

EDW End of Project Report

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Title: **DEVELOPMENT OF HCV GENOTYPING PROTOCOL BASED ON HIGH RESOLUTION MELTING ANALYSIS REAL-TIME PCR: A PILOT STUDY**

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Abstract: Determination of HCV genotype is important for the disease prognosis and prediction of treatment outcomes. Several methods of genotyping are commercially available and others are done in-house. Aims for the study were to elucidate nucleotide sequence of local HCV strains and to establish an in-house technique for HCV genotyping using DNA intercalating dye real-time PCR. HCV RNA was extracted and subjected to reverse transcription before amplification using SsoFast EvaGreen mix real-time PCR. Sequence analysis of the 5'UTR revealed two major groups of HCV genotypes (1 and 3). The GC content HCV genotype 1 and 3 were 57.14-58.44% and 55.19-57.14, respectively. HCV RNA was able to be detected with the PCR mix reaction. However, both main genotypes are indiscriminately clustered using high resolution melting analysis and melting temperature analysis. Further modification of the established method could result in better outcomes.

Key words: Hepatitis C virus, real-time PCR, high resolution melting curve analysis

Introduction

Hepatitis C is the major cause of liver disease worldwide, with 3-4 million people are infected each year. According to WHO, approximately 150 million people have chronic HCV infection and if untreated, can lead to liver cirrhosis and/or liver cancer (World Health Organization, 2017). In Malaysia, more than 400 000 people are agonized with the disease and currently it has become one of the major clinical and social burdens to the society (McDonald et al., 2014).

Hepatitis C virus is small (about 9.6 kb in size), positive sense single stranded RNA virus in the Flaviviridae family. The genome contains highly conserved untranslated regions (UTRs) at both the 5' and 3' termini which flank a single open reading frame encoding structural and non-structural proteins. Structural proteins include core protein, E1 and E2;

nonstructural proteins include NS2, NS3, NS4, NS4A, NS4B, NS5, NS5A, and NS5B. There are 7 major types of HCV, which can be further subdivided into more than 67 subtypes (Smith et al., 2014). The occurrence of HCV genotypes vary with geographic regions. Genotype 1, 2 and 3 are distributed globally while genotype 4, 5, and 6 are very common in geographic areas where chronic hepatitis C is highly prevalent. Meanwhile, in Malaysia, HCV genotype 1, genotype 3, genotype 4 and genotype 6 are commonly found among HCV seropositive persons (Ho, Ng, Kaur, & Goh, 2015; Mohamed, Rashid, Wong, Abdullah, & Rahman, 2013). The genotype of HCV is clinically important in determines the severity and aggressiveness of liver infection, as well as patient response to viral therapy and required duration of such therapy.

High resolution melt analysis (HRM analysis) is a new quantitative analysis of the melt curves for DNA fragments introduced in 2002 (Reed G, Kent J, & Wittwer C, 2007). HRM is the simplest method for genotyping, mutation scanning and sequence matching. High resolution melting assays require a real-time PCR detection system and HRM-dedicated software. The combination of PCR instrumentation and the next generation of saturating DNA-binding dyes permits the identification of small variations in nucleic acid sequences by the controlled melting of double-stranded PCR amplicons. These dyes (e.g., LCGreen, SYTO9, EVAGreen) were developed so that it can be used at concentration that result in saturation of all DNA binding sites without inhibiting the PCR reaction, which is much efficient than the second generation SYBR Green dye. Thus, reliable real-time PCR machine coupled with HRM-compatible software permit the rapid analysis of the resulting data sets and the discrimination of DNA sequences based on their composition, length, GC content, or strand complementary (Garritano et al., 2009).

In virology, HRM analysis for virus type determination has been reported elsewhere, such as HIV (Cousins et al., 2012), Norovirus (Tajiri-Utagawa, Hara, Takahashi, Watanabe, & Wakita, 2009) and human papillomavirus (Lee, Wu, Tseng, & Qiu, 2012). However, this technology application for the viruses and hepatitis C virus are in-house basis, which some are not reproducible due to different setting and availability of instruments (Athar et al., 2015; Li et al., 2010; Nazemi, Tazehabadi, Jafarpour, & Sharifi, 2011; Wu et al., 2017). Thus, we would like to conduct this study using our own methodology and local samples, in a pilot design, for a simple and efficient HCV genotyping technique establishment.

Objectives

1. To elucidate nucleotide sequences of local HCV strains.
2. To establish a HCV genotyping technique using third generation intercalating DNA dye high melting curve analysis.

Methodology

Patients

Forty (40) patients attending Medical Outpatient Departments, Tengku Ampuan Afzan Hospital were recruited for the study. Patients were confirmed their status as positive for anti-HCV. Blood specimens was collected in plain tubes and transferred to the Microbiology Laboratory, Kuliyah of Medicine, IIUM.

Sample processing

Serum samples were aliquoted and stored at -70°C until further used. Total RNA extraction was carried out using QIAamp Viral RNA extraction kit according to the manufacturer's instructions (Roche, Germany).

Reverse transcription

Briefly, a viral RNA was reverse-transcribed at 42°C for one hour in a final volume of 21.5 µl containing 10 µl of extracted RNA, 1 µl of random primer, 1 x of reaction buffer, 1 µl of 10 mM dNTP mixture, 200 U of SuperscriptTMIII reverse transcriptase, 20 U of recombinant RNasin ribonuclease inhibitor. The reaction was then denatured at 99°C for one minute to inactivate the reverse transcriptase.

Polymerase Chain Reaction (PCR) and HCV genotyping

Detail procedure for PCR, DNA sequencing and phylogenetic analysis has been described before (Hairul Aini, Mustafa, Seman, & Nasuruddin, 2012). Briefly, the reverse transcription products were amplified using conventional polymerase chain reaction (PCR). Later, the PCR products were clean up and send for DNA sequencing. The nucleotide sequences were then analysed to construct the phylogenetic tree for HCV grouping. The GC content for each sequence was calculated using software available online (<http://www.endmemo.com/bio/gc.php>)

Real-time Polymerase Chain Reaction and HRM analysis

PCR amplification was performed in a 96 well plates using CFX96 Real-Time PCR System (Bio-Rad, USA). Reaction conditions were modified to suit the real time PCR protocol as outlined by the SsoFast EvaGreen Supermix (Bio-Rad, Singapore) for the CFX96. The conditions were as follow: 30 sec at 95°C, followed by 35 cycles of 5 sec, melt at 95°C, and 5 sec anneal/elongation at 60°C.

The process was monitored after each elongation step by EvaGreen dye bound to the amplified 153 bp fragment products. Single fluorescence was detected in each cycle at 60°C. Following the final amplification step, specificity was verified using melting curve analysis by heating to 65°C, followed by slow heating (0.5°C per second) to 95°C with continuous fluorescence recording. Melting curves were recorded by plotting fluorescence signal intensity versus temperature.

High resolution melting (HRM) analysis was performed using the Bio-Rad Precision Melt Analysis software. This application software analyzes the HRM curve data to identify changes in the shape of the curve that indicate sequence polymorphisms, thus discriminate the different types of HCV nucleotide sequences.

Findings

The distribution of HCV genotypes in study samples

5'UTR-based sequencing assays were successful in 39 out of 40 isolates (97.5%) where 31 out of 40 (77.5%) isolates could be sequenced in NS5B-based assays (Table 1.0). However, there was one isolate with mixed-genotype infection found in this study where it was initially identified as genotype 1a via 5'UTR-based assay and later was identified as genotype 3a by NS5B-based assays. The GC content of HCV genotype 3 was range from 55.19-57.14 %, meanwhile the GC content of the HCV genotype 1 was 57.14-58.44%, respectively.

Table 1.0: Distribution of HCV genotypes and subtypes

Genotype and subtype	5'UTR-based assays [39/40] (%)	NS5B-based assays [31/40] (%)	Mono infection, 5'UTR + NS5B [30/40] (%)	Mixed-genotype infection, 5'UTR + NS5B [1/40]

				(%)
3a	28 (70)	24 (60)	23 (57.5)	1 (G1 + G3) (2.5)
1a	9 (22.5)	5 (12.5)	5 (12.5)	
1b	1 (2.5)	1 (2.5)	1 (2.5)	-
3b	1 (2.5)	1 (2.5)	1 (2.5)	-

Note: G1= Genotype 1, G3= Genotype 3

Real-time PCR analytical specificity and melting curve profile

Out of 40 samples, 38 were amplified using the Ssofast EvaGreen Mix kit to generate 153 bp of amplicon size. The specificity of primer sets used for the assay was initially evaluated using BLAST analysis, which confirmed that primers were homologous to HCV 5'UTR sequence only. The analytical specificity of the real time PCR was determined by evaluating the melting curves and the agarose gel electrophoresis for 4 specimens, including samples PAT 7 (genotype 3a), 20 (genotype 3a), 22 (genotype 3a), and 23 (genotype 1a), that are co-infected with HIV and HBV.

The melting curve profiles revealed a single mean peak, indicating that primers targeted a single gene and yielded one amplicon only, regardless the genotype of HCV or the presence of HIV and HBV (Figure 1.0). The agarose gel electrophoresis confirmed the specificity of amplification where amplicons separation by electrophoresis demonstrated single bands of the expected size (153 bp), which verify the absence of non-specific amplification (Figure 2.0). The homology of primers and single amplicons shown in the melting curves and gel electrophoresis demonstrate the high level of the specificity of the assay.

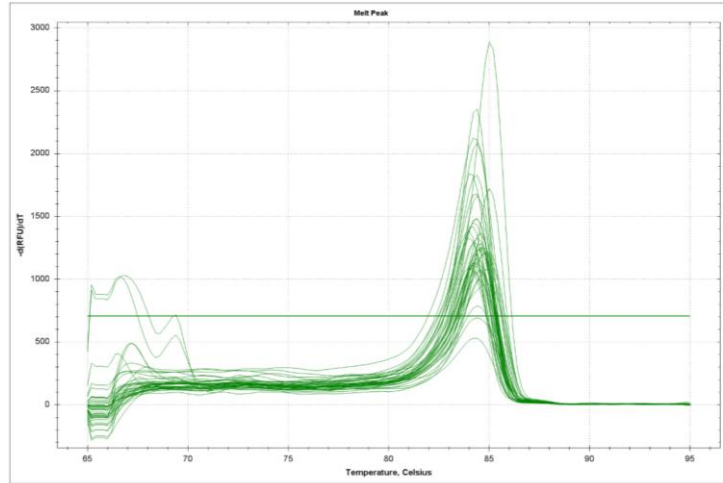


Figure 1.0: Analysis of melting curves of semi-nested real time PCR.

Amplicon dissociation of HCV-positive samples co-infected with HIV and HBV showed single peaks around 85°C, indicating the amplification of a single target gene.

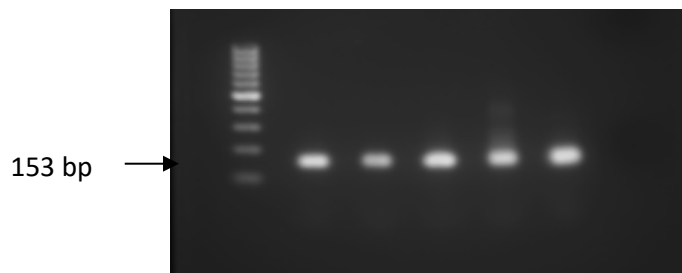


Figure 2.0: Agarose gel electrophoresis of semi-nested real time PCR products.

Analysis of amplicons on agarose gel showed single bands at the correct size (153 bp). Lane 1: 100 bp DNA Ladder (Thermo Fisher Scientific, USA); Lanes 2 to 5: HCV-positive samples co-infected with HIV and HBV; Lane 6: Positive control; Lane 7: Negative control.

Melting temperature analysis and HRM assessment

Specific melting temperature for each HCV strain was analysed to get particular feature of the melting temperature and HCV genotypes. Out of 40 samples, 35 gave substantial data (>95% confidence) for analysis. HCV strains were grouped into two categories, which were genotype 3 and genotype 1. Regardless of the groups, the range of melting temperature was 84.0°C – 85.0°C. There was no unique melting temperature point

which could differentiate the two groups. This is supported by the high resolution precision melting analysis, which the study panel could be clustered into 4 groups; with cluster 1 was the majority regardless of the genotypes.

Discussion

HCV genotypes 1 and 3 are the most common genotypes found globally. In Malaysia, these two genotypes are encountered circulate among HCV-infected patients (Ho et al., 2015; Mohamed et al., 2013; Wasitthankasem et al., 2015). Similarly, in this study, both of these HCV genotypes were detected (Table 1.0) using 5'UTR and NS5B-based assay and DNA sequence analysis.

The technique used was sensitive enough to amplify the template without much optimization, despite having 2 samples unamplified. This makes the Ssofast EvaGreen Mix (BioRad, US) convenient to be used as the amplification kit for HCV RNA detection. The turn-round time for complete amplification until melting curve generation was also rapid, which is about 1 hour. Thus, the kit is suitable for HCV detection and should be considered for HCV viral load application.

The real time PCR was performed to detect the HCV RNA through continuous monitoring of the accumulated amplicon using EvaGreen as an intercalating dye. EvaGreen dye has shown high reaction efficiencies, low PCR inhibition and low cost when compared to SYBR Green I (Eischeid, 2011; Mao et al., 2007). However, efficiency of SYBR Green I was not evaluated in this study. EvaGreen has recently been used for quantitation of some viruses and shown high sensitivity, specificity, and reproducibility supporting its usefulness as an intercalating agent in real time PCR (Hernández-Arteaga & López-Revilla, 2008; Wei et al., 2014). Moreover, EvaGreen dye, unlike SYBR Green I, is non-mutagenic due to its inability to cross cellular membranes and to reach the genomic DNA, thus it is considered a safe alternative to SYBR Green I dye. Ethidium bromide is a known mutagenic dye, while EvaGreen is non-mutagenic, non-cytotoxic and safe for direct disposal in the drain. Involving EvaGreen dye in our study has eliminated the need to apply ethidium bromide dye for amplicon visualization on the gel electrophoresis, thus critically reduce toxic burden on the lab personnel and the environment (Mao et al., 2007; Ohta, Tokishita, & Yamagata, 2001; Singer, Lawlor, & Yue, 1999).

In this study, the analytical sensitivity, specificity, and accuracy of the developed method were evaluated. Since EvaGreen dye non-specifically binds to any double-stranded DNA, the specific and non-specific amplification products were investigated to evaluate the analytical specificity of the developed assay. Melting curves analysis was carried out for all tested samples, combined with agarose gel electrophoresis for those co-infected with HBV and HIV to confirm the presence of the specific amplicons only. Single peaks and single bands of the correct size obtained by melting curves analysis and agarose gel electrophoresis, respectively, confirm the absence of non-specific amplification. The assay showed a high level of analytical specificity which was similar to that demonstrated by other methods. Furthermore, as compared to other real-time PCR chemistries, the fluorescence dye is the cheapest to use as genotyping.

Melting point temperature has been described as the temperature where 50% of the DNA amplicons are dissociated from the double stranded form to single stranded. In the melting temperature analysis, the EvaGreen dye is the parameter which was investigated. As the amplicon dissociate, the EvaGreen dye molecules are release out from the double stranded molecule and are no longer fluoresce. The changes in the fluorescence state is monitored and charted in real time, generating melting curve graph. There are several elements that contribute to the features of melting temperature of amplicons. Firstly, it is relying on the guanine and cytosine (GC) content of the amplicon. Melting point rises with the increase of GC content of the amplicon. The GC content of HCV genotype 3 was range from 55.19-57.14 %, meanwhile for the HCV genotype 1, it was slightly higher than that of genotype 3 (57.14-58.44%). However in this study, the GC content was not depicted in the melting curve analysis. The GC content also may have slight variation in interassays due to PCR condition, PCR kit used, and quality of initial sample. Secondly, is the length of the amplicon, which the melting state is increasing with the length of the amplicon. In this experiment, the amplicon size is more than 150 bp with 85°C melting point. Primer dimers are shorter in size resulting in lower melting temperature (60°C-70°C). Others factors include instrument type and reporting dye used.

Different real-time PCR instrument recorded difference melting temperature of similar amplicon. Gelaye et al (2017) has reported a comprehensive cross-platform assay to determine types of poxviruses (Gelaye et al., 2017). Many researchers utilised Lightcycler 480 (Roche, USA) (Yu et al., 2015) for HRM assay. This is because, theoretically, Lightcycler using air for ramping temperature, which could change temperature dramatically

during the PCR reaction. However, there are only slight differences between different instruments available in the market as reported by Gelaye et al., (2017).

In conclusion, based on the 5'UTR nucleotide sequence analysis, our local HCV strains are categorized mainly into HCV genotype 3 and genotype 1. All strains were easily detected using Ssofast EvaGreen Mix on the CFX96 instrument (BioRad, US). There was different in term of GC content between the two categories; however it does not depicted in both melting temperature and high resolution melting analysis using EvaGreen Real-time PCR. The technique can be improvised for better genotype discrimination outcomes.

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