Optimization of one-step real-time PCR for the X-tail target of HCV as a diagnostic test

Franciéli Pedrotti Rozales[1], Fernanda de-Paris[2], Alice Beatriz Mombach Pinheiro Machado[2], Cintia Costi[3], Afonso Luís Barth[1]

Clin Biomed Res. 2014;34(2):164-168

1 Laboratório de Pesquisa em Resistência Bacteriana (LABRESIS), Centro de Pesquisa Experimental, Hospital de Clínicas de Porto Alegre (HCPA) – Porto Alegre, RS, Brazil.

2 Unidade de Microbiologia e Biologia Molecular, Serviço de Patologia Clínica, HCPA – Porto Alegre, RS, Brazil.

3 Centro de Desenvolvimento Científico e Tecnológico, Fundação Estadual de Produção e Pesquisa em Saúde (CDCT/FEPPS) – Porto Alegre, RS, Brazil.

Corresponding author:
Fernanda de-Paris
E-mail: fparis@hcpa.ufrgs.br
Unidade de Microbiologia e Biologia Molecular – Rua Ramiro Barcelos 2350, 90035-903 – Porto Alegre, RS, Brazil

ABSTRACT

Infection with hepatitis C virus (HCV) is a global public health issue. The bloodborne nature of HCV transmission poses a substantial risk to healthcare workers, due to occupational exposure to needlestick injuries and blood and other body fluids containing the virus. Undiagnosed HCV infection, including in healthcare workers, represents a growing problem worldwide as the infected population ages, and HCV-related mortality and morbidity is expected to rise substantially over the coming decades. Consequently, diagnostic tests for HCV play an important role in this scenario. The aim of this study was to standardize a one-step RT-PCR assay for detection of HCV. The test demonstrated reproducibility, sensibility (100%), and the limit of detection was set at 100IU/mL. Our study indicates that this assay can be used as a diagnostic tool to follow up healthcare workers after occupational exposure.

Keywords: HCV; diagnostic test; X-tail; acute disease

Infection with hepatitis C virus (HCV) is a global public health issue. More than 170 million persons worldwide are infected with HCV[1]. Furthermore, the bloodborne nature of HCV transmission poses a substantial risk to healthcare workers, due to occupational exposure to needlestick injuries and blood, and other body fluids containing the virus[2,3]. Undiagnosed HCV infection, including in healthcare workers, represents a growing problem worldwide as the infected population ages, and HCV-related mortality and morbidity is expected to rise substantially over the coming decades[4]. Consequently, diagnostic tests for HCV play an important role in this scenario. Molecular assays (nucleic acid tests, NATs) are considered the gold standard for diagnosis of HCV infection[5]. In many countries, these tests are part of routine screening programmes, as they allow detection of infection before the immune response, which is the target of serological testing[6,7]. Furthermore, qualitative NATs are used to confirm viremia, which aids in the diagnosis of acute HCV infection[8]. Thus, qualitative HCV NATs play an extremely important role in the follow-up of occupational exposures to blood. In occupational exposures, as in acute infections, NAT results will become positive within 1 to 3 weeks — several weeks earlier than any serological test[8].

Real-time PCR (RT-PCR) is one of the most recent NAT assays for diagnosis of HCV RNA[9]. Several NATs have used the 5´NCR region of the HCV genome as a target for primers and probes[10]. As a highly conserved region, this is the target of choice for many in-house and commercial tests. However, there is disagreement as to the performance of the 5´NCR region

as a target in different HCV genotypes[11]. Recent studies have proposed the use of a new target region of the HCV genome, an additional element adjacent to the poly (U) tract of the 3´UTR known as the 3´-X-tail, or simply the X tail. This region is also highly conserved, and is believed to enable detection of all HCV genotypes[12,13].

The aim of this study was to standardize a qualitative NAT assay, specifically a one-step RT-PCR assay targeting the X-tail region of the HCV genome, for use as a diagnostic test for acute HCV infection.

METHODS

The one-step RT-PCR assay was run on 30 human plasma samples previously evaluated by means of a HCV NAT test (COBAS Amplicor HCV system Assay 2.0, Roche). Fifteen samples were positive and 15 were negative. The positive samples were sequenced for detection of the HCV genotype (1, 2, and 3). All plasma specimens used in the study were obtained from the collection of the "Centro de Desenvolvimento Científico e Tecnológico - Fundação Estadual de Produção e Pesquisa em Saúde", Porto Alegre, state of Rio Grande do Sul, Brazil. Specimens were stored at -80°C until use.

RNA was extracted with a QIAamp Mini Kit (Qiagen, Valencia, CA, USA) using 500 µL of plasma, 11.2 µg/ sample of carrier RNA, and 30 µL of elution buffer. Modifications in the sample volume, in carrier RNA quantity, and elution buffer volume were introduced in the QIAamp Mini Kit manufacturer's protocol to improve the sensitivity of RNA extraction. Primers (XTF5 – 5'GTGGCTCCATCTTAGCCCTAGT3' and HCMgR2 – 5'TGCGGCTCACGGACCTTT3' at 400 nM by Invitrogen, Carlsbad, CA, USA) and probe (HCVMGB2 5'FAM- CACGGCTAGCTGTG-MGB3' at 200 nM by Applied Biosystems, Foster City, CA, USA) were used to amplify the X-tail region as described by Drexler et al. The reaction was performed using a SuperScript™ III Platinumâ One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, USA) containing 6 mM of MgCl. Detection of a human gene (RNase P) was incorporated as the internal control. Thermal cycling was performed in a 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA) as follows: 50°C for 30 min, 95°C for 2 min, 45 cycles at 94°C for 15 s, and 55°C for 35 s.

To evaluate the test reproducibility and LOD of our assay, we performed six runs of a commercial panel of HCV RNA samples (HCV RNA Linearity Panel PHW804 by BBI Diagnostics - SeraCare Life Sciences, Gaithersburg, MD, USA). This panel comprises 10 samples (PHW01 to PHW10) of HCV genotype 1. The HCV load of these samples had been quantified previously by three different techniques (Cobas Amplicor HCV Monitor V.2.0 - Roche; Versant HCV RNA bDNA 3.0 - Bayer; HCV RNA Reference Lab Home Brew Real Time PCR).

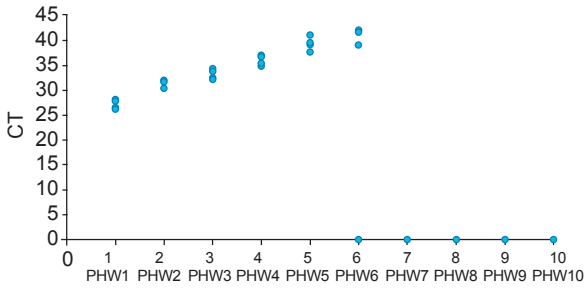
We performed serial dilutions of the PHW04 sample followed by one-step RT-PCR to evaluate the limit of detection of the assay. These were tested in 16 replicates for determination of the LOD.

RESULTS

The one-step RT-PCR technique described herein proved to be reproducible across the six replicates of each PHW sample (1 to 10), as the %CV obtained for each positive panel member was 3.39% for PHW1, 2.08% for PHW2, 2.94% for PHW3, 1.91% for PHW4, and 2.07 for PHW5. There was satisfactory linearity as tested with the HCV RNA Linearity Panel – PHW804 (Figure 1).

The PHW04 sample (20000 IU/mL) was diluted serially to obtain HCV concentrations of 1000 IU/mL, 500 IU/mL, 200 IU/mL, 100 IU/mL, 50 IU/mL, and 10 IU/mL. One-step RT-PCR was consistently able to detect all 16 replicates of each tested concentration up to 100 IU/mL.

All 30 clinical samples previously evaluated in the COBAS Amplicor HCV exhibited the same result on one-step RT-PCR, whether positive or negative. In fact, all three genotypes presented positive results, indicating that the X-tail region is an adequate target for HCV detection (Table 1).



CT: Cycle Threshold

Figure 1: Reproducibility and linearity of the panel sample across six repetitions of each sample (1 to 10).

Table 1: Results of COBAS Amplicor HCV system Assay 2.0 – Roche and the one-step Real Time PCR HCV assay.

Sample	Genotype	COBAS Amplicor test	Cobas Internal Control	RT-PCR in house	Internal Control
160	2	+	-	+ (CT 32.9)	+ (CT 33.8)
177	3	+	+	+ (CT 37.1)	+ (CT 32.9)
157	1	+	+	+ (CT 32.2)	+ (CT 34.3)
168	3	+	+	+ (CT 32.4)	+ (CT 34.9)
153	1	+	+	+ (CT 28.9)	+ (CT 35.7)
139	3	+	-	+ (CT 30.9)	+ (CT 34.1)
159	1	+	+	+ (CT 33.2)	+ (CT 35.3)
162	2	+	+	+ (CT 31.5)	+ (CT 34.7)
109	3	+	+	+ (CT 36.4)	+ (CT 34.6)
164	1	+	+	+ (CT 32.7)	+ (CT 32.7)
143	3	+	-	+ (CT 30.4)	+ (CT 32.7)
142	2	+	-	+ (CT 34.7)	+ (CT 32.2)
174	3	+	+	+ (CT 26.1)	+ (CT 33.0)
171	3	+	+	+ (CT 30.5)	+ (CT 33.7)
146	3	+	+	+ (CT 36.0)	+ (CT 33.6)
110	-	-	+	-	+ (CT 33.7)
97	-	-	+	-	+ (CT 33.9)
120	-	-	+	-	+ (CT 35.0)
123	-	-	+	-	+ (CT 35.6)
122	-	-	+	-	+ (CT 35.8)
115	-	-	+	-	+ (CT 35.4)
125	-	-	+	-	+ (CT 34.9)
128	-	-	+	-	+ (CT 36.7)
101	-	-	+	-	+ (CT 41.0)
108	-	-	+	-	+ (CT 35.1)
97	-	-	+	-	+ (CT 34.3)
124	-	-	+	-	+ (CT 32.7)
95	-	-	+	-	+ (CT 34.9)
120	-	-	+	-	+ (CT 34.9)
99	-	-	+	-	+ (CT 35.1)

CT: Cycle Threshold.

DISCUSSION

The aim of this study was to standardize a one-step RT-PCR assay to the X-tail region of HCV as a qualitative NAT for use in the setting of acute infection. This is a highly relevant issue in health care facilities, as many protocols for follow-up of occupational exposure to HCV require NATs with good specificity and sensitivity. In the acute infection period, most patients develop asymptomatic infection but will often have high-level viremia and high alanine aminotransferase (ALT) levels. HCV RNA can be detected 1 to 3 weeks after infection, approximately 1 month earlier than anti-HCV antibodies[8]. Furthermore, direct detection of HCV RNA has become an essential tool for the diagnosis of hepatitis C acute infection.

In this study, the one-step RT-PCR assay using the X-tail region of HCV as a target was able to detect all samples previously confirmed as positive by means of the COBAS Amplicor HCV test. Notably, all HCV genotypes (1, 2, and 3) were detected equally by both techniques. As these genotypes (1 to 3) are the most prevalent in Brazil[15,16]. RT-PCR exhibited high sensitivity and, therefore, is adequate for use as a diagnostic test in our country. This test also proved to be highly specific, as none of the 15 previously negative samples tested positive.

In addition, this assay incorporates RNAse P gene amplification as an internal control. A positive RNAse P reaction ensures that the quality of the sample is acceptable, and the internal-control is positive while target-negative reactions ensure a true negative result. Furthermore, RNAse P amplification performed as a separate reaction has shown better performance than the internal controls used by commercial assays. The use of RNAse P could thus reduce the costs and turnaround time of HCV NAT.

The LOD was established as 100 IU/mL, which is similar to that of other in-house assays for PCR-based HCV detection[5,17]. Many commercial assays report a LOD of 50 IU/mL. However, our one-step RT-PCR assay was designed for patient monitoring in the acute setting; this HCV NAT will be applied mainly in protocols for follow-up of health care workers after occupational exposure. In this period of HCV infection, patients exhibit high-level viremia[8,18], and a nucleic acid test with a LOD of 100 IU/mL is acceptable.

Our findings suggest that the one-step RT-PCR assay proposed herein is reproducible, specific, and sensitive for use as a HCV NAT in the acute setting. Therefore, this assay can be used as a diagnostic tool to follow up health care workers after occupational exposure.

ACKNOWLEDGEMENTS

This work was supported by *Fundo de Incentivo à Pesquisa e Ensino, Hospital de Clinicas de Porto Alegre* (FIPE/HCPA), *Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul* (FAPERGS), and *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq).

REFERENCES

1. WHO. World Health Organization. Hepatitis C. Fact sheet No. 164. Revised October 2000 [2 July 2014]. Available from: http://www.who.int/mediacentre/factsheets/fs164/en/.
2. Henderson DK. Managing occupational risks for hepatitis C transmission in the health care setting. Clin Microbiol Rev. 2003;16:546-68.
3. Pedrosa PB, Cardoso TA. Viral infections in workers in hospital and research laboratory settings: a comparative review of infection modes and respective biosafety aspects. Int J Infect Dis. 2011;15:e366-76.
4. Lee SR, Kardos KW, Schiff E, Berne CA, Mounzer K, Banks AT, et al. Evaluation of a new, rapid test for detecting HCV infection, suitable for use with blood or oral fluid. J Virol Methods. 2011;172:27-31.
5. Clancy A, Crowley B, Niesters H, Herra C. The development of a qualitative real-time RT-PCR assay for the detection of hepatitis C virus. Eur J Clin Microbiol Infect Dis. 2008;27:1177-82.
6. Berg T, Sarrazin C, Herrmann E, Hinrichsen H, Gerlach T, Zachoval R, et al. Prediction of treatment outcome in patients with chronic hepatitis C: significance of baseline parameters and viral dynamics during therapy. Hepatology. 2003;37:600-9.
7. Terrault NA, Pawlotsky JM, McHutchison J, Anderson F, Krajden M, Gordon S, et al. Clinical utility of viral load measurements in individuals with chronic hepatitis C infection on antiviral therapy. J Viral Hepat. 2005;12:465-72.
8. Scott JD, Gretch DR. Molecular diagnostics of hepatitis C virus infection: a systematic review. JAMA 2007;297:724-32.
9. Sizmann D, Boeck C, Boelter J, Fischer D, Miethke M et al. Fully automated quantification of hepatitis C virus (HCV) RNA in human plasma and serum by the the COBAS AmpliPrep/COBAS TaqMan system. J Clin Virol. 2007;38:326–33.
10. Germer JJ, Zein NN. Advances in the molecular diagnosis of hepatitis C and their clinical implications. Mayo Clin Proc. 2001;76:911-20.
11. Vermehren J, Kau A, Gartner BC, Gobel R, Zeuzem S, Sarrazin C. Differences between two real-time PCR-based hepatitis C virus (HCV) assays (RealTime HCV and Cobas AmpliPrep/Cobas TaqMan) and one signal amplification assay (Versant HCV RNA 3.0) for RNA detection and quantification. J Clin Microbiol. 2008;46:3880-91.
12. Drexler JF, Kupfer B, Petersen N, Grotto RM, Rodrigues SM, Grywna K, et al. A novel diagnostic target in the hepatitis C virus genome. PLoS Med. 2009;6:e31.
13. Friebe P, Bartenschlager R. Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. J Virol. 2002;76:5326-38.
14. WHO - World Health Organization. CDC protocol of realtime RTPCR for swine influenza A (H1N1) 2009 [2 July 2014]. Available from: http://www.who.int/csr/resources/publications/swineflu/CDCrealtimeRTPCRprotocol_20090428.pdf.

15. Santos BF, de Santana NO, Franca AV. Prevalence, genotypes and factors associated with HCV infection among prisoners in Northeastern Brazil. World J Gastroenterol. 2011;17:3027-34.

16. Silva CM, Costi C, Krug LP, Ramos AB, Grandi T, Gandolfi VL, et al. High proportion of hepatitis C virus genotypes 1 and 3 in a large cohort of patients from Southern Brazil. Mem Inst Oswaldo Cruz. 2007;102:867-70.

17. Davis GL. Hepatitis C. In: Schiff ER, Maddrey WC, editors. Schiff's Diseases of the Liver. Philadelphia, EUA: Lippincott-Raven Publishers; 1999. p. 793-836.

18. Hourfar MK, Schmidt M, Seifried E, Roth WK. Evaluation of an automated high-volume extraction method for viral nucleic acids in comparison to a manual procedure with preceding enrichment. Vox Sang. 2005;89:71-6.

Received: 17/04/2014
Accepted: 29/05/2014