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Multiplex real-time quantitative RT-PCR assay for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus type 1

Daniel Candotti [a,*], Jillian Temple [b], Shirley Owusu-Ofori [c], Jean-Pierre Allain [b]

[a] National Blood Service Cambridge, Long Road, Cambridge CB2 2PT, UK
[b] Department of Haematology, Division of Transfusion Medicine, University of Cambridge, Cambridge CB2 2PT, UK
[c] Transfusion Medicine Unit, Department of Medicine, Komfo Anokye Teaching Hospital, Kumasi, Ghana

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Abstract

A multiplex real-time quantitative reverse transcription (RT)-PCR assay was developed for simultaneous detection, identification and quantification of hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV-1) in plasma or serum samples. Genomic amplification of one virus was unaffected by the simultaneous amplification of the other two. Competition between HCV and HIV-1 amplifications slightly affected the yield of HIV-1 amplification. However, quantitation was possible when a single virus was present. The 95% detection limits were 30, 167 and 680 IU/ml for HBV DNA, HCV RNA and HIV-1 RNA, respectively. The multiplex assay detected with similar efficiency strains of HBV genotypes A–F, HCV genotypes 1–6, and HIV-1 subtypes A–G. Applied to 267 pools of 10 plasmas from blood donors, multiplex screening indicated that the assay was reproducible, sensitive, and specific. This assay has the potential to be used for large-scale nucleic acid testing (NAT) of blood donations.

Keywords: Multiplex real-time quantitative RT-PCR; HBV; HCV; HIV-1; Blood donors; Viral load

1. Introduction

The risk of transfusion-transmitted infection with pathogenic blood-borne viruses such as hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus type 1 (HIV-1) has been reduced by improving donor selection and by the development of sensitive serological tests to screen for HBV surface antigen (HBsAg) and antibodies to HCV and HIV-1 (Schreiber et al., 1996). However, a residual risk of viral infection persists related to the pre-seroconversion window period, infection with immunovariant viruses, immunosilent carriage, or occult carriage in the case of HBV infection (Allain et al., 1999; Candotti et al., 2000; Loussert-Ajaka et al., 1994; Soldan et al., 2003; Widell et al., 1996). Over the past decade, nucleic acid testing (NAT) methods for detection of viral nucleic acids (DNA or RNA) have been developed to reduce this risk. NAT combines the advantage of direct, highly sequence-specific, detection of an infectious agent genome with an analytical sensitivity several orders of magnitude higher than that of antigen detection or virus isolation methods.

Although the feasibility and the high level of performance of NAT in screening donated blood for transfusion-transmitted viruses have been demonstrated (Candotti et al., 2001, 2003a), their use for large-scale routine screening remains limited and controversial considering a poor cost-effectiveness (Jackson et al., 2003). To lower the cost of NAT, two non-mutually exclusive approaches have been proposed. First, applying NAT to pools of plasmas of various sizes as is currently implemented in Western countries and Japan. However, clinical trials have documented a lack of sensitivity due to dilution of samples with low viral load (Stramer et al., 2000), or high rate of false-positive results when attempting to concentrate viral particles by ultracentrifugation (Roth et al., 1999). Positive pools need resolution to identify infected individual donations lengthening the delay of products availability. A second approach is the development of multiplex NAT assays detecting several viruses simultaneously. Multiplex assays have the potential not only to improve cost-effectiveness but also to save time

* Corresponding author. Tel.: GRO-C
fax: GRO-C
E-mail address: dc241@ GRO-C (D. Candotti).

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and effort without compromising the test efficiency and feasibility (Elnifro et al., 2000).

Previous studies have demonstrated the practicality of identifying viral pathogens in various clinical and epidemiological settings with multiplex PCR assays (Elnifro et al., 2000, for review). Only a few multiplex assays have been developed for large-scale blood screening (Defoort et al., 2000; Giachetti et al., 2002; Meng et al., 2001; Mercier et al., 1999; Vet et al., 1999). Recently, two commercial semi-automated multiplex assay systems detecting HBV, HCV and HIV-1 simultaneously in blood donations were described: AMPLINAT MPX test utilises TaqMan real-time PCR on the ABI PRISM 7700 Analyser (Meng et al., 2001; Mine et al., 2003), and the transcription-mediated amplification-based Procleix Ultrio assay is under field evaluation (Linnen et al., 2002).

A sensitive and specific multiplex NAT assay is described below for the simultaneous detection, identification, and quantitation of HBV, HCV and HIV-1 in plasma or serum samples.

2. Material and methods

2.1. Clinical specimens and viral standards

Candidate blood donors from the Komfo Anokye Teaching Hospital (KATH) Blood Bank in Kumasi, Ghana, were tested pre-donation for HBsAg with a dipstick assay (VEDAlab, Alençon, France) according to the manufacturer's instruction. The limit of sensitivity provided by the manufacturer was 5 ng/ml of HBsAg. In parallel, anti-HIV-1, HIV-2, HIV-O screening was performed with Determine (Abbott Laboratories, Delkenheim, Germany) and anti-HCV was from Intec (Hong Kong, PRC) as previously described (Allain et al., 2003). Plasmas collected from serologically non-reactive donors were decanted and 200 μl of each plasma were mixed in a plasma pool of 10 samples and stored at −80 °C. The remaining individual plasmas were kept frozen at −20 °C. Both pools and individual samples were transferred to the Laboratory of Molecular Virology, Division of Transfusion Medicine, Cambridge University, for further molecular and serological investigations. This study was approved by the University of Science and the Technology School of Medical Sciences committee on human research publication and ethics, Kumasi, Ghana.

Working reagents for nucleic acid amplification techniques from the National Institute for Biological Standards and Controls (NIBSC, Potters Bar, UK) were used to establish the analytical sensitivity of the multiplex assay. These calibrated reagents contained 500 IU/ml of the Eurohep HBV genotype A standard reference 1 (code 98/780), 710 IU/ml of a HCV genotype 3 donation (code 01/408) and 3,630 IU/ml of a HIV-1 subtype B isolate (code 99/634-002), respectively. In addition, a NIBSC Multiplex Working Reagent (code 99/732) containing HBV genotype A (1,000 IU/ml), HCV genotype 3 (710 IU/ml) and HIV-1 subtype B (3,630 IU/ml) was used as a multiplex standard. HBV genotype A–F panel (HBsAg subtyped panel) was obtained from the Institut National de Transfusion Sanguine, France. Two genotype panels including calibrated HCV genotypes 1–6 (NIBSC; code 02/202) and dilutions of HIV-1 subtypes A–G (HIV-1 RNA prototype subtype panel, FDA/CBER, USA) were also tested.

2.2. Viral nucleic acid isolation

HBV DNA, HCV RNA and HIV-1 RNA were isolated from 200 μl of plasma using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Viral nucleic acids were eluted from the filter column with 50 μl of nuclease-free double distilled water and stored at −80 °C until further use.

2.3. Primers and probes

The sequences of the probe BS-1 and the primers HBV-Taq 1 and HBV-Taq 2 were designed from the conserved regions of HBV surface gene as previously described (Weinberger et al., 2000). The fluorogenic probe was 5′-labelled with Cy5 dye and 3′-labelled with Black Hole Quencher 2 (BHQ-2) (PROLIGO France SAS, Paris, France). HCV-specific primers MAD-1 and MAD-2 and probe MAD-3 were designed from the 5′-UTR region (Candotti et al., 2003b). Primers LTR-F (5′-TAAAGCTTGCCTTGAGTGCT-3′) and LTR-R2 (5′-GTCTGAGGGATCTCTAGTTACCAG-3′), and probe LTR-P (5′-AGTAGTGTGTGCCCGTCTGTTGTGTG-3′) are located in the LTR region of HIV-1 genome. MAD-3 and LTR-P probes were 5′-labelled with FAM and VIC, respectively, and 3′-labelled with TAMRA (Applied Biosystems, Warrington, UK).

2.4. Multiplex real-time RT-PCR

Viral DNA and RNA were amplified using the Mx4000™ Multiplex Quantitative PCR System (Stratagene, La Jolla, CA, USA). Amplification was performed with the Brilliant™ Two-Step Quantitative RT-PCR Core Reagent kit (Stratagene, La Jolla, CA, USA). The reverse transcription (RT) reaction contained 1× core RT buffer, 0.5 mM each dNTP, 20 ng/μl random primers, 20 units StrataScript RT®, 20 units RNasin® and 10 μl of template nucleic acid preparation per 20 μl reaction. The reaction was carried out at 25 °C for 5 min, 45 °C for 60 min and 95 °C for 3 min. The Q-PCR reaction contained 1× core PCR buffer, 5 mM $MgCl_2$, 0.3 mM each dNTPs, 0.8 μM each HCV and HIV-1 primers, 1 μM each HBV primers, 0.2 μM each fluorogenic probe, 0.3 μM reference dye ROX, 2.5 U SureStart Taq polymerase and 10 μl of template cDNA preparation per 50 μl reaction. After an initial incubation at 95 °C for

10 min, 50 two-step cycles of 1 min at 60 °C and 30 s at 95 °C were carried out.

2.5. Viral nucleic acid quantification

HBV DNA and HCV RNA were quantified individually as previously described (Allain et al., 2003; Candotti et al., 2003a,b). HIV-1 RNA was quantified using the Mx4000™ Multiplex Quantitative PCR System and the Brilliant™ Two-Step Quantitative RT-PCR Core Reagent kit (Stratagene). A RT reaction contained 1× core RT buffer, 0.5 mM each dNTP, 2 μM LTR-R2 primer, 20 units StrataScript RT®, 20 units RNasin® and 10 μl of template RNA preparation per 20 μl reaction. The reaction was carried out at 25 °C for 5 min, 45 °C for 60 min and 95 °C for 3 min. The Q-PCR reaction contained 1× core PCR buffer, 5 mM $MgCl_2$, 0.2 mM each dNTPs, 0.4 μM LTR-F and LTR-R2 primers, 0.2 μM VIC-labeled LTR-P probe, 0.3 μM reference ROX dye, 2.5 U SureStart Taq polymerase and 10 μl of template cDNA preparation per 50 μl reaction. After an initial incubation at 95 °C for 10 min, 50 two-step cycles of 1 min at 60 °C and 30 s at 95 °C were carried out. For each run duplicates of a 10-fold serial dilution of WHO International Standard for HIV-1 RNA for NAT assays 97/656 (NIBSC) containing 4–4000 IU of HIV-1 genome per RT reaction were analyzed. The sensitivity of the method was estimated using the WHO standard 97/656 and the Probit sofware at 400 IU/ml.

3. Results

3.1. Optimization of a multiplex real-time RT-PCR

Three previously developed individual real-time Q(RT)-PCRs were combined to generate a multiplex real-time RT-PCR for simultaneous detection and quantification of HBV, HCV and HIV-1 genomes. Optimum conditions for the multiplex RT-PCR assay were achieved by doubling the amount of reverse transcriptase (20 U per reaction) for cDNA preparation and the concentration of dNTPs (1.2 mM) in the amplification reaction. Increasing the concentration of polymerase did not significantly improve the amplification reaction. It was maintained at 2.5 U per reaction. Particular attention was paid to the concentration of $MgCl_2$ as the optimal concentrations were different in the single virus Q-PCR. The $MgCl_2$ concentration was balanced to 5 mM that provided optimal HBV and HCV amplification whereas the efficiency for HIV-1 was reduced. A 0.5–2 cycles delay of the C_t values was observed for HIV-1 samples with viral loads below 10^4 IU/ml. Concentrations of primers and probes also required adjustments that resulted in the final concentrations indicated in Section 2. Increasing the concentration of HIV-1 specific primers or probe did not compensate totally for the slight loss of efficiency related to the suboptimal $MgCl_2$ concentration.

3.2. Simultaneous detection of HBV DNA, HCV RNA and HIV-1 RNA

To determine the ability of the multiplex HBV/HCV/HIV-1 assay to identify individual viruses, working reagents for nucleic acid amplification techniques from NIBSC were tested in parallel. Serial dilutions were prepared in human plasma negative for all three viruses, and four replicates of each dilution were tested. The fluorescence emitted by each virus-specific probe being of different wavelength, viral DNA or RNA was directly identified. Analysis of the C_t values obtained for the three independent dilution series showed linear curves for each virus with a regression coefficient >0.96.

The ability of the multiplex assay to simultaneously detect HBV DNA, HCV and HIV-1 RNA in the same sample was evaluated using serial dilutions of a mixture of three plasmas from patients infected with a single virus. In this experiment, viral loads were similar and ranged between 50 and 10^4 IU/ml. Virus specific fluorescence signals (FAM, VIC and Cy5) developed in each reaction and a regression coefficient over 0.86 was observed for each virus (Fig. 1).

The ability of the multiplex assay to detect low concentrations of viral genome in the presence of another abundant viral genome was assessed. Multiplex real-time RT-PCR was performed on samples containing a range of identical concentrations of two viruses (50 to 10^3 IU/ml of viral DNA or RNA) in the absence or presence of a relatively high concentration of a third virus (10^4, 10^5 and 10^6 IU/ml of HIV-1 RNA, HCV RNA and HBV DNA, respectively). Irrespective of the presence of the other viruses, no reproducible amplification signal was obtained below 10^2 IU/ml HCV RNA and 5×10^2 IU/ml HIV-1 RNA. In contrast, HBV DNA detection was not affected by the co-amplification of high load of either HCV or HIV-1 RNA. Similarly, HCV and HIV-1 RNA detection was not affected by the presence of a high HBV DNA load. The presence of high HIV-1 RNA concentration slightly increased the number of thermal cycles required to detect 10^2 IU/ml of HCV RNA (+1 C_t value) but it had no effect on the detection of higher HCV RNA loads. In contrast, irrespective of the HIV-1 RNA concentration tested, the HIV-1-specific fluorescent signal was significantly delayed (+2 to 3 C_t values) in the presence of a high HCV RNA load, and the signal was no longer correlated with the number of HIV-1 targets initially present in the reaction. However, the sensitivity of the multiplex assay was not affected by the presence of high viral loads as indicated by the clearly detectable signals still generated by 50, 100 and 500 IU/ml of HBV DNA, HCV RNA and HIV-1 RNA, respectively.

3.3. Sensitivity of the multiplex assay and detection of viral variants

The analytical sensitivity of the multiplex HBV/HCV/HIV-1 assay was initially established separately for each of

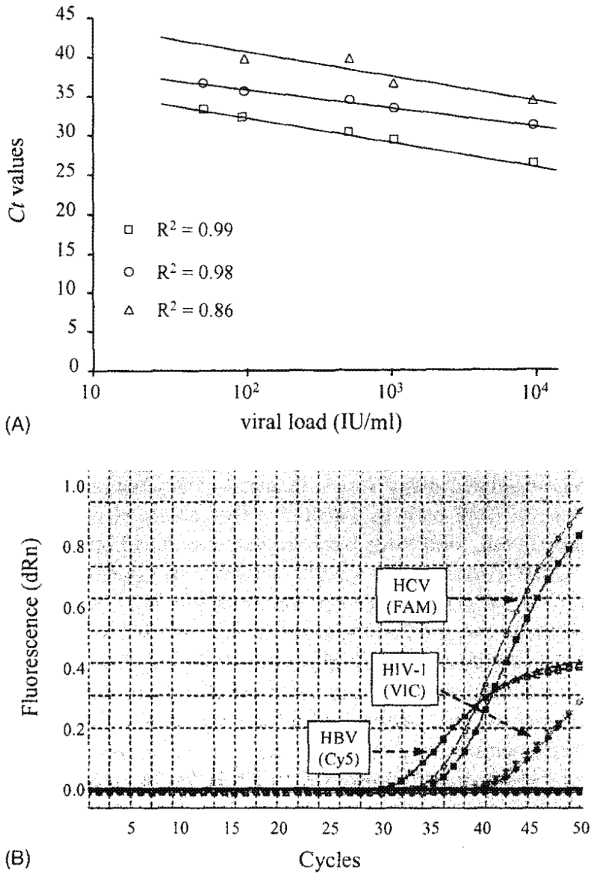

Fig. 1. Simultaneous amplification of HBV DNA, HCV RNA and HIV-1 RNA. (A) Plot of threshold cycle number (C_t) vs. serial dilutions ($\log_{10}$) of a mixture of three plasmas from patients infected with a single virus (□: HBV, ○: HCV, Δ: HIV-1); dilutions ranging from 50 to 10,000 IU/ml of viral genomes were tested; regression coefficient for each virus is indicated. (B) Amplification plots generated within the Mx4000TM Multiplex Quantitative PCR System instrument (Stratagene) from a sample containing 500 IU/ml of each HBV DNA (Cy5 signal), HCV RNA (FAM signal) and HIV-1 RNA (VIC signal); The *x*-axis is the time from the initiation of the amplification; the *y*-axis is the increase in fluorescence (dRn); threshold fluorescence is shown as the horizontal line.

the three viruses using the individual Working Reagents from NIBSC (codes 98/780, 01/408 and 99/634-002) and Probit software. The 95% detection limit was 47 IU/ml for HBV DNA, 132 IU/ml for HCV RNA and 674 IU/ml for HIV-1 RNA. The detection limit of the multiplex assay when the three viruses were simultaneously present was determined using dilutions of a multiplex Working Reagent from NIBSC (code 99/732). The 95% detection limit was 30 IU/ml for HBV DNA, 167 IU/ml for HCV RNA and 680 IU/ml for HIV-1 RNA.

To assess the ability of the multiplex assay to detect the whole range of HBV, HCV and HIV-1 genotypes, three panels were tested. The HBV genotype A–F panel, the HCV genotype 1–6 and the HIV-1 subtype A–G were tested. All genotypes in each panel were detected with similar efficiency. The detection limit observed ranged from 25 to 50 IU/ml for the HBV panel, from 50 to 200 IU/ml for the HCV panel, and from 540 to 875 IU/ml for the HIV-1 panel.

3.4. Intra-/inter-assay variability

Intra-assay variability was determined by testing eight replicates of three samples each containing 100, 500 or 10^4 IU/ml of HBV, HCV and HIV-1. Variation of the C_t values ranged from 26.1 ± 0.2 to 32.9 ± 0.3 (0.8–1% variation) for HBV, from 30.3 ± 0.2 (0.8–2.4% variation) for HCV, and from 33.5 ± 0.5 to 38 ± 1.3 (1.6–3.4% variation) for HIV-1. Inter-assay variability was based on data collected from six independent nucleic acid purification and amplification reactions conducted on 10-fold dilutions of a different mixture of the three viruses due to volume limitation. The inter-assay variability expressed as % C_t ranged between 3.4 and 3.6% at 10^4 and 10^7 IU/ml for HBV, between 2.6 and 2.9% at 10^3 and 10^5 IU/ml for HCV, and between 7 and 3.4% at 3×10^2 and 3×10^4 IU/ml for HIV-1.

3.5. Viral load quantification using multiplex real-time RT-PCR

Three plasma samples containing either HBV, HCV or HIV-1 were calibrated against the WHO standards and mixed in order to generate a preparation containing 10^4 IU/ml of each virus to be used as an in-house multiplex standard. Serial dilutions of this preparation were tested with the multiplex assay and reference curves were generated for HBV (slope: 1.59; $R^2 = 0.99$), HCV (slope: 1.19; $R^2 = 0.95$) and HIV-1 (slope: 1.28; $R^2 = 0.95$). Serial dilutions of three independent plasmas, each of them containing only one virus, were tested in parallel. The regression curves obtained for all dilution series showed similar slopes when compared to the corresponding reference curves (1.57, 1.44 and 1.33 for HBV, HCV and HIV-1 dilutions, respectively). The regression coefficient was 0.99 for HBV and HCV, and 0.95 for HIV-1. These results suggested that the multiplex assay was suitable for viral genome quantification when only one virus was present in the sample tested.

Twenty-four HBV-containing samples (genotypes A–F; viral load ranging from 15 to 10^5 IU/ml), 14 HCV-containing samples (genotype 1–6; viral load ranging from 300 to 10^5 IU/ml) and 39 samples containing HIV-1 (subtypes A–G; viral load ranging from 200 to 2×10^5 IU/ml) were quantified using the multiplex assay and results were compared to viral loads previously determined with single real-time Q(RT)-PCRs as previously described (Allain et al., 2003; Candotti et al., 2003a,b). As shown in Fig. 2, viral loads determined with the multiplex assay and the single virus Q(RT)-PCR were closely correlated for HBV ($R^2 = 0.94$ and $P < 0.0001$), HCV ($R^2 = 0.95$ and $P = 0.0017$), and HIV-1 ($R^2 = 0.87$ and $P < 0.0001$). For each virus, discrepancies between multiplex and single quantitative assays increased below 500 IU/ml.

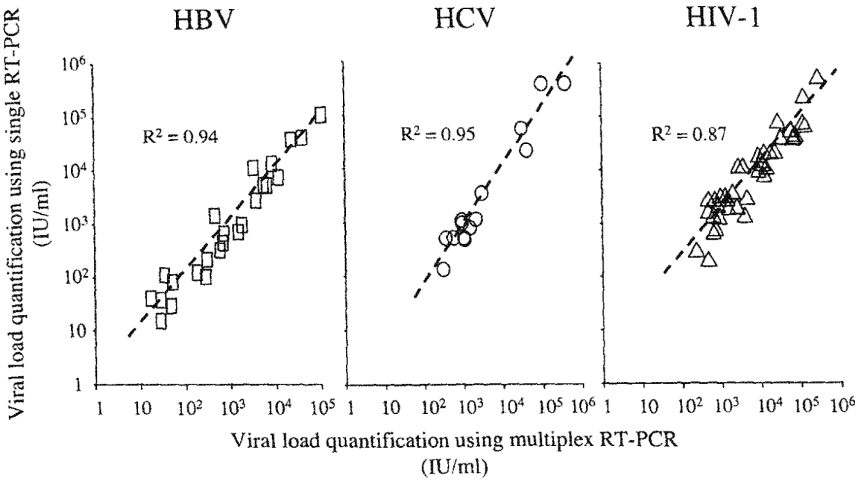

Fig. 2. Relationship between viral loads quantified with multiplex RT-PCR and single (RT)-PCR. Twenty-four HBV-containing samples of genotypes A–F with viral load ranging between 15 and 10^5 IU/ml (□), 14 HCV genotypes 1–6 containing samples with viral load ranging between 3×10^2 and 10^5 IU/ml (○), and 39 HIV-1 subtypes A–G containing samples with viral load ranging between 2×10^2 and 2×10^5 IU/ml (Δ) were tested.

3.6. Defining virus-specific cut-offs for the multiplex assay in pools of 10 plasmas

The multiplex assay was used to screen for HBV DNA, HCV RNA and HIV-1 RNA in minipools of 10 plasmas from Ghanaian blood donors negative for serological viral markers using rapid screening tests as previously described (Allain et al., 2003). To define a cut-off number of C_t for each individual virus signal when using the multiplex assay to screen clinical samples, results obtained with 44 multiplex positive plasma pools were analysed. Forty-one of these 44 plasma pools (93%) tested HBV DNA positive. All these 44 pools were then resolved by testing the individual donations included in these pools with HBV Q-PCR. One to 10 HBV positive individual donations were detected in 37 of the 41 positive pools. No individual sample in four positive pools (average C_t: 43 cycles, range: 41–46 cycles) was reactive by HBV Q-PCR and these pools were considered false positive. Similarly non-reproducible low fluorescence signal was randomly observed in 19 out of 88 (21.5%) HBV-, HCV-, HIV-1-negative controls (average C_t: 44 cycles, range: 38–49 cycles) tested in different multiplex runs. Two HCV (2%) and one HIV-1 (1%) false positive results were also obtained with C_t values of 38 and 47 cycles, respectively. No specific amplicon was detected in these samples suggesting that the fluorescence signal did not result from minor contamination event but rather from background fluorescence due to the degradation of the probe above 38 cycles of amplification. The analysis of the ratio between confirmed positive and false positive samples at each amplification cycle indicated that an HBV signal detected below 40 cycles of amplification had a predictive value over 90% and a viral load = 10 IU/ml. Due to the lack of data, the same analysis could not be performed for HCV and HIV-1. However, according to data accumulated on low viral load standards when assessing assay sensitivity, a signal obtained below 37 cycles for HCV and below 40 cycles for HIV-1 had a predictive value >95% and a viral load = 167 IU/ml and = 680 IU/ml, respectively. As a result of this analysis, cut-offs of 40, 37 and 40 cycles were used for HBV, HCV and HIV-1 NAT with the multiplex assay, respectively.

A total of 267 pools of 10 plasmas were tested with the multiplex assay using the cut-offs defined above (Table 1). HBV DNA was detected in 116 pools (43%). Twenty-four

Table 1
Multiplex assay testing of 10-plasmas minipools

	HBV reactive	HCV reactive	HIV-1 reactive
Multiplex testing of 267 pools (%)	116 (43.4%)	8 (3%)	1 (0.4%)
Pool resolution			
No. of pools resolved	24	7	1
No. of individual plasmas tested	235	70	10
No. of reactive individual plasmas (%)	79 (34%)	4 (5.7%)	1 (10%)
No. of reactive plasmas per pool (range)	1 – 10	1	1
Viral load range (IU/ml)	$6 - 2.4 \times 10^4$	$4 \times 10^5 - 2.6 \times 10^6$	10^5
No. of pools confirmed reactive (%)	24 (100%)	4 (57%)	1 (100%)

positive pools were resolved and HBV DNA was detected in 79 of 235 individual plasmas (34%) with viral load ranging between 6 and 2.4×10^4 IU/ml. One to 10 HBV DNA containing plasmas were found in the positive pools. We further obtained evidence that positive pools containing more than three HBV DNA containing individual samples corresponded to contamination occurring at the initial donor sampling level. Individual samples considered true positive contained anti-HBc. Eight pools (3%) were positive for HCV RNA (average C_t: 32.5 cycles, range: 30–36) and seven of these pools were resolved using HCV Q-RT-PCR. Four positive pools (C_t of 27–30 cycles) contained one HCV positive individual plasma with viral load ranging from 4×10^5 to 2.6×10^6 IU/ml. These samples were anti-HCV positive by EIA. No HCV positive sample was detected in the other three pools. These three pools had borderline C_t numbers (36–36.5 cycles) and were considered false positive. Finally, one pool (0.4%) was identified as HIV-1 positive and contained one positive donation with a viral load of 10^5 IU/ml. Retrospective investigations revealed that all the HCV-infected donations had been included in the pool study without having been tested for anti-HCV antibodies due to a temporary break in test supply and the HIV-1-infected donation was missed by EIA (Innogenetics) during a short-time interruption of the rapid test supply.

Although a rare occurrence, the multiplex assay performance in a sample serologically reactive for HBsAg, anti-HCV and anti-HIV was tested. HBV DNA and HIV-1 RNA were simultaneously detected and this result was confirmed by specific individual (RT)-PCR assays. No HCV RNA was amplified using the multiplex assay or HCV single QRT-PCR indicating that the patient had recovered from HCV infection as frequently found in West Africa (Candotti et al., 2003b). Finally, five plasmas from previously documented HCV/HIV-1 dual-infected asymptomatic patients were tested with the multiplex assay. Both viral genomes were detected in all samples.

4. Discussion

In this study, we investigated a multiplex real-time RT-PCR assay for the simultaneous detection and identification of HBV DNA, HCV RNA and HIV-1 RNA. The development of such multiplex NAT is made difficult by the different enzymes and ion optimal concentrations required by each target virus amplification and detection. To overcome these difficulties, the optimization of the multiplex assay was focused first on the design of three sets of primers and probes.

This optimization process resulted in a multiplex real-time RT-PCR assay that detected and differentiated HBV DNA, HCV RNA and HIV-1 RNA simultaneously in infected plasmas. Although a multiplex assay for detecting HBV, HCV and HIV-1 has been previously reported, the whole process involved two separate amplification and detection steps that were labor-intensive and time-consuming (Defoort et al., 2000). In contrast, the use of real-time RT-PCR presented the benefits of increased speed due to reduced cycle time, removal of post-PCR detection procedures minimizing the risk for carry-over contamination, and using fluorogenic labels and sensitive signal detection (Mackay et al., 2002). The AMPLINAT MPX test based on TaqMan real-time PCR on the ABI PRISM 7700 Analyser (Meng et al., 2001) and the transcription-mediated amplification-based Procleix Ultrio assay (Linnen et al., 2002) were unable to discriminate between the three viruses. A secondary diagnostic step using virus-specific amplification was necessary to identify the origin of the test signal. The multiplex assay developed here provided direct, single step, identification of amplified viral genome by taking advantage of two recent technical improvements in the field of real-time PCR. First, the development of dark or 'black-hole' non-fluorescent quenchers that increased the number of fluorophores available with minimum overlap between emission spectra, and second, a real-time PCR platform (Mx4000™, Stratagene) that incorporated a tungsten light source emitting light over a broad range of wavelengths (350–750 nm). By combining these two technical innovations, it became possible to include in the reaction three spectrally discernable fluorescent oligoprobes and, consequently, to achieve immediate identification of the amplified target.

Analysis of the amplification data obtained with calibrated multiplex standard reagents and dual-infected clinical samples demonstrated differences in primers and probes compatibility, as well as in efficiency when simultaneously used for amplification. RNA virus genomic amplification showed higher intra- and inter-assay variations compared to DNA virus amplification suggesting that the RT step was the main source of variability in this assay as previously reported for quantitative RT-PCR (Freeman et al., 1999). No major interference was observed between the three simultaneous amplifications. A similar sensitivity was obtained for each virus when present alone or co-amplified with the other two. Our data also showed that the efficiency of HBV DNA amplification was not affected by the simultaneous amplification of HCV or HIV-1 RNA and vice-versa, as previously reported (Mercier et al., 1999). In contrast, there was evidence of competition between the two viral RNA amplifications that affected mainly the yield of HIV-1 amplification. However, HIV-1 detection was delayed rather than inhibited.

The competition between HCV and HIV-1 amplification appears to be reduced when the two viruses are present in similar amount as indicated by the parallel slopes of the standard curves generated using a solution of the three viruses as standard (Fig. 1). This result suggested that quantification might be possible at least when a single virus is present. Indeed, the calculated viral load from either HBV-, HCV- or HIV-1-infected samples after HBV PCR, HCV or HIV-1 RT-PCR correlated well with the viral load obtained from the same samples after the multiplex RT-PCR (Fig. 2). However, in agreement with the competition data,

poor correlation was found for HCV and HIV-1 viral load in plasmas samples co-infected with HCV and HIV-1 tested by individual RT-PCR or multiplex RT-PCR. When used in area of high endemicity for both HCV and HIV-1 such as some part of Sub-Saharan Africa, this competition may affect the performance of the multiplex assay as a quantitative but not a qualitative assay.

Compared to AMPLINAT MPX and Procleix Ultrio assays, the multiplex RT-PCR assay showed a lower analytical sensitivity especially for HIV-1. The difference in sensitivity between these three assays can be in part explained by differences in the volume of plasma used for nucleic acid extraction and the final amount of DNA/RNA target molecules loaded in the amplification reaction. The Procleix Ultrio and the AMPLINAT MPX assays are performed with 500 and 200 μl of plasma, respectively, and the totality of the extracted nucleic acids is used in the amplification reaction. Consequently, the amount of target molecules loaded in each amplification reaction is 12.5 and 5 times higher in the Procleix Ultrio and the AMPLINAT MPX, respectively, than in this multiplex real-time RT-PCR. The sensitivity of this assay can be improved by increasing the volume of plasma tested as previously reported for the Amplis-creen assay (Roche Diagnostic Systems) (Jongerius et al., 2001). Considering the multiplex assay format, the volume of plasma should be increased to at least 1 ml to increase significantly the input of target molecules in the reaction. The manual nucleic acid purification method used in this study was not designed for such high volumes. Automated methods recently developed to accommodate larger volume of plasma are becoming available. The reduced HIV-1 sensitivity may also be explained by the fact that the reaction conditions are not optimal for HIV-1 amplification in the multiplex assay. A 95% limit detection of 400 IU/ml is achieved when a HIV-1 single real-time RT-PCR was performed using the same primer-probe set (data not shown).

HCV and HIV-1 are characterized by a high genetic variability with several genotypes (6 HCV genotypes) or subtypes (11 subtypes in HIV-1 major group M) differing from each other by ~30% over the complete genome (Janssens et al., 1997; Zein, 2000). Seven HBV genotypes have also been identified with 8–15% divergence between the groups (Kidd-Ljunggren et al., 2002). In this study, the multiplex RT-PCR assay detected with similar efficiency reference strains of HBV genotypes A–F, HCV genotypes 1–6, and HIV-1 subtypes A–G. A similar range of detection of HBV, HCV and HIV-1 genetic variants has been reported for the AMPLINAT MPX (Meng et al., 2001).

The window period preceding seroconversion remains the main cause of the residual risk of viral transmission in blood transfusion. Although the sensitivity of the serological tests has improved in recent years, nucleic acid testing provides the most effective mean to reduce the window period. Viral loads of HBV DNA, HCV RNA and HIV-1 RNA ranged between 10^1 and 10^4 IU/ml (or 10^2 and 10^5 geq/ml), 10^5 and 10^6 IU/ml, and 2×10^2 and 10^5 IU/ml (or copies per ml), respectively (Biswas et al., 2003; Meng et al., 2001). In addition, the ramp up doubling time was calculated at 0.5 and 1 day for HCV and HIV-1, respectively, indicating that, for these two viruses, the difference in window period time reduction with 50 or 500 IU/ml test sensitivity was less than a day. The multiplex assay HCV sensitivity will be sufficient to detect HCV window period donations when tested individually or in various pool sizes. Due to a lower sensitivity for HIV-1, the assay is likely to be slightly less effective in reducing the HIV-1 window period. Although blood donations are commonly tested for HBV surface antigen, HBV transmission remains the main residual risk of transfusion-associated viral infection (Soldan et al., 2003). With HBV, the residual risk is not only related to window period new infections before HBsAg becomes detectable, but also to chronic carriers with anti-HBc but no detectable HBsAg (Allain et al., 1999; Soldan et al., 2003). Previous studies have shown that such HBsAg-negative, anti-HBc-reactive donations contain low level of HBV DNA (Kleinman et al., 2003; Sato et al., 2001). Consequently, multiplex assays require high HBV DNA sensitivity to reduce the risk of both the window period and chronic infections.

The preliminary data presented in Table 1 indicates a very high frequency of low level HBV DNA in Ghanaian blood donors testing negative with a 5 ng/ml sensitive HBsAg rapid test. The low viral load detected in some individual samples of resolved positive pools suggests that 10-plasmas minipool screening might not be sufficiently effective as suggested by others (Biswas et al., 2003). A false-positive rate of 0.17% has been reported using the AMPLINAT MPX (Mine et al., 2003). In the preliminary study presented here, 100% specificity was observed for HBV and HIV-1 amplification and 98.9% for HCV amplification (three false positive results among 266 samples tested). These three samples were borderline positive indicating that the risk of false positive result may increase with the number of amplification cycles as previously suggested by other groups (Lanciotti and Kerst, 2001; Schutten et al., 2000).

The inclusion of an internal control in the assay remains an approach to achieve a higher degree of confidence in negative results. The internal control should be added prior to sample processing to control for nucleic acid recovery, presence of inhibitors in the extracted samples, and technical errors during the testing process (Rosenstraus et al., 1998). Plasmid DNA or in vitro RNA transcript have been previously used as synthetic internal control (Drosten et al., 2001; Pasloske et al., 1998), but a more relevant approach will be to add an unrelated RNA virus as internal control during the sample processing as previously suggested (Cleland et al., 1999). A passive reference dye is actually included in the multiplex amplification reaction to compensate for non-PCR related variations in fluorescence and to provide a stable baseline to which samples are normalized. The addition of a reference dye is optional and its removal from the reaction will liberate a fourth channel in the Mx4000 platform with excitation and emission wavelengths of 584 and 612 nm, respectively.

A real-time multiplex system has been developed for the simultaneous detection and identification of HBV, HCV and HIV-1. Despite multiplexing, quantification remains possible due to limited interference between amplification reactions. Assay performance with both standards and clinical samples indicates that the assay is reproducible, sensitive and specific. With further improvements, this assay has the potential to be used for large-scale virus screening of blood donations.

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