Development of SYBR Green-based Multiplex Real-time PCR Assay for Simultaneous Detection of HIV-1 and HCV in Co-infected Patients

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Abstract
Currently, hepatitis C virus (HCV)-related liver disease is the leading causes of death among patients with human immunodeficiency viruses (HIV). This high prevalence, to a large extent, can be attributed to overlapping transmission routes, that is, via body fluids. HIV co-infection is observed to cause a quicker progression of HCV and increase the risk of liver cirrhosis and hepatocellular carcinoma. Because both HIV and HCV therapies are expensive and present high health risks, effective and efficient clinical monitoring is vital during the treatment. The objective of this research is to develop an in-house SYBR green-based multiplex real-time PCR assay as a means of sensitive, but cheaper test to detect HIV-1 and HCV. The researchers designed primer pairs targeting the HIV-1 RNAse gene and HCV 5'UTR. Each of them amplifies 153-bp fragment with Tm of 80.0 ± 0.4°C and 87-bp fragment with Tm of 83.5 ± 0.4°C respectively.

The lower detection limits of this assay for HIV-1 and HCV are 10^5 and 10^4 copies/mL respectively. Although this assay has not met the WHO diagnostic standards for the clinical management of HIV-1 and HCV infection, it is still useful as a prototype for development of monitoring tools for the patients co-infected with HIV/HCV, especially in the rural areas where the commercial viral load assays are largely unaffordable.

Keywords: Co-infection, HIV-1, HCV, Multiplex, Real-time PCR, SYBR Green.

Introduction

In the era of highly active antiretroviral therapy (HAART) where there is a considerable decrease in the mortality rate due to the opportunistic infections caused by the patients' compromised immune system, hepatitis C virus (HCV)-related liver disease is currently the leading cause of death among the patients infected with human immunodeficiency viruses (HIV)[6].

The prevalence of HCV infection in the individuals with HIV is around 40%[7] with up to 2.3 million people globally estimated to be suffering from HIV-1/HCV co-infection[10]. This high prevalence to a large extent can be attributed to overlapping transmission routes, that is, bodily fluids especially through the parenteral routes such as injection or blood transfusion[4,6,9]. HIV co-infection is observed to elevate the HCV replication rate, further resulting in a faster progression of disease and an increased risk of liver cirrhosis as well as hepatocellular carcinoma[4,6].

HIV and HCV therapies are expensive and present high health risks. HCV infection has also been observed to increase the risk of hepatotoxicity caused by HAART[4,6], thereby denoting that an effective and efficient way of monitoring is essential. The more common and widely used diagnostic methods are serological tests that are more cost-effective. However, these methods are less suitable for monitoring because serological tests, which are based on patients' antibody production, are not in direct proportion with the actual viral load (VL).

Thus, these tests could provide high positive results even though with successful therapy and when the VL number has fallen below the sustained virological response threshold. In fact, these tests are also not sensitive enough to detect a smaller change in concentration that could indicate ineffective treatment and the need to discontinue therapy[2,9].

The current gold standard for VL detection is real-time PCR. The real-time PCR technique enables real-time monitoring, further resulting in reduced time and labor needed in post-PCR processing. The technique also allows for the amplification and differentiation of shorter fragments which will ensure higher reaction efficiency. The fluorescence spectroscopy detection method enables more sensitive assay and VL quantitation[8]. However, the currently available commercial tests are probe-based real-time PCR assays that require specific fluorescent-labeled oligonucleotide for each target such as RealTime HCV Viral Load (Abbott), Amplicor HIV-1 Monitor (Roche), or the multiplex assay cobas® MPX (Roche).

Labeled probes are expensive and introduce additional complexity to the design of the assay. An alternative is to use fluorescent dyes that will nonspecifically intercalate into DNA minor grooves such as SYBR Green. These dyes are less expensive and simpler to incorporate into the assay design[1].

The objective of this experiment is to develop a sensitive assay for the clinical management of HIV-1/HCV co-infection therapy that is more affordable and applicable for use in the rural areas. To reduce further costs, multiplex PCR technique was used. In a multiplex PCR reaction, more than

one target sequence can be amplified in one reaction. This will reduce the materials, time and labor needed during testing.

Material and Methods

Preparations of standard solutions: Plasmids containing HIV-1 RNAseH and HCV 5'UTR target sequences were used as the positive controls. The standard solutions for HIV-1, HCV and HIV-1/HCV mixture were prepared from the serial dilutions of HIV-1 and HCV plasmid stock solutions. Plasmid concentration in $\mu g \cdot mL^{-1}$ was determined by using UV spectrophotometry. Thereafter, it was converted to copy number/mL.

Oligonucleotide primers: This assay was designed based on the HIV-1 isolate HXB2 (GenBank: K03455.1) and HCV isolates commonly found in Indonesia. Primers for HIV-1 RNAseH target sequences are HIV1_F (5'-TGGGAAACATGGTGGACAGAG-3') and HIV1_R (5'-CCTGTTAGCTGCCCCATCTAC-3'). This pair amplified a 153-bp fragment.

Primers for HCV 5'UTR target sequences are HCV3_F (5'-GGAGAGCCATAGTGGTCTGC-3') and HCV3_R (5'-GCATTGAGCGGGTTTATCCAAG-3') which resulted in an 87-bp amplicon fragment. All oligonucleotides were designed and analyzed by using Primer3Plus, Oligo Analyzer Tool and uMelt.

SYBR Green real-time PCR assay: HIV-1/HCV SYBR Green-based multiplex real-time PCR assay was performed in 20-µL reaction mixtures containing 10 µL of 2X LightCycler® 480 SYBR Green I Master (Roche), 0.3 µM HIV-1 primer pair, 0.1 µM HCV primer pair and 5 µL standard solution containing varying amount of HIV-1, HCV, or HIV-1/HCV positive control plasmids.

The PCR profile for the assay begun with an activation stage at 95 °C (5.0 °C/s) for 5 min which was followed by 45 cycles of 95 °C (5.0 °C/s) for 10 s, 53 °C (2.2 °C/s) for 10 s, 72 °C (5.0 °C/s) for 20 s and 78 °C (5.0 C/s) for 17 s. At the end of the amplification cycles, a single melting temperature analysis stage was performed by 0.1 °C/s increase in temperature from 70 °C to 95 °C. The resulting peaks were analyzed to identify the HIV-1 and/or HCV specific amplicons. Amplification and analysis were performed on MyGo Pro Real-Time PCR (IT-IS Life Science) by using software from the same provider.

Results and Discussion

The assay developed in this research was evaluated qualitatively. The primary focus was on maximizing sensitivity through the optimization of primer design and concentration. Before further analysis, each primer and amplicon was investigated for melting temperature (Tm) and peak specificity. The results show that the amplicon fragments of both HIV-1 and HCV produce a specific Tm peak. HIV-1 and HCV amplicon Tm were found to be 80.0 ± 0.2 °C and 83.5 ± 0.2°C respectively (Fig. 1a–b). Electrophoresis by using 10 % polyacrylamide gel confirmed that both bands are the desired targets (Fig. 1c). Artifacts of both primer pairs including dimers and secondary structure formation, also produced Tm below 78 °C and were completely distinguished from the amplicons (Fig. 1a–b). This ensures that those artifacts will not interfere with the melting analysis of the amplicons.

To investigate the assay sensitivity, single plex real-time PCRs were performed on 10^7–10^2 copies/mL standard solution of each HIV-1 and HCV. It turns out that this assay appears to favor the amplification of HCV sequence. As seen in table 1, positive specific peaks were detected for up to 10^5 copies/mL for both HIV-1 and HCV. However, only HCV specific peak was detected at 10^4 copies/mL. No positive result was detected for HIV-1 or HCV below 10^3 copies/mL.

The finding was further supported by the observations in multiplex assays. To evaluate the ability of the assay for the simultaneous detection of HIV-1 and HCV, multiplex real-time PCRs were performed on the standard solutions containing 10^7, 10^6 and 10^3 copies/mL of each HIV-1 and HCV positive control. Successful co-amplification should display a melting peak profile as shown in fig. 2. Table 2 shows the results.

As seen in fig. 2, the endpoint amplification of HCV provides a higher fluorescence intensity relative to that of HIV-1. After optimization (data not shown), it became evident that a higher HIV-1-to-HCV primer ratio is required to obtain a decent result in the multiplex assay.

Table 1
Singleplex HIV-1 and HCV real-time PCR to investigate assay sensitivity.

Copy number/mL	HIV-1 Amplification	Mean Cq	Copy number/mL	HCV Amplification	Mean Cq
$1{,}0 \times 10^7$	+	30.318	$1{,}0 \times 10^7$	+	25.991
$1{,}0 \times 10^6$	+	35.054	$1{,}0 \times 10^6$	+	29.661
$1{,}0 \times 10^5$	+/-	39.753	$1{,}0 \times 10^5$	+	33.249
$1{,}0 \times 10^4$	-		$1{,}0 \times 10^4$	+	37.76
$1{,}0 \times 10^3$	-		$1{,}0 \times 10^3$	-	
$5{,}0 \times 10^2$	-		$5{,}0 \times 10^2$	-	
$1{,}0 \times 10^2$	-		$1{,}0 \times 10^2$	-	

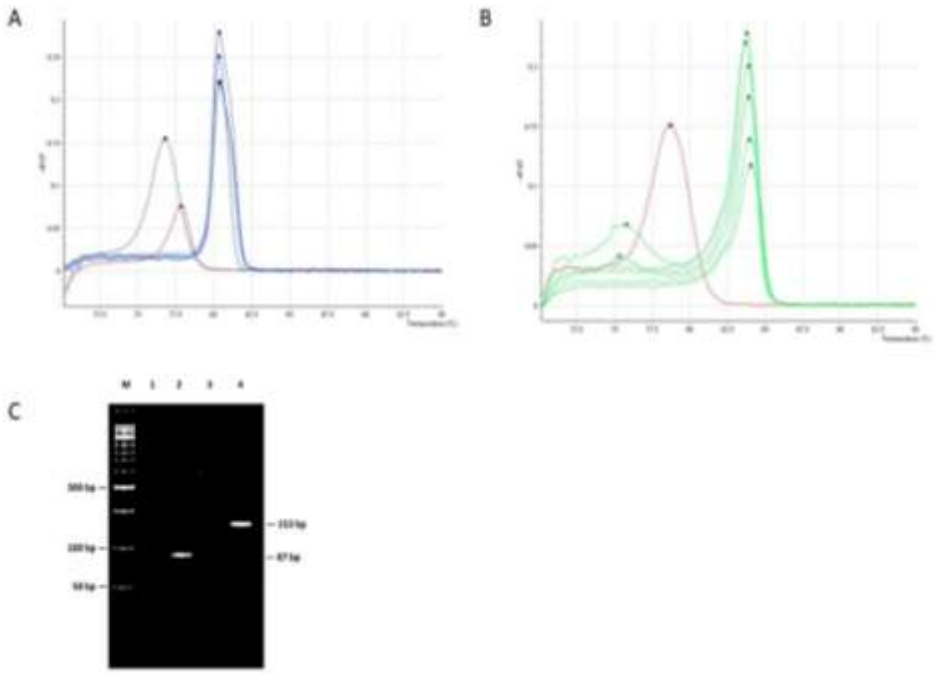

Figure 1: Both HIV-1 and HCV amplicon fragments display melting peak at specific temperatures. Melting peak below 80°Care primer dimers and artifacts as indicated by the observed peaks in NTC (red). (a) HIV-1 amplicon fragment has a specific Tm of 80 ± 0.22 °C, while that of (b) HCV is 83.54 ± 0.28^{0}C. Both fragments are confirmed with (c) 10% polyacrylamide gel electrophoresis

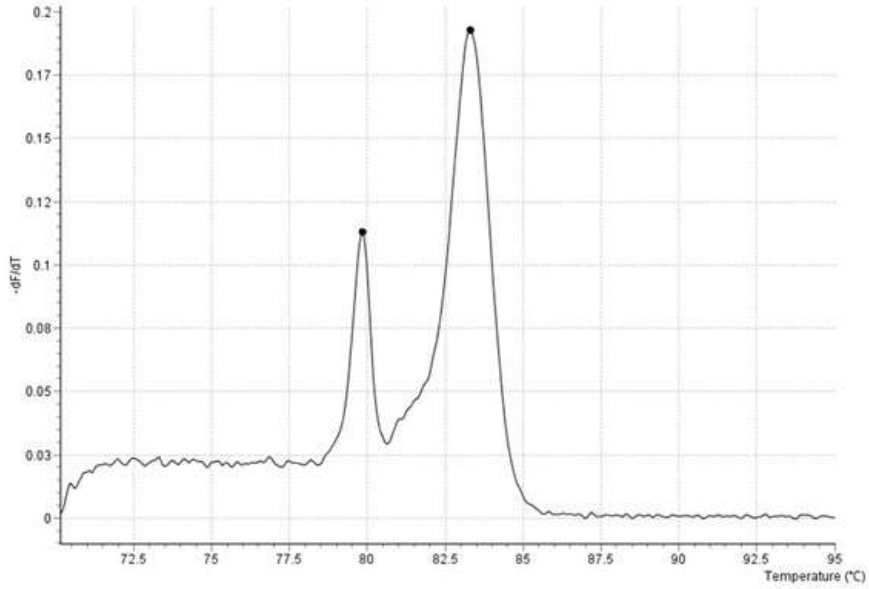

Figure 2: Melting peak profile generated by a successful multiplex real-time PCR in this assay. The specific peak for each HIV-1 and HCV can be clearly distinguished. HCV amplification gave greater fluorescence signal as indicated by the higher peak compared to that of HIV-1.

Table 2
Positive detection of HIV-1/HCV using multiplex real-time PCR assay developed in this research.

HIV-1/HCV (copies/mL)	Positive replicates	
	HIV-1	HCV
1,0 x 10^7	100 %	100 %
1,0 x 10^6	60 %	100 %
1,0 x 10^3	0 %	12.86 %

HCV amplicon fragment was also consistently detected in the earlier cycles as compared to HIV-1 based on the singleplex assays (Table 1).

There are several possibilities that may lead to this finding. First, the HCV oligonucleotide primers may be of higher quality than HIV-1 ones. Primarily, this concerns primer dimers and secondary structure formations. These structures will hinder the primer attachment on its complementary strand which negatively affects the reaction efficiency and sensitivity[3].

Another factor contributing to assay quality is the amplicon fragment profile. Shorter amplicons generally result in more efficient amplifications. They are also less susceptible to slight variations in the reaction conditions such as Ta or salt concentrations[3]. This is a very vital aspect to consider in the multiplex PCR assay because there is more than one target sequence with different primer profiles. Hence, it is unlikely to create a fully optimized reaction condition for each target. Instead, there will be adjustments to compromise each of the target sequences, which can likely result in relatively suboptimal reaction conditions for polymerase activity. Generally, the amplicon length should fall between 80 bp and 150 bp, although 150–200-bp-long amplicon is acceptable and many successful results had been reported with it[5,6].

Oligonucleotide primers designed for HIV-1 detection in this assay produce an amplicon fragment of 153 bp in length, whereas HCV primers produce a fragment of only 87 bp. Previous experiments (data not shown) demonstrated that the HCV primers were more adaptable to different Ta, ramp rates, master mixes, as well as primer concentrations and combinations.

On the contrary, HIV-1 primers demonstrated to have a considerably narrower range in those aspects. The difficulty of designing a HIV-1 target sequence with the shortest size possible, while still considering the dimers and secondary structures, is due to the low GC content of the target gene, that is, RNAseH. The RNAseH target sequence contains 46 % GC, whereas that of HCV contains 57 % GC. Using a shorter sequence of the HIV-1, RNAse will produce amplicon fragment with a lower melting temperature than 80 °C. This risks indistinguishable melting peaks among the amplicon and the primer artifacts[3].

Other than primer and amplicon profile, the structural hindrance coming from the plasmids used as the positive controls may contribute to the imbalance of the amplification. For the records, HIV-1 positive control plasmid is 7043 bp in size, whereas HCV positive control plasmid is only 2812 bp. Naturally, larger DNA strand is more prone to an incomplete dissociation and a higher structure formation. However, there is currently no evidence to support this argument, thereby warranting a further investigation. There is also a possibility of imbalance due to SYBR Green bias toward HCV amplicon fragments, but this seems unlikely.

Bustin and Huggett[3] observed that SYBR Green has a greater affinity toward AT-rich sequences. As it is explained, the RNAseH target sequence used in this research contains more AT base pairs. Moreover, the longer fragment facilitates the intercalation of more SYBR molecules[3]. If there is a possibility of bias, then it should be against HCV.

Finally, it is important to note that the reaction mixture also contributes to assay performance. Using this master mix with no modification provides the described results. This assay might perform differently by using different master mixes[3]. Increasing the polymerase concentration and adjusting salt concentration and/or composition are some of the ways to enhance the assay sensitivity without redesigning a new primer set or switching target genes[3].

Despite optimization, this assay still has not met the diagnostic standard required by the WHO for the clinical management of HIV-1 and HCV infection. The WHO requires assay with lower detection limit as little as 50 IU/mL (100–200 copies/mL) for HCV and 50–200 copies/mL for monitoring of HIV treatment[9]. Validation with real samples from patients and approved commercial assays is needed for the further development and characterization. However, it is still useful as a prototype for developing the monitoring tools for the patients co-infected with HIV/HCV, especially in rural areas where commercial viral load assays are largely unaffordable.

Conclusion

This in-house SYBR Green-based real-time PCR assay has a detection limit of 10^5 copies/mL and 10^4 copies/mL for HIV-1 and HCV respectively. HCV oligonucleotide primers display good primer qualities whereas that of HIV-1 need improvement. Replacing the amplification target for HIV-1 and modification of reaction mixture appear to be the most effective steps for further development.

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