIV-1 positive) and C (HCV positive) with viral load over 1,000 copies/ml and 100 copies/ml, respectively were detected. Finally, analysis of group D (seronegative plasma samples) did not show any positive signal, confirming the specificity of the assay.

Discussion

The paper describes an approach for simultaneous detection of HIV-1 and HCV in plasma samples. The method uses a molecular beacon to run multiplex PCR in real-time. Application of advanced immunological method is limited during the seronegative window period as well as in cases of delitescence and non-immunogenic forms of HCV and HIV-1 infections. The most advanced techniques for detection of transfusion transmitted viruses such as HIV-1 and HCV are base on real-time detection, as a NAT

Fig. 3 Conserved genomic sequences were applied for HIV-1 primers and probe design

Accession Number	Forward Primer Region	Reverse Primer Region	Probe Region
AM000053	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AY521629	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AY521631	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AY521630	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
DQ396400	ATACAGTGCAGGGGAAAG	CCAGAGTAGTCTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
DQ676872	GTACAGTGCAGGGGAAAG	CCAGAGAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
DQ207944	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGAGACAGCAGA
AF457052	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF457053	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTTCAGGGACAGCAGA
AF457055	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF457066	GTACAGTGCAGGGGAAAG	CCAAAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF457067	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF457068	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTCTATTACAGGGACAGCAGA
AF457069	GTACAGTGCAGGGGAAAG	CCAGAGCAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AM000055	GTACAGTGCAGGGGAAAG	CCAGAGTAGCTTTGCAGGTC	GGTTTATTACAGGGACAGCAGA
AF457070	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF457077	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTTCAGGGACAGCAGA
AF457079	GTACAGTGCAGGGGAAAG	CCAGAGCAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF457080	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF457081	GTACAGTGCAGGGGAAAG	CCAAAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AY322185	GTACAGTGCAGGAGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AY322190	GTACAGTGCAGGGGAGAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF004885	GTACAGTGCAGGGGAAAG	CCAGAATAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AY322185	GTACAGTGCAGGAGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF069670	GTACAGTGCAGGAGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AY322193	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
EU110097	GTACAGTGCAGGGGAAAG	CCAAAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
EU110092	GTACAGTGCAGGGGAAAG	CCAAAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
EU110094	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
EU110085	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
EF545108	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGAGACAGCAGA
AY500393	GTACAGTCCAGGGGAAGA	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGAGACAGCAGA
AB253421	GTACACTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AB287376	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AB287379	GTACAGTGCAGGGGAAAG	CCAGAGTAGCTTTGCTGGTC	GGTTTATTACAGAGACAGCAGA
AY713406	GTACAGTGCAGGGGAAAG	CCAAAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF069671	ATACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF069669	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF069673	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF107771	GTACAGTGCAGGGGAAAG	CCAAAGCAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AY253305	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AY253306	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AY253314	GTACAGTGCAGGAGAAAG	CCAGAGTAGTCTTGCTGGTC	GGTTTATTACAGAGACAGCAGA
AF361872	GTACAGTGCAGGAGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA
AF413987	GTACAGTGCAGGGGAAAG	CCAGAGTAGTTTTGCTGGTC	GGTTTATTACAGGGACAGCAGA

method, which essentially shorten the duration of analysis, increase the sensitivity and specificity, and provide simultaneous quantification as well. In contrast to a single-target format, the multiplex format of the HIV-1/HCV assay developed in the present study has advantages with respect to both time and cost [21, 22]. The time needed to complete initial screening is decreased due to the ability to amplify multiple targets simultaneously in one tube. Multiplex assays using molecular beacon probes save cost of testing not only by reducing consumption of reagents but also by enabling its use in systems such as NASBA[2] [1, 22], strand displacement amplification, and rolling-circle amplification which avoids necessity of expensive equipment such as thermal cycler [21].

The use of nonspecific DNA binding probes such as SYBR Green I is limited to detection of total amount of all amplification products synthesized in on reaction tube and melting curve analysis is required for multiplex detection [13]. So amplification detection base on specific probes is desired. Although several commercial techniques such as COBAS TaqMan HIV-1 or HCV (Roche Molecular Systems, Inc.) are FDA approved real-time PCR but they are monoplex and more expensive than in house designed multiplex real-time PCR. In this study, the use of molecular

[2] nucleic acid sequence-based amplification.

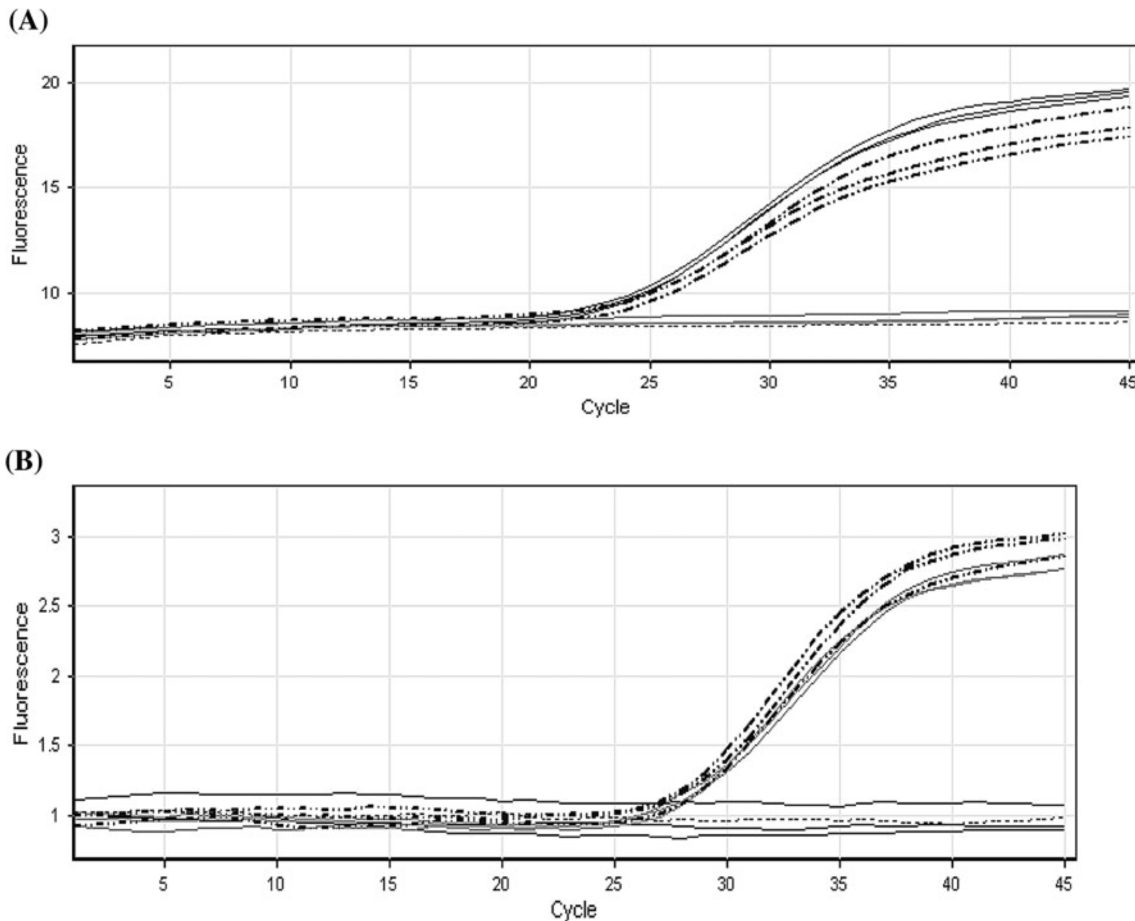

Fig. 4 Real-time detection of two different viral genomes in a multiplex format. Each reaction contained two sets of PCR primers specific for unique HIV-1 and HCV nucleotide sequences and two molecular beacons, each specific for one of the two amplicons and labeled with a differently colored fluorophore. HCV is plotted in *solid black*, multiplex format plotted in *dotted–dashed black*, HIV is plotted in *solid gary* and Non template control is plotted in *dotted gary line*. **a** FAM/Syber window for HCV detection **b** JOE/Tet window for HIV-1 detection

Table 1 Sensitivity of multiplex real-time RT-PCR

[a]HIV RNA copies/ml	Number detected	Percent detected
10^6	8/8	100
10^5	8/8	100
10^4	8/8	100
10^3	8/8	100
10^2	5/8	62.2
[b]HCV RNA copies/ml	Number detected	Percent detected
10^6	8/8	100
10^5	8/8	100
10^4	8/8	100
10^3	8/8	100
10^2	8/8	100

Four experiments in duplicate were performed. The replicates were considered positive when HIV-1 or HCV were revealed

[a] Sensitivity of assay for HIV-1 virus

[b] Sensitivity of assay for HCV virus

beacons was chosen as the probe of choice due to their advantages such as quenching efficiency over other probes such as Taqman and Hyprobe. In molecular beacon, the close distance between the dye and the quencher decreases the likelihood of their detachment, leading to lower background fluorescence resulting in enhanced detection and higher sensitivity [21]. Molecular beacons are especially suitable for identifying point mutations since they can distinguish targets with only a single nucleotide difference in equivalent length [18, 20, 21]. Molecular beacons have also been used for quantification of pathogens, virus replication, and gender detection in embryos. With the use of molecular beacons in real-time RT-PCR, no post-amplification handling is required and the chance of contamination of samples with amplicons which leads to false-positive results decrease. The hairpin structure of molecular beacons in comparison to Taqman made it more specific than corresponding conventional linear probes [19].

Table 2 Multiplex real time RT-PCR sensitivity evaluated in different ratios of standard plasma dilution conditions

HCV copies/ml	HCV positive replicates	HIV-1 copies/ml	HIV-1 positive replicates
10^6	4/4	10^6	4/4
10^6	4/4	10^5	4/4
10^6	4/4	10^4	4/4
10^6	4/4	10^3	4/4
10^6	4/4	10^2	1/4
10^6	4/4	10^6	4/4
10^5	4/4	10^6	4/4
10^4	4/4	10^6	4/4
10^3	4/4	10^6	4/4
10^2	4/4	10^6	4/4

Two experiments in duplicate were performed. The replicates were considered positive when both HIV-1 and HCV were revealed

Specificity and sensitivity of 100 % for oligonucleotide microarray for real-time PCR detection of HIV-1, HBV and HCV was reported by Khodakov et al. [23]. The results obtained using the developed assay in the present study demonstrates that a specificity of 100 % and sensitivity of 96 % can be achieved. This sensitivity was due to two negative clinical samples in 50 samples. Actually, we could not determine the reason of this negative result as we did not have access to the patient for resampling.

The detection limit for HCV-RNA was demonstrated as 100 copies/ml and for HIV-1 it is 1,000 copies/ml that is better than results obtained by De Crignis et al. That were used SYBR Green I [8]. Our data was obtained using mono-infected as well as co-infected samples. These results are in concomitance with previously reported results [9].

All subtypes detection of any virus is reliant on finding the most conserved region of the viral genome by the use of a complete databank of aligned sequences of the virus genomes. The design of primers set was carried out to cover every known polymorphism in the genomes of viral subtypes. Furthermore, all the primer and probe sets were tested against a panel of anonymous samples previously found positive for viruses such as HBV, HTLV-1, TTV, B19, HSV-1, HSV-2, HHV-6, HHV-8, HCMV and EBV. Using these primers set and probes, no other genomes were detected.

It can therefore be concluded that application of molecular beacons to enable multiple target detection can improve reliability, speed, cost, and ease of diagnostic clinical assays [1]. The assay introduced here is demonstrated to be suitable for concomitant detection of HCV and HIV-1 in plasma or serum samples for clinical diagnosis with a further possible use in blood screening.

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Conflict of interest The authors declare that they have no conflict of interest.

References

1. Lau LT, Feng XY, Lam TY, Hui HK, Yu AC (2010) Development of multiplex nucleic acid sequence-based amplification for detection of human respiratory tract viruses. J Virol Methods 168(1–2):251–254
2. Templeton KE, Scheltinga SA, Beersma MF, Kroes AC, Claas EC (2004) Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza a and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. J Clin Microbiol 42(4):1564–1569
3. LaBarre P, Hawkins KR, Gerlach J, Wilmoth J, Beddoe A, Singleton J, Boyle D, Weigl B (2011) A simple, inexpensive device for nucleic acid amplification without electricity-toward instrument-free molecular diagnostics in low-resource settings. PLoS One 6(5):e19738. doi:10.1371/journal.pone.0019738
4. Mabey D, Peeling RW, Ustianowski A, Perkins MD (2004) Diagnostics for the developing world. Nat Rev Microbiol 2(3):231–240. doi:10.1038/nrmicro841
5. Ngom B, Guo Y, Wang X, Bi D (2010) Development and application of lateral flow test strip technology for detection of infectious agents and chemical contaminants: a review. Anal Bioanal Chem 397(3):1113–1135. doi:10.1007/s00216-010-3661-4
6. Peeling RW, Mabey D, Herring A, Hook EW 3rd (2006) Why do we need quality-assured diagnostic tests for sexually transmitted infections? Nat Rev Microbiol 4(12):909–921
7. Tucker JD, Bu J, Brown LB, Yin YP, Chen XS, Cohen MS (2010) Accelerating worldwide syphilis screening through rapid testing: a systematic review. Lancet Infect Dis 10(6):381–386
8. De Crignis E, Re MC, Cimatti L, Zecchi L, Gibellini D (2010) HIV-1 and HCV detection in dried blood spots by SYBR Green multiplex real-time RT-PCR. J Virol Methods 165(1):51–56
9. Giachetti C, Linnen JM, Kolk DP, Dockter J, Gillotte-Taylor K, Park M, Ho-Sing-Loy M, McCormick MK, Mimms LT, McDonough SH (2002) Highly sensitive multiplex assay for detection of human immunodeficiency virus type 1 and hepatitis C virus RNA. J Clin Microbiol 40(7):2408–2419
10. Jarvis L, Becker J, Tender A, Cleland A, Queiros L, Aquiar A, Azevedo J, Aprili G, Bressan F, Torres P, Nieto S, Ursitti A, Montoro J, Vila E, Ramada C, Saldanha J (2008) Evaluation of the Roche cobas s 201 system and cobas TaqScreen multiplex test for blood screening: a European multicenter study. Transfusion 48(9):1853–1861
11. Meng Q, Wong C, Rangachari A, Tamatsukuri S, Sasaki M, Fiss E, Cheng L, Ramankutty T, Clarke D, Yawata H, Sakakura Y, Hirose T, Impraim C (2001) Automated multiplex assay system for simultaneous detection of hepatitis B virus DNA, hepatitis C virus RNA, and human immunodeficiency virus type 1 RNA. J Clin Microbiol 39(8):2937–2945. doi:10.1128/JCM.39.8.2937-2945.2001
12. Schmidt M, Pichl L, Jork C, Hourfar MK, Schottstedt V, Wagner FF, Seifried E, Muller TH, Bux J, Saldanha J (2010) Blood donor screening with cobas s 201/cobas TaqScreen MPX under routine conditions at German Red Cross institutes. Vox Sang 98(1):37–46

13. Gibellini D, Gardini F, Vitone F, Schiavone P, Furlini G, Re MC (2006) Simultaneous detection of HCV and HIV-1 by SYBR Green real time multiplex RT-PCR technique in plasma samples. Mol Cell Probes 20(3–4):223–229
14. Busch MP, Kleinman SH, Jackson B, Stramer SL, Hewlett I, Preston S (2000) Committee report. Nucleic acid amplification testing of blood donors for transfusion-transmitted infectious diseases: report of the Interorganizational Task Force on Nucleic Acid Amplification Testing of Blood Donors. Transfusion 40(2):143–159
15. Palella FJ Jr, Delaney KM, Moorman AC, Loveless MO, Fuhrer J, Satten GA, Aschman DJ, Holmberg SD (1998) Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N Engl J Med 338(13):853–860. doi:10.1056/NEJM199803263381301
16. Sherman KE, Rouster SD, Chung RT, Rajicic N (2002) Hepatitis C virus prevalence among patients infected with human immunodeficiency virus: a cross-sectional analysis of the US adult AIDS Clinical Trials Group. Clin Infect Dis 34(6):831–837
17. Sulkowski MS, Mast EE, Seeff LB, Thomas DL (2000) Hepatitis C virus infection as an opportunistic disease in persons infected with human immunodeficiency virus. Clin Infect Dis 30(Suppl 1):S77–S84
18. Goel G, Kumar A, Puniya AK, Chen W, Singh K (2005) Molecular beacon: a multitask probe. J Appl Microbiol 99(3): 435–442
19. Marras SA, Tyagi S, Kramer FR (2006) Real-time assays with molecular beacons and other fluorescent nucleic acid hybridization probes. Clin Chim Acta 363(1–2):48–60
20. Monroe WT, Haselton FR (2003) Molecular beacon sequence design algorithm. Biotechniques 34(1):68–70, 72–63
21. Vet JA, Majithia AR, Marras SA, Tyagi S, Dube S, Poiesz BJ, Kramer FR (1999) Multiplex detection of four pathogenic retroviruses using molecular beacons. Proc Natl Acad Sci USA 96(11):6394–6399
22. Tong Y, Lemieux B, Kong H (2011) Multiple strategies to improve sensitivity, speed and robustness of isothermal nucleic acid amplification for rapid pathogen detection. BMC Biotechnol 11:50
23. Khodakov DA, Zakharova NV, Gryadunov DA, Filatov FP, Zasedatelev AS, Mikhailovich VM (2008) An oligonucleotide microarray for multiplex real-time PCR identification of HIV-1, HBV, and HCV. Biotechniques 44(2):241–246, 248