Chapter 10

DETECTION OF HEPATITIS C VIRUS RNA USING REVERSE TRANSCRIPTION PCR

S.F. Yap
Department of Allied Health Sciences,
Faculty of Medicine, University of Malaya,
Kuala Lumpur, Malaysia

10.1. INTRODUCTION

Hepatitis C virus (HCV) is a major aetiologic agent of non-A, non-B (NANB) post-transfusion hepatitis. It is a positive stranded RNA virus of -10 000 nucleotides. The viral genome comprises a 5' non-translated region (NTR) and a 3' NTR flanking a continuous single open reading frame (ORF) encoding a single polyprotein. The polyprotein is divided into a 5' structural region comprising putative core and envelope proteins, and a 3' region comprising non-structural (NS1 to NS5) proteins. Based on its genomic organization, the virus resemble most closely members of the flaviviridae family. HCV is classified into a series of 'genotypes' on the basis of differences in the nucleotide sequence of various regions of the genome particularly the core and NS5 regions. Some genotypes show worldwide distribution whereas others are more restricted geographically. The most common genotypes are types 1, 11, 111, IV and V (Mori/Okamoto) which corresponds to Simmonds types la, lb, 2a, 2b and 3a respectively.

Routine laboratory diagnosis is dependent on detection of anti-HCV antibodies that are produced in response to the infection. Commercially available diagnostic assays utilize a series of recombinant HCV antigens whose amino acid sequences are deduced from the nucleotide sequence of different regions of the viral genome. The objective of inclusion of several antigenic proteins in the assay, particularly those from regions that are less influenced by genotype variation, is improvement of the assay sensitivity in view of the significant genomic heterogeneity of the virus.

Detection of the viral genome (HCV RNA) is by a combination of cDNA synthesis and PCR followed by gel analysis and/or hybridization assay. In principle, cDNA is synthesized using the viral RNA as template and the enzyme, reverse transcriptase. The cDNA is then amplified by PCR and the product detected. Agarose gel electrophoresis provides a rapid and simple detection method; however, it is non-quantitative. Further, nested PCR is necessary to achieve the required sensitivity. To improve on the sensitivity as well as the specificity, various hybridization assays are employed for detection. Format that allow for quantitation of the viral load have also been described. HCV RNA detection has been applied to the diagnosis of acute HCV infection, to monitor response to anti-viral therapy and to supplement serological testing. The assay protocol described in this paper is adapted from that published by Chan et al. Comments on various aspects of the assay are based on experience with the method in our laboratory.

10.2. HCV RNA ASSAY

10.2.1. Specimen handling

Care must be taken when handling samples to ensure stability of the HCV RNA. Blood is collected into sterile plain tubes and centrifuged as soon as possible (within 4 hours) and the

serum stored in suitable aliquot. If analysis is delayed for more than 24 hours, it is recommended that the serum be stored frozen. Care must also be taken to ensure that there is no cross-contamination of specimens.

10.2.2. Materials and reagents

All materials and reagents employed must be sterile and RNase free. All reagents employed are molecular grade.

Primers are derived from the highly conserved 5' non-translated region of the viral genome. Primer sequences are as follows:

Primer	Region	5' base position	Polarity #	Sequence 5' to 3'
209	5'NTR	8	-	ATACTCGAGGTGCACGGTCTACGAGACCT
211	5'NTR	29	-	CACTCTCGAGCACCCTATCAGGCAGT
939	5'NTR	297	+	CTGTGAGGAACTACTGTCTT
940	5'NTR	279	+	TTCACGCAGAAAGCGTCTAG

Orientation of primer sequence (+ = sense; - = anti-sense)

10.2.3. Reverse transcriptase

Activity: 5' to 3' DNA polymerase

Substrate: RNA or DNA template with an RNA or DNA primer bearing a 3'hydroxyl group

Reaction:

$$DNA_{OH} \text{ or } RNA_{OH} \xrightarrow[Mg^{++}\ dATP,dTTP,dGTP,dCTP]{RT} DNA\text{-}({}_pdN)_n + nPP_i$$
$$RNA\text{-}({}_pdN)_n + nPP_i$$

Reference: Sambrook p 5.55

The enzyme employed in the assay is Superscript II RNase H Reverse Transcriptase from BRL. The enzyme is preferred as it is devoid of both RNase A and RNase H activity. If enzyme employed carry RNase H activity, it is necessary to include an RNase inhibitor in the reaction to prevent degradation of the template during reverse transcription.

10.3. HCV RT-PCR PROTOCOL

10.3.1. Sample preparation

Reagents required:
Trizol LS reagent from BRL
Chloroform

Isopropyl alcohol
75% ethanol
RNase free water

Equipment required:
Microcentrifuge
Microfuge tubes
Micropipettes and plugged tips

Procedure:

Unless otherwise stated, the following procedure is carried out at room temperatur:

(1) Add TRIZOL LS reagent to serum in a ratio of 3:1 and incubate for 5 minutes.

(2) Add 0.2 mL of chloroform for every 0.75 mL of TRIZOL LS reagent. Shake vigorously for 15 seconds and let stand for 2 to 15 minutes.

(3) Spin at 12000 × g for 15 min at 4°C.

(4) Transfer the upper clear aqueous phase to a fresh clean tube and precipitate the RNA with 0.5 mL isopropyl alcohol per 0.75 mL of TRIZOL LS reagent.

(5) Incubate for 10 min and centrifuge at 12000 × g for 10 min at 4°C.

(6) Discard supernatant and wash RNA pellet with 75% ethanol adding at least 1 mL of 75% ethanol for every 0.75 mL TRIZOL LS reagent. Mix and centrifuge for 5 min at 4°C.

(7) Discard ethanol and air-dry RNA pellet.

(8) Resuspend pellet in 20 µL RNase free water.

10.3.2. Reverse transcription

Reagents required:
Reverse transcriptase (Superscript II)
RNase free water
Primer for cDNA synthesis (primer 1, p2O9)
dNTPs

Equipment required:
Waterbath
Micropipette and plugged tips

Procedure:

(1) Transfer 2-5 µL of RNA into a fresh clean tube.

(2) Add primer I (0.3) µM) and water to 10 µl.

(3) Heat at 90°C for 2 min and then cool on ice.

(4) Add 4 µL 5X cDNA synthesis buffer; final conc.:

50 mM Tris-HCl pH8.3
75 mM Kcl
3 mM $MgCl_2$

2 µL 0.1 M DTT
3 µL dNTPs (10 mM stock) and
water to 19 µl

(5) Equilibrate at 37°C for 2 min and add 1 µL of reverse transcriptase, mix gently and incubate at 37°C for 45 min to 1 hour. Place on ice or freeze.

10.3.3. Amplification

Reagents required:
10 × PCR buffer (supplied with Taq)
dNTPs
Outer primers, 209 & 939 (for 1st round PCR)
Inner (nested) primers, 211 & 940 (for 2nd round PCR)
Taq DNA polymerase
RNase free water
Mineral oil

Equipment required:
Micropipette and filter tips
Thermal cycler

Procedure:

First round PCR:

1. Prepare PCR premix as follows:

Reagent	Final concentration
10 × PCR buffer	1x
dNTPs	0.2 mM each
Primers	0.3 µM each
Taq	1 unit
Water to 25 µl	

Final reaction volume = 30 µl

2. Overlay the premix with oil and add 5 µL of cDNA to the PCR mix and cycle using the following parameters:

Temperature (oC)	Time
94	10 min
94	1 min
50	1 min
72	2.5 min
72	10 min

Total number of cycles = 30 µl

Second round PCR:

Use 2-5 µL of the 1st round PCR product with the same cycling parameters and PCR mix except that the primers are the inner nested ones and the final volume is 50 µl.

10.3.4. Detection

Reagents required:
Agarose
Ethidium bromide (10 mg/mL)
1 × TAE or TBE buffer
Sample loading dye (bromophenol blue)
Molecular weight marker (eg. I 00 bp ladder)

Equipment required:
Gel tank and power pack
Magnetic stirrer with hot plate or microwave oven
UV transilluminator

Procedure:

(1) Prepare a 1.5–2% agarose gel using either 1 × TAE or TBE buffer.

(2) Add 4 parts of PCR product to 1 part of loading dye, mix and load into the gel.

(3) Load about 200 ng of molecular weight marker into the first lane of the gel.

(4) Run at 80–100 V.

(5) View the bands on a UV transilluminator.

Quality control

(1) Include in each run a known positive and negative control serum.

(2) Include a 'reagent control' that contains water instead of sample.

(3) Observe all recommended procedures and precautions to avoid amplicon contamination.

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