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Simultaneous detection of HCV and HIV-1 by SYBR Green real time multiplex RT-PCR technique in plasma samples

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Abstract

This paper describes the development of a SYBR Green-based multiplex real time RT-PCR for the simultaneous detection of HCV and HIV-1 genomes in plasma samples. Viral genomes were identified in the same sample by their distinctive melting temperature (Tm) which are 81.6 and 86.5 °C for HIV-1 gag 142 bp amplicon and HCV 5′-NCR region 226 bp amplicon, respectively. Analysis of known scalar concentrations of reference plasma indicated that the multiplex procedure detects at least 500 copies/ml of both HIV-1 and HCV. In addition, we also assayed HIV-1 and HCV viral load in 30 co-infected patients and in 15 blood donors, confirming the sensitivity and specificity of the assay.

This method may represent a useful alternative method for the detection of HIV-1/HCV co-infection, reliable for a rapid and relatively inexpensive screening of blood donors.

Keywords: HIV-1; HCV; SYBR Green; Multiplex RT-PCR

1. Introduction

HIV-1 and HCV co-infection is a relatively common clinical occurrence in Europe and in USA affecting approximately 25% of HIV-1–infected patients and 10% of HCV-infected individuals, respectively [1–3]. This high prevalence of HIV-1/HCV co-infection is closely related to common transmission routes such as blood and blood products. In particular, the prevalence of HCV infection in HIV-1 positive patients is higher in parental transmission by injecting drug use (about 90%) and by transfusion of blood or blood products (70%), whereas it is relatively lower after sexual or vertical transmission [4–6]. Although HAART treatment of HIV-1 positive patients has clearly decreased the incidence of mortality and the rate of opportunistic infection, the clinical importance of HIV-1/HCV co-infection is increasing because HCV-related liver disease has emerged as a major cause of morbidity and mortality in HAART-treated HIV-1–infected patients [7,8]. Indeed, HCV infection is associated with an increased risk of developing hepatotoxicity in response to HAART and it has negative effects on HIV disease progression [9,10]. On the other hand, HIV-1 co-infection accelerates HCV-related liver diseases, inducing a more rapid evolution to cyrrosis and hepatocarcinoma in these patients [11,12]. Besides the direct clinical impact of HIV-1 and HCV co-infection, HCV and HIV-1 are two of the most important infectious agents transmitted by blood transfusion and so, reliable and sensitive screening of blood units through serological and molecular procedures is essential [13]. Although sensitive serological tests were developed successfully, a residual risk of viral infection persists, since the HIV or HCV infection may be transmitted either during the so-called window period, when viremia precedes seroconversion, or in the presence of immunovariant virus infection, where the serological tests may be ineffective [13]. To minimize this possibility, several nucleic acid tests (NAT) have been developed with a dramatically improved analytical sensitivity in comparison with direct antigen detection or virus isolation [13,14]. However, NAT technology has a major drawback of high cost and consequent poor cost-effectiveness. Moreover, the pooling of samples employed by several NAT approaches determines a relatively high rate of false-positive results, a loss of sensitivity when a low-positive sample is diluted into the pool and the need to recognize infected unit in the pool with a delay of blood availability particularly severe for some cellular

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products such as platelets [13,14]. To overcome these weaknesses, previous studies developed sensitive and rapid multiplex PCR assays that can detect the genomes of different viruses simultaneously [15–17]. In particular, some multiplex real-time PCR, approaches were developed employing TaqMan technology for viral co-infection detection [18,19]. Recently, multiplex real-time PCR, with melting curve analysis, has been described as a simple, reliable, and rapid assay for the detection and identification of certain bacteria, protozoa, and viruses [20–25].

This paper describes a RT-PCR multiplex real-time technique based on SYBR Green as fluorochrome for simultaneous detection of HIV-1 and HCV genomes in clinical plasma samples.

2. Materials and methods

2.1. Patients

Thirty HIV-1/HCV co-infected patients and 15 HIV-1/HCV seronegative blood donors were enrolled in our study after informed consent, following the Helsinki declaration. HIV-1/HCV seropositive and healthy blood donor plasma samples were obtained from patients living in Italy. Sequence analysis [26] indicated that HIV-1 seropositive patients were infected by HIV-1 subtype B, whereas HCV genotype analysis of HCV-infected patients showed different genotypes such as 1a, 1b, 3a, and 4 HCV genotypes.

2.2. Branched DNA HIV-1 and HCV RNA viral load determination

HIV-1/HCV co-infected patients' plasma was extracted and quantified by Quantiplex HIV-1 RNA 3.0 assay branched DNA kit (Bayer, Tarrytow, NJ) or HCV RNA 3.0 assay branched DNA kit (Bayer) using Bayer Diagnostics Quantiplex bDNA System 340 (Bayer) following the manufacturers' instructions. The amount of HIV-1 RNA is expressed as the number of copies per millilitre of plasma. The lower detection limit is determined at 50 copies/ml for HIV-1 and 3200 copies/ml for HCV.

2.3. Viral RNA extraction and purification from plasma

Whole blood samples were collected from HIV-1/HCV-infected and blood donor patients by venepuncture in EDTA-containing tubes. The samples were centrifuged at 2500 rpm for 20 min and the plasma was stored at −80 °C until use. In order to extract and purify HIV-1 RNA genome, one millilitre of plasma obtained from HIV-1/HCV-infected patients or healthy blood donors was processed by QIAamp Ultrasens Virus kit (Qiagen, Hilden, Germany) following the manufacturers' instructions. Purified RNA was eluted in 40 μl of kit elution buffer and stored at −80 °C until use. In parallel, scalar reference plasma dilutions were treated by the same procedure.

2.4. Assessment of HIV-1 and HCV reference sera

The standards for HIV-1 and HCV RNA analysis were assessed by scalar dilution of HIV-1 positive control plasma used for the HIV-1 or HCV b-DNA technique shown above (Chiron). The HIV-1 stock virus concentration contained 6.5×10^5 RNA copies/ml, whereas the HCV stock virus, concentration contained 2.5×10^6 RNA copies/ml. Prior to RNA purification procedure, the HIV-1 and HCV stocks were diluted in HIV-1 and HCV-negative plasma and mixed to achieve several HIV-1/HCV scalar dilutions (from 5×10^5 copies to 50 copies/ml).

2.5. Oligonucleotide primers

We used HIV-1 *gag* and HCV 5′-NCR region specific primers to avoid major mismatches due to HIV-1 and HCV genome variability.

HIV-1 *gag* primers are: 5′-TGCTATGTCAGTTCCCCT-TGGTTCTCT-3′ and 5′-AGTTGGAGGACATCAAGCAGC-CATGCAAAT-3′. The amplification with this pair of oligonucleotides yielded a 142 bp *gag* fragment.

HCV 5′-NCR primers are: 5′-GTCTAGCCATGGCGT-TAGTATGAG-3′ (77–100; HCVP1) and 5′-ACCCTAT-CAGGCAGTACCACAAG-3′ (302–280; HCVP2). The length of HCV-specific amplified fragment obtained is 226 bp. The oligonucleotide sequences were analysed by Oligo 6.0 (Med Probe, Oslo, Norway) for their compatibility in a multiplex RT-PCR assay.

2.6. Viral genomes retrotranscription

Following plasma RNA extraction, the samples were retrotranscribed by Thermoscript RT-PCR System (Invitrogen, San Diego, CA). Five microliters of extracted RNA were added to 1 μl SK431 (2 μM), 1 μl HCVP2 (2 μM), 2 μl dNTP (10 mM), and 3 μl distilled water and incubated at 65 °C for 5 min. The samples were chilled in ice and 4 μl 5× RT-Buffer (Invitrogen), 1 μl DTT (0.1 M), 1 μl RNaseOUT (40 U/μl), 1 μl distilled water, and 1 μl Thermoscript RT (15 U/μl) were added. The cDNA synthesis was carried out at 55 °C for 30 min and then at 85 °C for 5 min. The RNA template was removed by RNase H treatment at 37 °C for 20 min as described by the manufacturer. The cDNA was stored at −80 °C until use.

2.7. Determination of HIV-1 and HCV RNA viral load by SYBR Green real time PCR

HIV-1/HCV duplex SYBR Green real time PCR assay was performed in 20 μl PCR mixture volume containing 10 μl of 2× Quantitect SYBR Green PCR Master Mix (Qiagen), 2.5 mM $MgCl_2$, 0.2 μM of each HIV-1 and HCV oligonucleotide primer, 2U of Faststart Taq (Roche, Mannheim, Germany), and 4 μl of cDNA obtained from plasma samples or from scalar dilutions of plasma standard virus stock.

The co-amplification was performed as follows: activation step at 95 °C for 15 min and 45 cycles in four steps: 94 °C for 5 s, 60 °C for 15 s, 72 °C for 30 s, and 78 °C for 3 s. A single

fluorescence detection was performed in each cycle at 78 °C to reveal the positive samples. The analysis of fluorescence signal at 78 °C rules out interference by any dimer primers artefacts, even though this option was randomly observed especially after the 40th amplification cycle and almost exclusively in negative samples. At the end of the amplification cycles, melting temperature analysis was performed by a slow increase in temperature (0.1 °C/s) up to 95 °C. Amplification, data acquisition, and analysis were performed on a LightCycler instrument (Roche) using LightCycler 5.3.2 software (Roche). The melting peaks were analyzed to distinguish HCV and/or HIV-1-specific amplicons. All samples were run in duplicate. All amplicons derived from clinical samples tested were run in a 3% agarose gel electrophoresis.

3. Results

3.1. SYBR Green-based real time multiplex RT-PCR optimization to detect HCV and HIV-1 genomes

We assessed a SYBR Green-based real time multiplex RT-PCR for the simultaneous detection of HIV-1 and HCV viral load in plasma samples. We selected HIV-1 and HCV specific oligonucleotides pairs able to detect all significant HIV-1 subtypes and HCV genotypes. We chose two classical HIV-1 specific oligonucleotides, represented by SK 431 and SK 462, that recognize a well-conserved region in the HIV-1 *gag* gene amplifying a 142-bp fragment. This pair of oligos is known to be effective in the amplification of major HIV-1 subtypes and has been successfully employed for classical PCR and real-time RT-PCR techniques [27,28]. On the other hand, the HCV-specific oligonucleotides (HCVP1 and HCVP2) were designed to amplify a 226-bp fragment within the 5′ non-coding region (5′-NCR) of HCV genome. These primers [29,30] match the well-conserved sequences among different HCV genotypes (Fig. 1) but do not display significant interactions with viral or human sequences.

Optimal conditions for the SYBR Green multiplex real time RT-PCR assay were achieved by the use of Quantitect SYBR Green PCR Master Mix modified by means of a further addition of 2.5 mM of magnesium chloride and 2U of FastStartTaq polymerase. Moreover, concentrations of primers were adjusted, as described in Section 2.7, to obtain maximum sensitivity.

3.2. SYBR Green real time multiplex RT-PCR and melting curve analyses

We evaluated the performances of SYBR Green real time multiplex RT-PCR assay by HCV and HIV-1–positive reference plasma analysis. This approach is basically focused on real time detection of a fluorescent-positive signal followed by melting curve analysis in order to disclose specific ampicons. In the first set of experiments, specific HIV-1 and HCV-positive plasma dilutions at the same concentrations (from 500.000 to 50 copies/ml for each virus) were co-purified,

Genotypes	Accession number	HCV region 77-100	HCV region 280-302
		GTCTAGCCATGGCGTTAGTATGAG	CTTGTGGTACTGCCTGATAGGGT
1a	M62321	----------	----------
1a	M67463	----------	----------
1b	D00832	----------	----------
1b	AY460204	----------	----------
1c	AY051292	----------	----------
2a	D10075	----------	----------
2a	AB047639	- C----------	----------
2b	D10077	----------	----------
2c	D50409	----------	----------
2e	D49745	----------	----------
2f	D49754	- C----------	----------
2k	AB031663	----------	----------
3a	AF046866	- C----------	----------
3b	D49374	----------C-------	----------
3k	D49747	----------C-------	----------
4a	D45193	----------	----------
5a	D50466	----------	----------
6a	D88469	----------	----------
6b	D37841	----------	----------
6e	D88474	----------	----------
6f	D37846	----------	----------
6g	D49748	----------	----------
6h	D88466	----------	----------
6i	D37849	----------	----------
6j	D37848	----------	----------
6k	D84264	----------	----------
6l	D88471	----------	----------

Fig. 1. Comparison between HCV sequences of several genotypes and oligonucleotides sequences. The complementary reverse oligo sequence was analyzed. The forward primer is described by Komourian [29], whereas the reverse primer partially represents the reverse oligo by Defoort [30]. The oligonucleotide sequences were analyzed by Oligo 6.0 software for their compatibility.

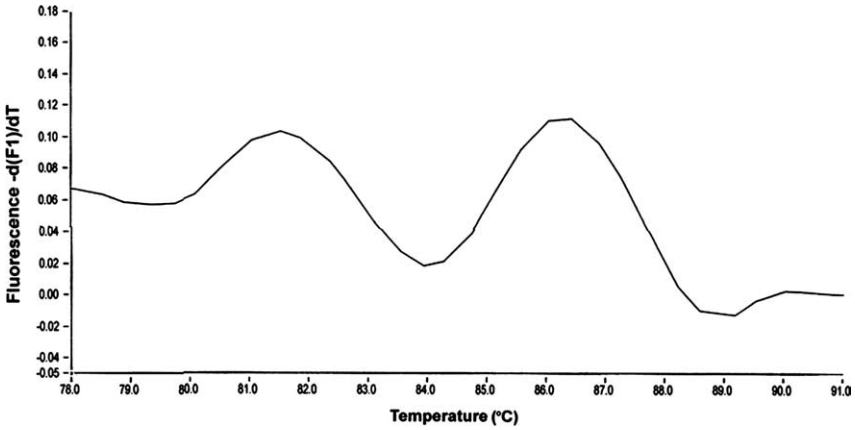

Fig. 2. A typical melting curve analysis of HCV and HIV-1 reference plasma. The HCV and HIV-1-specific melting curves are on the right and left of the picture respectively.

retrotrascribed, and analyzed by LightCycler instrument. Since SYBR Green fluorochrome effectively binds all amplicons without establishing a direct differentiation between HCV and HIV-1-specific products, multiplex RT-PCR fragments are detected by melting curve analysis. Hence, HIV-1 and HCV amplicons can easily be distinguished by specific Tm values due to the different length and compositions of two amplicons. In these experimental conditions, the HIV-1 melting curve displays a Tm = 81.6 °C, whereas the HCV curve showed a Tm = 86.5 °C (Fig. 2). In our hands, HCV and HIV-1–related positive specific fluorescent peaks were detected in all samples analyzed in duplicate until 500 copies/ml of both viruses. These data may be approximately considered the assay sensitivity limit (Table 1). In particular, if we consider the extraction, purification, and retrotranscription at optimal efficiency, the sensitivity of the assay may be considered about 10 copies/reaction for each virus. As shown in Fig. 3, agarose gel electrophoresis confirmed the exact length of the amplicons: two bands of 142 and 226 bp, specific for HIV-1 and HCV, respectively, were detected in all the co-infected samples analyzed. In addition, the specificity of multiplex was determined by a lack of any specific signal when HIV-1 and HCV-negative plasmas were analyzed.

In the next series of experiments, we evaluated the ability of the multiplex assay to reveal the two specific Tm peaks, when a low amount of viral genome is simultaneously co-amplified with a high yield of other viral nucleic acid. SYBR Green real-time multiplex RT-PCR was carried out on HIV-1 and HCV plasma reference sample, where scalar concentrations of HIV-1 from 100.000 to 50 copies/ml were amplified in the presence of 100.000 copies/ml of HCV and vice versa. In these experimental conditions, HCV and HIV-1-specific melting curves were constantly detected when 500 copies/ml of HIV-1 were amplified with 100.000 copies/ml of HCV (Table 2). The two melting curve peaks were also detected when 500 copies/ml of HCV genomes were amplified with 100.000 copies/ml of HIV-1. These data demonstrated that the specific Tm detection in this multiplex RT-PCR assay is not significantly affected even when co-infection with very different viral load occurs.

Table 1
SYBR Green multiplex real time RT-PCR analytical sensitivity evaluated by standard plasma dilution analysis

HIV-1 (copies/ml)	HCV (copies/ml)	Replicates
500.000	500.000	10/10
50.000	50.000	10/10
5.000	5.000	10/10
1.000	1.000	10/10
500	500	10/10
200	200	5/10
100	100	0/10
50	50	0/10

Five experiments in duplicate were performed for complessive 10 replicates. The replicates were considered positive when both HIV-1 and HCV were revealed.

3.3. Analysis of patients' plasma by SYBR Green real-time multiplex RT-PCR

In order to test the SYBR Green real time multiplex RT-PCR on co-infected patients, we selected 30 seropositive

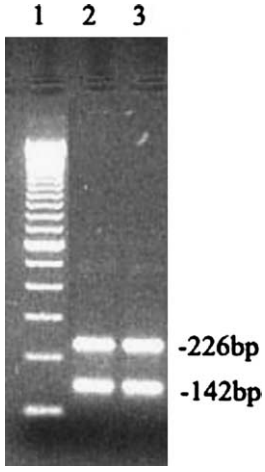

Fig. 3. Agarose gel electrophoresis analysis of HIV-1 and HCV co-amplified samples after SYBR Green multiplex real time RT-PCR. The HIV-1–specific amplicon is at 142 bp, whereas the HCV amplicon is at 226 bp. Lane 1 represents molecular markers. Lanes 2 and 3 showed a co-amplification of 5000 copies/ml of both HCV and HIV-1 reference plasma.

Table 2
SYBR Green multiplex real time RT-PCR analytical sensitivity evaluated in different ratio of standard plasma dilution conditions

HIV-1 (copies/ml)	HCV (copies/ml)	HIV-1 and HCV positive replicates
100.000	100.000	10/10
50.000	100.000	10/10
5.000	100.000	10/10
1.000	100.000	10/10
500	100.000	10/10
200	100.000	3/10
100	100.000	0/10
50	100.000	0/10
100.000	100.000	10/10
100.000	50.000	10/10
100.000	5.000	10/10
100.000	1.000	10/10
100.000	500	10/10
100.000	200	4/10
100.000	100	0/10
100.000	50	0/10

Five experiments in duplicate were performed for complessive 10 replicates. The replicates were considered positive when both HIV-1 and HCV were revealed.

HIV-1/HCV co-infected patients and 15 blood donors. These HIV-1/HCV positive samples were previously quantified by a gold standard method represented by HCV or HIV-1–specific b-DNA assay. The results, shown in Table 3 and in Figs. 4 and 5, demonstrated that all 17 HIV-1 positive samples with a viral load over 500 copies/ml were detected by the assay. Moreover, the 11 HIV-1 positive samples with viral load $<$50copies/ml and two samples with HIV-1 genomes between 100 and 180 copies/ml were not detected. On the other hand, all the 27 HCV co-infected samples with a HCV b-DNA detectable viral load were revealed by the assay, whereas one of three samples with viral load not determined by b-DNA was detected by the multiplex assay. Moreover, patients infected with different HCV genotypes such as 1a, 1b, 3a, and 4 genotypes were disclosed by the multiplex technique, confirming the good performance of specific oligos. The melting curve analysis allowed a reliable identification of HCV and HIV-1 amplicons with an HIV-1 and HCV Tm with values of 81.6 and 86.5 °C, respectively, albeit slight melting temperature variations due to different amplification fragment internal sequences were sometimes noticed. Finally, the analysis of 15 healthy blood donors did not show any positive signal, confirming the specificity of the assay.

4. Discussion and conclusions

This report describes the development of a qualitative SYBR Green-based multiplex real time RT-PCR for the simultaneous detection of HCV and HIV-1 RNA in plasma samples. This multiplex RT-PCR format was assessed using an HIV-1 and HCV-specific oligonucleotide pair that encompassed RNA sequences in viral conserved genome regions in order to yield primers able to detect all the HCV genotypes and HIV-1 major subtypes. Moreover, the optimization of this

Table 3
Positive detection of HIV-1/HCV infection patients by SYBR Green multiplex real time RT-PCR

Patient	b-DNA HIV-1 (copies/ml)	b-DNA HCV (copies/ml)	Multiplex HIV-1 positive	Multiplex HCV positive
1	1.8×10^2	1.6×10^7	−	+
2	<50	3.2×10^7	−	+
3	5.0×10^5	4.1×10^4	+	+
4	8.3×10^2	9.0×10^6	+	+
5	<50	6.1×10^6	−	+
6	<50	9.4×10^5	−	+
7	9.3×10^2	3.5×10^5	+	+
8	1.0×10^2	1.8×10^6	−	+
9	5.4×10^2	7.2×10^5	+	+
10	<50	1.7×10^7	−	+
11	1.7×10^4	2.4×10^4	+	+
12	1.9×10^5	6.2×10^6	+	+
13	<50	8.6×10^6	−	+
14	2.0×10^3	1.2×10^7	+	+
15	<50	2.5×10^4	−	+
16	<50	<3200	−	−
17	1.5×10^4	9.8×10^4	+	+
18	1.5×10^4	5.7×10^6	+	+
19	2.9×10^3	6.4×10^5	+	+
20	<50	6.8×10^5	−	+
21	<50	2.5×10^5	−	+
22	<50	<3200	−	−
23	3.7×10^5	5.1×10^6	+	+
24	1.1×10^4	9.0×10^6	+	+
25	<50	2.5×10^5	−	+
26	6.5×10^3	2.5×10^6	+	+
27	5.4×10^3	1.6×10^5	+	+
28	4.3×10^3	1.1×10^4	+	+
29	9.2×10^2	1.2×10^4	+	+
30	5.8×10^2	<3200	+	+

assay focused initially on the compatibility of oligonucleotide pairs during the amplification to minimize potential non-specific interactions such as dimer primers. In particular, the assessment of primer concentration, the increase in magnesium chloride concentration and the Taq enzyme amount are pivotal factors to enhance sensitivity and specificity. The SYBR Green real time multiplex RT-PCR assay is based on melting curve analysis to specifically determine the target. The Tm of HCV and HIV-1 melting curve was detected at 86.5 and 81.6 °C with slight modifications due to sequence variability between positive patients. This difference in melting allows an unambiguous identification of specific virus amplicons.

The first intriguing multiplex approach for detecting HIV-1 and HCV was based on two separate amplification and flow cytometry-based detection steps, but it was labor intensive and time consuming [30]. Other multiplex approaches such as AMPLINAT MPX test [15], TMA-based assay [17,32], and classical multiplex RT-PCR [19] are sensitive methods for HIV-1 and HCV analysis, but they were unable to distinguish directly between the target viruses, and a further virus detection specific step was necessary to discriminate the correct signal source. In contrast, the real-time RT-PCR offers several advantages such as the increased speed due to reduced cycle time, the removal of post-PCR detection, high sensitivity,

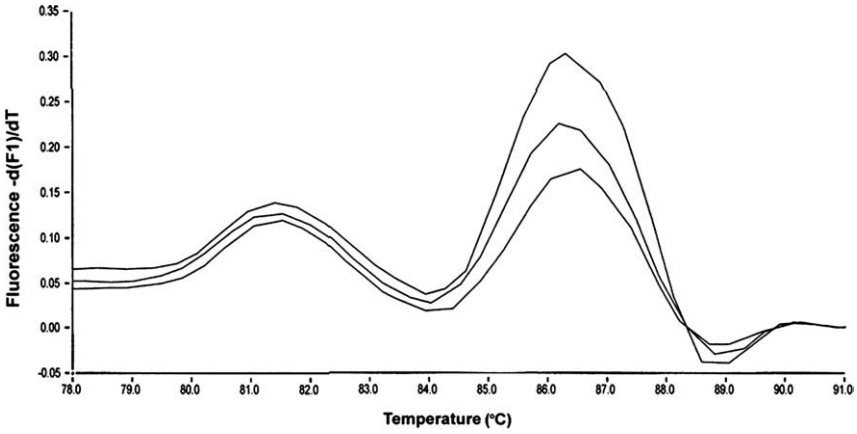

Fig. 4. A typical melting curve analysis of three HCV and HIV-1 positive plasma patients.

and the relatively simple technical feasibility [31]. On the other hand, a multiplex real-time RT-PCR based on TaqMan strategy was recently developed [18] for HCV and HIV-1 detection and this technique provided a direct identification of specific amplicons employing probes labeled by different fluorophores with distinct emission spectra [31]. Our assay detects HCV and HIV-1 genomes in the plasma by melting curve analysis of amplified products through SYBR Green, a fluorescent molecule that has three main advantages over other real time PCR detection formats such as labelled probes. Firstly, SYBR Green is a low-cost fluorochrome, whereas labelled probes are expensive and add complexity to both the oligonucleotide design and the parameters of the multiplex or classical amplification reaction [33]. Secondly, in spite of using specific probes during real-time PCR (TaqMan), PCR artefacts may be noted especially at amplification cycles beyond the 30th [34,35] and the selected sequence targets may more readily undergo specific mutations. Thirdly, the SYBR Green-based technique allows a technically simpler approach and PCR artefacts can be ruled out by analysis of the amplified melting curve [36].

Although SYBR Green-based multiplex real time RT-PCR assay is a qualitative technique, analysis of known HIV-1 and HCV-positive reference plasma dilution end point can give an approximate indication of its sensitivity. In fact, the analytical sensitivity of this technique is determined at 500 copies/ml both for HCV and HIV-1. This sensitivity is comparable to

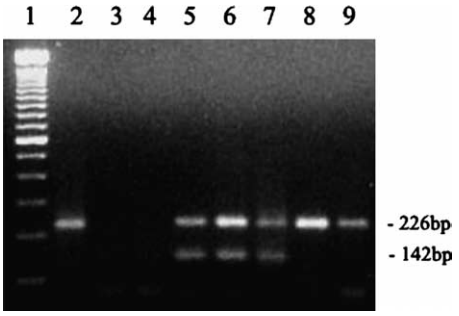

Fig. 5. Agarose gel electrophoresis analysis of patients (lanes 2 and 5–9) and healthy donors (lanes 3 and 4) plasma amplified by SYBR Green multiplex real time RT-PCR.

other multiplex PCR formats for HCV and HIV-1 detection [18,19], whereas the technique described by Meng and co-workers is more sensitive for HIV-1 detection only [15]. It is noteworthy that this SYBR Green multiplex assay may potentially be employed as an alternative method for screening blood samples. The window period of HCV or HIV-1 infection represents the main cause of the residual risk of viral transmission in blood transfusion [6,15,37,38]. Viral loads between 10^5 and 10^6 copies/ml for HCV and 2×10^2–10^5 copies/ml for HIV-1 have been reported during the window period [13,15]. In addition, the ramp up doubling time in the window period was calculated as 0.1 and 1 day for HCV and HIV-1, respectively, suggesting that multiplex assay HCV sensitivity will then be sufficient to detect HCV genome in window period donations, whereas HIV-1 assay sensitivity (500 copies/ml) will show a window period reduction as described in other assays [13,15,18].

In conclusion, besides a good level of sensitivity and specificity, the easy handling, relatively low cost, rapid analysis (approximately four hours), and effective features of the SYBR Green real-time multiplex assay could represent further advantages for single sample screening in every laboratory. Moreover, this assay may be a useful and a rapid approach for simple and effective concomitant detection of HCV and HIV-1 in plasma samples, with a further possible use in assessing virus infection in blood unit screening.

Acknowledgements

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