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HIV-1 and HCV detection in dried blood spots by SYBR Green multiplex real-time RT-PCR

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A B S T R A C T

Dried blood spot (DBS) is a reliable method of blood collection used for the diagnosis of several human diseases. DBS is particularly useful for diagnosing children and for the screening of high-risk populations especially in countries where health facilities are not readily accessible. This report describes a qualitative SYBR Green-based real-time multiplex RT-PCR for the simultaneous detection of hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV-1) genomes in DBS. Specific viral amplicons were identified in the same sample by their distinctive melting temperatures. The analysis of scalar concentrations of the reference samples indicated that this multiplex procedure detects at least 2500 copies/ml of HCV and 400 copies/ml of HIV-1. HIV-1 and HCV viral loads in 20 patients infected with HIV-1 and/or HCV and in 5 healthy blood donors were also tested, confirming the sensitivity and specificity of the assay.

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

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1. Introduction

It has been estimated that 10 million individuals are co-infected with HIV and HCV worldwide (Thomas, 2008). These two viruses cause chronic progressive diseases, characterized by long clinical latency and immune system hyperactivation. The availability of highly active antiretroviral therapy (HAART) has increased the life expectancy for HIV patients and, since this crucial change in the management of HIV infection, HCV co-infection has become a major cause of death in HIV-infected patients. In addition, HIV infection exacerbates the morbidity of HCV infection itself, resulting in a decreased likelihood of spontaneous self-clearance (Nelson and Thomas, 2001).

HIV and HCV share the same routes of transmission: parenteral, sexual and mother-to-child, although to a different extent for each virus (Rockstroh and Spengler, 2004). Since the parenteral route is the most effective in transmitting HCV, injecting drug users (IDU) represent the main subset of the population with HCV infection. Otherwise, the prevalent route of transmission in subjects infected with HIV depends on the geographical zone (Kilmarx, 2009). In co-infected mothers, the rate of HIV and HCV transmission to newborns ranged from 13.3 to 30% and from 5.4 to 23%, respectively. The transmission rate from co-infected mothers is higher than that from mothers infected with HIV or HCV (England et al., 2006; Thomas, 2008).

Diagnosis of HIV and HCV infection is achieved by identifying specific antibodies in sera. However, the serological procedures may be misleading prior to seroconversion and in infants born to HIV seropositive women. In these conditions, PCR is the method of choice for the diagnosis of HIV and/or HCV infection. The main drawback of currently implemented molecular tests is the need for complicated processing and low temperature storage and transportation. These requirements constitute a major barrier for PCR use in limited-resource settings where blood borne virus epidemics are particularly severe.

Dried blood spots can overcome several obstacles limiting access to molecular tests: DBSs represent an easy blood collection procedure allowing safe refrigeration-free shipment (Cassol et al., 1992; Li et al., 2004) to a reference centre where molecular tests can be performed. Moreover, DBS is simpler to collect than venous blood because a few drops of blood on filter paper can eliminate the need for a larger volume of blood sample. From 1963 when Guthrie and Susi (1963) used this procedure for the first time to measure phenylalanine in newborns suspected of having phenylketonuria, DBS demonstrated its feasibility in several clinical and biological fields (Mei et al., 2001). Many papers have

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demonstrated a wide application of PCR procedures in viral detection using DBS, such as HIV, HBV, enterovirus and herpesvirus, because the blood specimens spotted on filter paper may be stored at room temperature for relatively long periods without consistent loss of targets and, when the blood spots are dried, samples can be easily handled and processed (Gupta et al., 1992; Barbi et al., 2000; Lewensohn-Fuchs et al., 2003; Distefano et al., 2004; Jardi et al., 2004; Uttayamakul et al., 2005; Jacob et al., 2008; Smets et al., 2008; Soetens et al., 2008; Zhang et al., 2008; Lloyd et al., 2009; Reigadas et al., 2009). DBSs are also suitable for antiretroviral genotypic resistance testing (Dachraoui et al., 2008) and in monitoring HIV-RNA during antiretroviral therapy (Marconi et al., 2009).

This paper assessed a qualitative multiplex real-time RT-PCR technique for the simultaneous detection of HIV-1 and HCV genomes on DBS to couple RT-PCR sensitivity and flexibility with filter paper support. This method may represent an alternative method to detect HIV-1 and HCV viruses and overcome the difficulties of blood sampling and handling evidenced in screening analysis, children and patients in developing countries.

2. Materials and methods

2.1. HIV/HCV infected patients and healthy donors

Twenty-five blood samples from adult individuals were analyzed: 13 were collected from patients that were co-infected with HIV-1 and HCV, 4 from patients infected with HIV-1, 3 from patients infected with HCV and 5 from healthy blood donors. Both the patients and healthy blood donors gave their informed consent, following the Helsinki declaration. Sequence analysis showed that all HIV-1 seropositive patients were infected by HIV-1 subtype B, whereas HCV genotype analysis of the patients infected with HCV disclosed genotypes 1a, 1b, 2a and 3a. All HIV and/or HCV positive plasma samples were tested by Versant HIV-1 RNA 3.0 b-DNA Assay and Versant HCV RNA 3.0 b-DNA Assay (Siemens, Munich, Germany) gold-standard techniques following the manufacturer's instructions. The lower detection limit is determined at 50 copies/ml for HIV and 3200 copies/ml for HCV.

2.2. DBS preparation and nucleic acid extraction procedure

Blood spots were prepared dropping 50 μl of whole blood onto Whatman no. 3 filter paper (Whatman, Maidstone, UK). The spots were dried at 37 °C for 1 h before nucleic acid extraction. Three punches (each one 3 mm in diameter), entirely covered with the blood sample, were excised by puncher from DBSs. To avoid carryover contaminations, the puncher was thoroughly cleaned with 70% ethanol followed by PBS wash and by repeated punching of clean filter paper between uses.

Nucleic acids were extracted by QIAamp DNA Mini kit (Qiagen, Hilden, Germany) using the protocol specific for DBS. QIAamp spin mini columns (Qiagen) effectively bind both RNA and DNA in clinical samples as described by the manufacturer and by Read (2001). Samples were eluted from the filter column with 30 μl of nuclease-free water and stored at −80 °C until use.

2.3. DBS standard panels

To assess the sensitivity and specificity of method, DBSs were prepared using plasma HIV-1 and HCV calibrated virus standards (Siemens). Prior to the extraction procedure, the HIV-1 and HCV viral stocks were added to HIV-1 and HCV negative blood samples and mixed together to achieve HIV-1 or HCV scalar dilutions from 5×10^5 to 50 copies/ml and from 5×10^6 to 50 copies/ml, respectively. Hence, 50 μl were then spotted on filter paper and extracted as described above.

2.4. HIV-1 and HCV detection by multiplex SYBR Green real-time RT-PCR

Highly conserved regions of HCV and HIV-1 genome were selected to be the target of oligonucleotide pairs used in PCR to avoid major mismatches due to HIV-1 and HCV genome variability. HIV-1 primers were: 5′-TGCTATGTCAGTTCCCCTTGGTTCTCT-3′ and 5′-AGTTGGAGGACATCAAGCAGCCATGCAAAT-3′. This pair of oligonucleotides amplified a 142 bp sequence in the HIV-1 *gag* gene (Christopherson et al., 1997). HCV primers were: 5′-GTCTAGCCATGGCGTTAGTATGAG-3′ (HCVP1) and 5′-ACCCTATCAGGCAGTACCACAAG-3′ (HCVP2; Komurian-Pradel et al., 2001). The amplification performed with these primers yielded a 226 bp fragment located in the 5′ NCR region of HCV.

HIV-1/HCV multiplex SYBR Green real-time RT-PCR assay was performed in a 20 μl PCR mixture containing 10 μl of 2× Quantitect SYBR Green PCR Master Mix (Qiagen), 0.4 μl of 10 mM dNTPs mix (Roche, Mannheim, Germany), 0.4 μl of Taq polymerase (5 U/μl; Invitrogen, Carlsbad, USA); 0.2 μl of 25 mM $MgCl_2$, 0.4 μM of HIV-1 oligonucleotide primers, 0.2 μM of HCV oligonucleotide primers, 0.25 μl of RT Mix and 8 μl of extracted sample. The co-amplification was performed as follows: RT step at 50 °C for 20 min, 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. Single fluorescence detection was performed in each cycle at 78 °C to minimize the interference of dimer primer artefacts. At the end of the amplification cycles, melting temperature analysis was performed by a slow increase in temperature (0.4 °C/s) up to 95 °C. Amplification, data acquisition, and analysis were performed on a LightCycler instrument (Roche, Mannheim, Germany) using LightCycler 5.3.2 software. The melting peaks were analyzed to distinguish HCV and/or HIV-1-specific amplicons. All samples were run in three independent experiments performed in duplicate. All amplicons derived from clinical samples tested were run in a 1.5% agarose gel electrophoresis. The viral copies were expressed per millilitre of plasma. To control the amplification of clinical samples each DBS extract was amplified by beta-actin oligos as previously described (Gibellini et al., 2008).

3. Results

3.1. SYBR Green real-time multiplex RT-PCR optimization for HCV and HIV nucleic acid detection

The optimal conditions for the SYBR Green multiplex real-time RT-PCR were obtained using Quantitect SYBR Green PCR Master Mix modified by further addition of magnesium chloride (5 nmol), dNTPs (4 nmol) and Taq polymerase (2 units). Importantly, the concentrations of oligonucleotides were adjusted to 0.4 and 0.2 μM primer concentration for HIV-1 and HCV respectively to achieve the maximum sensitivity.

SYBR Green is a double strand DNA binding molecule that does not recognize specific dsDNA targets. Hence, the different amplicons were distinguished by the melting curve analysis. The values of melting temperature (Tm), due to different length and composition of the two amplicons, specifically disclosed the presence of HCV and HIV-1 in the samples. The HIV-1 and HCV melting curves display a Tm of 81.6 and 86.5 °C, respectively (Fig. 1). The chosen oligonucleotide pairs were checked by BLAST analysis. As previously reported (Komurian-Pradel et al., 2001; Gibellini et al., 2004, 2006), they did not show any relevant homology with viral

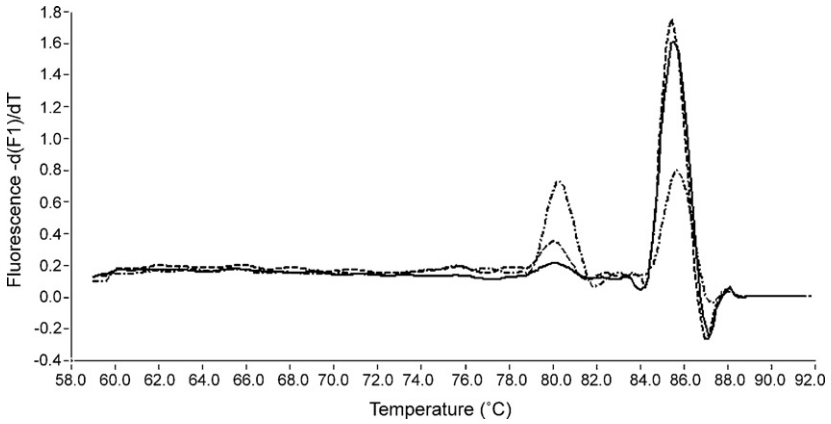

Fig. 1. Melting curve analysis of three HCV/HIV-1 co-infected samples. The HIV-1 and HCV Tm were determined at 81.6 and 86.5 °C, respectively.

or human sequences whereas they reveal all representative HIV-1 subtypes and HCV genotypes.

3.2. SYBR Green real-time multiplex RT-PCR assay sensitivity and specificity

In the first set of experiments, the assay was evaluated on HIV-1 or HCV positive reference plasma sample dilutions.

HCV or HIV-1 scalar dilutions were added to uninfected blood samples and a blood aliquot (50 μl) was spotted on filter paper. After extraction and real-time RT-PCR, the assay sensitivity limit, determined as the dilution where 100% of samples were revealed by our technique, was 2500 copies/ml for HCV and 400 copies/ml for HIV-1 (Table 1). The Ct values showed linear reference curves for both viruses ($r > 0.95$). Agarose gel electrophoresis analysis demonstrated HIV-1 142 bp and HCV 226 bp amplicons confirming the method's specificity (data not shown).

Hence, serial dilutions of HIV-1 and HCV reference samples mixture were used to test whether the assay will simultaneously detect HIV-1 and HCV in the same sample. In these experiments, scalar concentrations of both HIV-1 and HCV viral genomes to uninfected blood samples (Table 2) were added. In this context, HCV and HIV-1 were detected in 100% of samples at 2500 and 400 copies/ml, respectively, showing the same sensitivity displayed in the previous experiments performed with HCV or HIV-1 as single target.

Since some reports indicate that multiplex PCR sensitivity may be affected by the target ratio in the sample, it was evaluated whether multiplex assay could reveal low level of HIV-1 or HCV close to the sensitivity limit when co-amplified in the presence of high yields of the other viral nucleic acid. DBSs were prepared mixing either scalar concentrations of HIV-1 (from 5×10^5 to 50 copies/ml) with 10^6 copies/ml for HCV, or scalar concentrations of HCV (from 5×10^6 to 50 copies/ml) with 10^5 copies/ml of HIV-1. In these experiments, HCV and HIV-1-specific melting curves were constantly detected both when 400 copies/ml of HIV-1 were amplified with 10^6 copies/ml of HCV and 2500 copies/ml of HCV were amplified with 10^5 copies/ml of HIV-1 as reported in Table 3.

Table 1
SYBR Green multiplex real-time RT-PCR analytical sensitivity in DBSs.

HCV copies/ml	HIV-1 copies/ml	HCV or HIV-1 positive replicates
5×10^6	0	6/6
5×10^5	0	6/6
5×10^4	0	6/6
5×10^3	0	6/6
2.5×10^3	0	6/6
5×10^2	0	2/6
50	0	0/6
0	5×10^5	6/6
0	5×10^4	6/6
0	5×10^3	6/6
0	5×10^2	6/6
0	4×10^2	6/6
0	10^2	1/6
0	50	0/6

Table 2
SYBR Green multiplex real-time RT-PCR sensitivity analysis with similar HIV-1 and HCV genome concentration in DBSs.

HCV copies/ml	HCV positive replicates	HIV-1 copies/ml	HIV-1 positive replicates
5×10^6	6/6	5×10^6	6/6
5×10^5	6/6	5×10^5	6/6
5×10^4	6/6	5×10^4	6/6
5×10^3	6/6	5×10^3	6/6
2.5×10^3	6/6	4×10^2	6/6
5×10^2	1/6	5×10^2	6/6
4×10^2	1/6	4×10^2	6/6
2.5×10^2	0/6	2.5×10^2	3/6
50	0/6	50	0/6

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

To test the SYBR Green real-time multiplex RT-PCR on DBSs from infected patients, 20 patients infected with HIV-1 and/or HCV (13 individuals co-infected with HIV-1 and HCV, 4 infected with HIV-1 and 3 infected with HCV) and 5 healthy blood donors were selected.

Table 3
SYBR Green multiplex real-time RT-PCR sensitivity detection in presence of different viral genome concentration.

HCV copies/ml	HCV positive replicates	HIV-1 copies/ml	HIV-1 positive replicates
10^6	6/6	5×10^5	6/6
10^6	6/6	5×10^4	6/6
10^6	6/6	5×10^3	6/6
10^6	6/6	5×10^2	6/6
10^6	6/6	4×10^2	6/6
10^6	6/6	10^2	1/6
10^6	6/6	50	0/6
5×10^6	6/6	10^5	6/6
5×10^5	6/6	10^5	6/6
5×10^4	6/6	10^5	6/6
5×10^3	6/6	10^5	6/6
2.5×10^3	6/6	10^5	6/6
5×10^2	2/6	10^5	6/6
50	0/6	10^5	6/6

Table 4
Detection of HIV-1/HCV genomes by SYBR Green multiplex real-time RT-PCR assay in DBSs achieved from patients.

Patient	HIV-1 bDNA copies/ml	HCV bDNA copies/ml	SYBR Green multiplex HIV-1	SYBR Green multiplex HCV
1	1.7×10^3	1.2×10^6	+	+
2	9.3×10^4	3.1×10^6	+	+
3	1.5×10^5	4.9×10^5	+	+
4	6.0×10^4	5.5×10^5	+	+
5	8.7×10^3	8.6×10^5	+	+
6	$1{,}3 \times 10^5$	3.0×10^6	+	+
7	1.8×10^4	2.5×10^6	+	+
8	1.2×10^3	4.3×10^6	+	+
9	2.8×10^4	3.3×10^5	+	+
10	<50	1.7×10^7	–	+
11	<50	4.7×10^6	–	+
12	<50	1.2×10^4	–	+
13	<50	<3200	–	–
14	Negative	1.5×10^6	–	+
15	Negative	4.2×10^5	–	+
16	Negative	<3200	–	+
17	2.3×10^4	Negative	+	–
18	3.7×10^3	Negative	+	–
19	2.2×10^3	Negative	+	–
20	1.0×10^2	Negative	–	–
21[a]	Negative	Negative	–	–
22[a]	Negative	Negative	–	–
23[a]	Negative	Negative	–	–
24[a]	Negative	Negative	–	–
25[a]	Negative	Negative	–	–

[a] Healthy blood donors.

The patients' plasma were previously quantified by Versant HIV-1 RNA 3.0 b-DNA Assay and Versant HCV RNA 3.0 b-DNA Assay (Table 4).

Fifty microlitres of blood collected by venopuncture from these individuals were spotted onto filter paper and assayed by multiplex RT-PCR technique. HIV-1 genome was detectable in 12 out of 17 HIV-1 positive samples (9 co-infected with HIV-1 and HCV and 3 infected with HIV-1). It is noteworthy that the four negative samples showed a HIV-1 plasma viral load <50 copies/ml in three cases as documented by bDNA test whereas the last sample showed a plasma concentration of 100 copies/ml. On the other hand, HCV was revealed in 15 out of 16 samples analyzed. Interestingly, HCV was identified in one out of two patients with bDNA HCV viral plasma load <3200 copies/ml. Patients infected by different HCV genotypes were revealed by the multiplex technique thus demonstrating the good flexibility of HCV oligonucleotide pair. The analysis of the five healthy blood donors did not show any positive results, confirming the specificity of the assay.

4. Discussion

As a physical support for nucleic acid detection, DBS was first employed in 1987 when DNA was released from filter paper by micro-extraction for the screening of newborns (McCabe et al., 1987). This procedure is easier to perform than venepuncture so it requires fewer trained staff and is particularly useful when bleeding neonates for minimal invasion. DBSs represent a low infectious hazard since they decrease the handling of potentially infectious material in fact the drying process affects negatively the activity of enveloped virus. In addition, owing to their reduced infectious risk and room temperature stability, DBSs can be shipped by mail in low gas-permeable zip closure bags (Therrell et al., 1996). In this study, a multiplex real-time RT-PCR to detect HIV-1 and HCV genomes on DBSs was implemented successfully by applying this method of blood collection on nucleic acid amplification technique for testing children and for screening surveys, especially in developing countries. SYBR Green was selected as real-time RT-PCR reporter molecule because it is inexpensive and represents a simple technical approach in comparison to other real-time PCR formats such as TaqMan and beacons. HCV 5′NCR 226 bp and HIV-1 gag 142 bp amplicons are easily differentiated by the melting curve analysis where HCV and HIV-1 melting temperatures are detected at 86.5 and 81.6 °C, respectively.

Although this method is essentially qualitative, it is able to detect at least 2500 copies/ml for HCV and 400 copies/ml for HIV-1 on DBSs when tested on HIV-1 and HCV reference samples. These sensitivity limits represent a good performance for this technique because the HIV-1 and HCV plasma viremia in acute infections is generally between 2×10^2 and 10^5 copies/ml for HIV-1 and $>10^4$ copies/ml for HCV (Caudai et al., 2005; Cozzi-Lepri et al., 2002; Glynn et al., 2005; Zeuzem and Herrmann, 2002) suggesting an application of this method even during the early phases of HIV-1 or HCV infection. The results obtained with reference samples were confirmed in 20 patients infected with HIV-1 and HCV and 5 healthy blood donors. Although the number of screened samples is limited, the assay did not show any false positive specimens. Instead, it detected constantly HIV-1 and/or HCV infected samples with a viral load above the expected sensitivity limit.

The SYBR Green multiplex real-time RT-PCR displays similar sensitivity in respect to other nucleic acid amplification techniques applied on DBS when HIV genome is tested (Marconi et al., 2009; Nugent et al., 2009; Ou et al., 2007; Reigadas et al., 2009; Uttayamakul et al., 2005). Since the early 1990s several epidemiological studies have used DBS to evaluate the prevalence of HIV-1 (Amellal et al., 2008; Beck et al., 2001; Cassol et al., 1991; Creek et al., 2008; Jacob et al., 2008; Kane et al., 2008; Panteleeff et al., 1999; Reigadas et al., 2009; Sherman et al., 2005; Uttayamakul et al., 2005; Yourno and Conroy, 1992; Zhang et al., 2008) by analysis of the proviral HIV-1 DNA in PBMCs. Recently, RNA stability was also demonstrated (Reigadas et al., 2009) even though some loss, as a result of drying process, has been noticed (Ou et al., 2007; Nugent et al., 2009). However, RNA obtained from DBSs was suitable for molecular analysis for monitoring therapy efficacy by HIV-1 RNA quantitation (Marconi et al., 2009) or determining HIV-1 subtypes (Cassol et al., 1996) and drug-resistance mutations (Plantier et al., 2005; McNulty et al., 2007; Hallack et al., 2008; Youngpairoj

et al., 2008). On the other hand, HCV testing on DBS has been performed only for antibody detection (Judd et al., 2003; Croom et al., 2006; Hickman et al., 2008) and not for RT-PCR application although the possibility of extracting HCV RNA suitable for downstream applications from dried samples has been demonstrated recently (Lloyd et al., 2009). Lloyd et al. (2009) demonstrated the feasibility of HIV quantitation and HIV/HCV genotyping from dried blood plasma samples collected by a non-paper based matrix. However, the matrix used in that report had not been validated extensively as filter paper used for DBSs, which have already a wide number of applications (Mei et al., 2001; Fiscus et al., 2006; Sjoholm et al., 2007).

This SYBR Green real-time RT-PCR technique assay also proved to be flexible. The nucleic acid purification employed procedure is able to isolate DNA and RNA from blood at high efficiency (Read, 2001). Hence, this may be advantageous for HIV-1 detection in clinical samples because both HIV-1 proviral DNA and genomic RNA can be amplified and then the HIV sensitivity may improve (McNulty et al., 2007; Reigadas et al., 2009) even though the DNA contribution to HIV-1 specific amplification on DBS consistently varies with plasma viral load (Masciotra et al., 2007; McNulty et al., 2007; Reigadas et al., 2009). In addition, this whole acid nucleic purification procedure can lead potentially to the development of multiplex PCR with several DNA and/or RNA viruses as targets in the same samples. It is also noteworthy that the SYBR Green multiplex real-time RT-PCR technique can recognize different HCV genotypes: HCV genotyping, performed on samples tested, disclosed genotypes 1a, 1b, 2a and 3a. The HCV and HIV-1 oligonucleotide pairs employed encompass specific conserved regions so they are potentially able to detect all major HCV genotypes and HIV-1 subtypes.

In conclusion, the coupling between real-time SYBR Green multiplex RT-PCR and the filter format of DBSs may represent a flexible tool for the diagnosis of HCV and HIV-1 infection in specific patient groups and in the developing countries where blood volumes and/or the specific health organization does not allow classical analytical procedures.

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