Development and validation of a simple and rapid method for hepatitis C virus genotyping based on one-step RT-qPCR

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Abstract. Hepatitis C virus (HCV) infections caused by different subtypes require different treatments; therefore, rapid and cost-effective genotyping methods for the diagnosis of HCV are greatly needed. In the present study, a new method to diagnose HCV subtypes that depends on a one-step quantitative reverse transcription PCR (RT-qPCR) and TaqMan fluorescence probe technique is described. Five pairs of primers and five probes were designed, which were able to detect five genotypes in three reaction tubes. One reaction was used to detect the 1b subtype, one was used to detect the 2a and 6a subtypes, and the other was used to detect the 3a and 3b subtypes. Rigorous performance validation was implemented for five aspects: Precision, sensitivity, accuracy, specificity and anti-interference. The HCV subtype that infected 289 patients was evaluated in the present study via RT-qPCR and verified by sequencing. The results revealed that the 1b subtype accounted for 45% of infections, the 2a subtype accounted for 9% of infections, the 3a subtype accounted for 13% of infections, the 3b subtype accounted for 18% of infections, and the 6a subtype accounted for 15% of infections. The analytical sensitivity for the detection of each of the five HCV subtypes was 1,000 IU/ml. The new method performed well in the performance validation mentioned above, indicating its effectiveness as a HCV genotyping method. RT-qPCR has mitigated some of the former challenges of existing HCV genotyping methods, including the time commitment, expense, and inaccuracy of such methods. The performance validation of this new method showed that RT-qPCR is reliable enough to be widely applied in China for HCV genotyping.

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Introduction

Hepatitis C is one of the most common chronic liver diseases worldwide, and is caused by hepatitis C virus (HCV) infection (1). According to World Health Organization (WHO) guidelines published in 2016, an increasing number of deaths have been caused by HCV-related diseases annually (2), and patient mortality from HCV-associated cirrhosis and hepatic cell cancer will continue to increase unless more efficient therapies are applied in the clinic (3). In addition, a previous study showed that the projected prevalence of HCV in Asia is 2.8%, which accounts for over 60% of the estimated cases worldwide (4). Therefore, it is not surprising that there are >8.9 million people living with HCV infection in China, and that China has the highest burden of HCV infection worldwide (5,6). Currently, various direct-acting antivirals (DAAs) show significant advantages in the treatment of hepatitis C, including high potency, a higher barrier to resistance, a favourable tolerability profile, and many other aspects (7,8). However, for patients infected with different HCV subtypes, to achieve better antiviral effects, the use of DAA drugs differs (9). Therefore, accurate HCV genotyping results directly determine the therapeutic schedule and treatment effect.

To reduce the global burden of HCV infection and mortality, more accurate treatment is needed (2). To date, various therapies have been identified that treat HCV (10). However, HCV has seven main genotypes as primary divisions (11), and different subtypes of HCV infection exhibit different chronic disease progressions, have different responses to antitoxic therapy, and require different therapies (12). Therefore, a method that can offer a rapid and cost-effective HCV diagnosis is greatly needed. HCV genotyping is of great significance in guiding antiviral therapy, which not only is an important indicator in the diagnostic process, but also reflects the treatment effect.

Currently, HCV genotyping methods used in China and globally vary, such as the Versant HCV genotype assay (LiPA) 2.0, TaqMan PCR, sequencing, whole-genome deep

sequencing (WGS), and the NS5B-based microarray (13-17). Sequencing is considered the most accurate method for HCV genotyping (6). Nevertheless, it has many drawbacks, as it is time-consuming, expensive, etc. This coincides with the need for HCV antiviral therapy; therefore, a more efficient and accurate method for HCV genotyping is needed.

Based on reverse transcription-quantitative PCR (RT-qPCR) and regional HCV subtype distribution characteristics, a method based on a large number of clinical trials to distinguish five prevalent HCV subtypes in a one-step reaction was designed to meet clinical needs. For those classic HCV genotyping methods, the higher requirements for time and capital investment make them more difficult to promote and broadly apply. According to the performance validation, this method's favourable reproducibility, sensitivity, accuracy, specificity and anti-interference shows that it overcomes these problems. Therefore, this method could be put into clinical practice and be beneficial for the adjunct diagnosis and treatment of hepatitis C.

Materials and methods

Samples. The present study included 65 qualified clinical samples from hepatitis C patients (males, 61.5%) aged 18-76 years (median age, 49) who were referred to The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University, and 224 other qualified clinical samples from hepatitis C patients (males, 60.7%) aged 19-78 years (median age, 50) who were referred to the Department of First Generation Sequencing, Hangzhou DiAn Medical Laboratory, Zhejiang, China between January 2018 and January 2019. The concentration of HCV RNA in the above samples, quantified by the automatic nucleic acid quantitative detection system (AMPLLY Biotech Co., Ltd.), was above $1x10^3$ IU/ml (range: $1.0x10^3$-$5.7x10^8$ IU/ml). The present study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University. All patients provided written informed consent and agreed to the use of their samples in scientific research.

Materials. The HCV genotype assay kit used in the study was originally designed by the authors. Aiming at the five most prevalent HCV subtypes in China, a set of processes was designed that could detect each of the subtypes above, regardless of whether an infection was separate or complex. The kit is suitable for genotyping 1b, 2a, 3a, 3b and 6a HCV subtypes from clinical samples (plasma or serum). RT-qPCR was performed on the ABI 7500 instrument as described previously (18).

HCV RNA extraction. The RNA extraction kit was purchased from Taipu Biosciences (China) Co., Ltd. To extract HCV RNA, 550 μl lysate was added to several 1.5 ml centrifuge tubes. Next, 100 μl plasma samples was added to each tube, mixed for 20 sec, and then the tubes were allowed to stand for 10 min at 50˚C. Afterwards, a purification column was placed into a 2 ml collection tube. The mixture was then added to the purification column and centrifuged at 12,740 x g for 1 min at room temperature before the filtrate was discarded. The purification column was washed with RNA extraction buffer I diluted in ethanol and RNA extraction buffer II diluted in ethanol, successively, and then centrifuged at 12,740 x g for 1 min at room temperature before the filtrate was discarded. After 2 min centrifugation at 12,740 x g at room temperature, the purification column was placed at room temperature for 2-3 min. The purification column was transferred to a new centrifuge tube, and 50 μl eluant was added to the centre of the column and allowed to stand for 2 min. After centrifugation at 12,740 x g for 1 min at room temperature, the RNA solution was collected in a tube. The eluant was preheated at 65-70˚C, and if the time of elution was prolonged for 3 min or the eluant was added only once to the centre of the purification column and eluted again, the extraction efficiency was improved.

Primer and probe design for HCV genotyping. The primers and probes were originally designed by Haifeng Huang and synthesized by Sangon Biotech Co., Ltd. The fluorescence signal collection of the multi-fluorescence detector was set to the FAM (494 nm excitation and 522 nm emission wavelentghs) and JOE (520 nm excitation and 548 nm emission wavelengths) channels using the ABI 7500 instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.). The 5'-untranslated region (UTR) core of the HCV genome region was used to design the sequences of primers and probes using Primer Premier 5.0 (Premier Biosoft International) for HCV genotyping, and they were designed first without three consecutive G or C bases at the end of the primer, and by avoiding complementarity between themselves or the primers. The exact sequences of the primers and probes are listed in Table I. These primers and probes allowed HCV subtypes 1b, 2a, 3a, 3b and 6a to be distinguished with only three RT-PCR reaction tubes. The HCV 1b reaction tube confirmed the presence of HCV 1b subtype infection or a complex infection with the fluorescein FAM™. The HCV 2a/6a reaction tube confirmed the presence of HCV 2a subtype infection with fluorescein FAM™ and HCV 6a subtype infection with the fluorescein JOE™. The HCV 3a/3b reaction tube confirmed the presence of HCV 3a subtype infection with fluorescein FAM™ and HCV 3b subtype infection with fluorescein JOE™. The positive test results were confirmed when one fluorescein in one tube presented a typical S-type amplification curve or the cycle threshold (Ct) value was ≤26.5.

Preparation and optimization of the one-step RT-qPCR system. For RT-qPCR, the essential components of the HCV RT-PCR reaction are listed as follows: Thermal starter enzyme, reverse transcriptase, RT-PCR buffer, primer pair, probe and PCR enhancer. The single component addition optimization was then performed with a HiScript II One Step RT-PCR kit (Vazyme Biotech Co., Ltd.), which includes Champagne thermal starter enzyme, reverse transcriptase and RT-PCR buffer. As the cofactors of thermally stable DNA polymerase, the concentration of magnesium ions was carefully set, and a series of magnesium ion concentration gradients were established to verify the best concentration of magnesium ions. To better regulate the pH value of the system and increase the activity of the DNA polymerase, a Tris-based buffering reagent and a reagent containing potassium ions were added into the RT-PCR buffer. In addition, the 1% recommended

Table I. Reverse transcription-quantitative polymerase chain reaction primers and probes for HCV genotyping.

HCV subtype	Primer/probe	Sequence (5' to 3')
1b	Upstream primer	CTCGTAGACCGTGCACCATGA
	Downstream primer	CAGATCGTTGGTGGAGTTTACT
	Probe	FAM-GCACGAATCCTAAACCT-MGB
2a	Upstream primer	CTCGTAGACCGTGCACCATGA
	Downstream primer	CAGATCGTTGGCGGAGTATACT
	Probe	FAM-GCACGAATCCTAAACCT-MGB
6a	Upstream primer	CTCGTAGACCGTGCACCATGA
	Downstream primer	CAGATCGTTGGCGGAGTTTACT
	Probe	JOE-GCACTCTTCCAAAACCC-MGB
3a	Upstream primer	CTCGTAGACCGTGCACCATGA
	Downstream primer	CAGATCGTTGGTGGAGTATACG
	Probe	FAM-ACACCATCCGCCGCCCACA-MGB
3b	Upstream primer	CTCGTAGACCGTGCACCATGA
	Downstream primer	CAGATCGTTGGTGGAGTATATG
	Probe	JOE-ACACACCCCGTCGCCCACA-MGB

FAM and JOE represent the reporting dye; MGB represents the quenching dye. HCV, hepatitis C virus; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

concentrations of glycerinum and formamide were used as PCR enhancers to promote the amplification of templates with high GC content. The amounts of each primer pair and probe added per test were 150 and 50 pmol, respectively. The final reaction volume was set at 50 μl, containing 38 μl each subtype of HCV RT-PCR reaction reagent, 2 μl enzyme, and 10 μl RNA sample. The final reaction conditions were as follows: 42˚C for 30 min; 95˚C for 3 min; 10 cycles of 94˚C for 20 sec, 55˚C for 20 sec and 72˚C for 30 sec; followed by 30 cycles of 94˚C for 15 sec and 60˚C for 45 sec, with fluorescence signal collection at 60˚C.

Validation of RT-qPCR for HCV genotyping. For the sensitivity validation of RT-qPCR, five positive HCV subtype (1b, 2a, 3a, 3b and 6a) samples that could be detected by the kit were taken as the reference. These were diluted to near the minimum detection limit of $1x10^3$ IU/ml, which was confirmed by the automatic nucleic acid quantitative detection system (AMPLLY Biotech Co., Ltd). The detection of each subtype was repeated 10 times, and the detection rate was calculated.

To validate the accuracy of this new method, sequencing, as described by Tong *et al* (6), was undertaken for genotyping samples and compared with RT-qPCR. In the test, 11 HCV samples were tested by sequencing. The nucleic acid was extracted from these samples and sequenced, and the results compared with NCBI data to determine the genotype.

For the anti-interference validation of RT-qPCR, a jaundice sample, a lipid sample and a haemolysis sample were mixed with high Ct value HCV 3a subtype samples as interfering substances. The differences in Ct values between samples with interfering substances (9X the volume of specimens plus 1X the volume of the interfering substance) and samples without interfering substances (9X the volume of specimens plus 1X the volume of normal saline) were recorded.

For the within-run and between-run precision validation of RT-qPCR, five positive HCV subtype (1b, 2a, 3a, 3b and 6a) samples were diluted to a low concentration (~1 to $3x10^3$ IU/ml), with five tubes tested repeatedly three times to a total of 15 repetitions. The Ct value, SD value and coefficient variable (CV) value of each experiment were calculated.

RT-qPCR for clinical application. The reaction mixture was prepared in a 1.5 ml centrifuge tube; three centrifuge tubes were required for the three different reaction reagents. These centrifuge tubes were instantaneously mixed and centrifuged at 6,000 x g for 10 sec at room temperature. Next, 40 μl three reaction reagents were distributed in three different thin-walled PCR 8-tube strips, to which 10 μl RNA was subsequently added, including the treated specimens, negative RNA control samples (extracted from samples that tested negative from HCV) and positive RNA control samples (extracted from samples that tested positive for HCV). Furthermore, the thin-walled PCR tubes were covered, numbered and centrifuged instantaneously. After that, the reaction tubes were placed on the ABI 7500 instrument, and the PCR conditions were set as follows: 42˚C for 30 min; 95˚C for 3 min; 94˚C for 20 sec, 55˚C for 20 sec, and 72˚C for 30 sec for 10 cycles; 94˚C for 15 sec and 60˚C for 45 sec for 30 cycles, with fluorescent signal collection at 60˚C. The results were analysed after RT-qPCR according to the qPCR amplification curve and Ct value per sample.

Statistical analysis. The CV was calculated by dividing the SD by the mean value. Data analysis, including data compilation and percentage calculation, was performed using SPSS software version 22.0 (IBM Corp.).

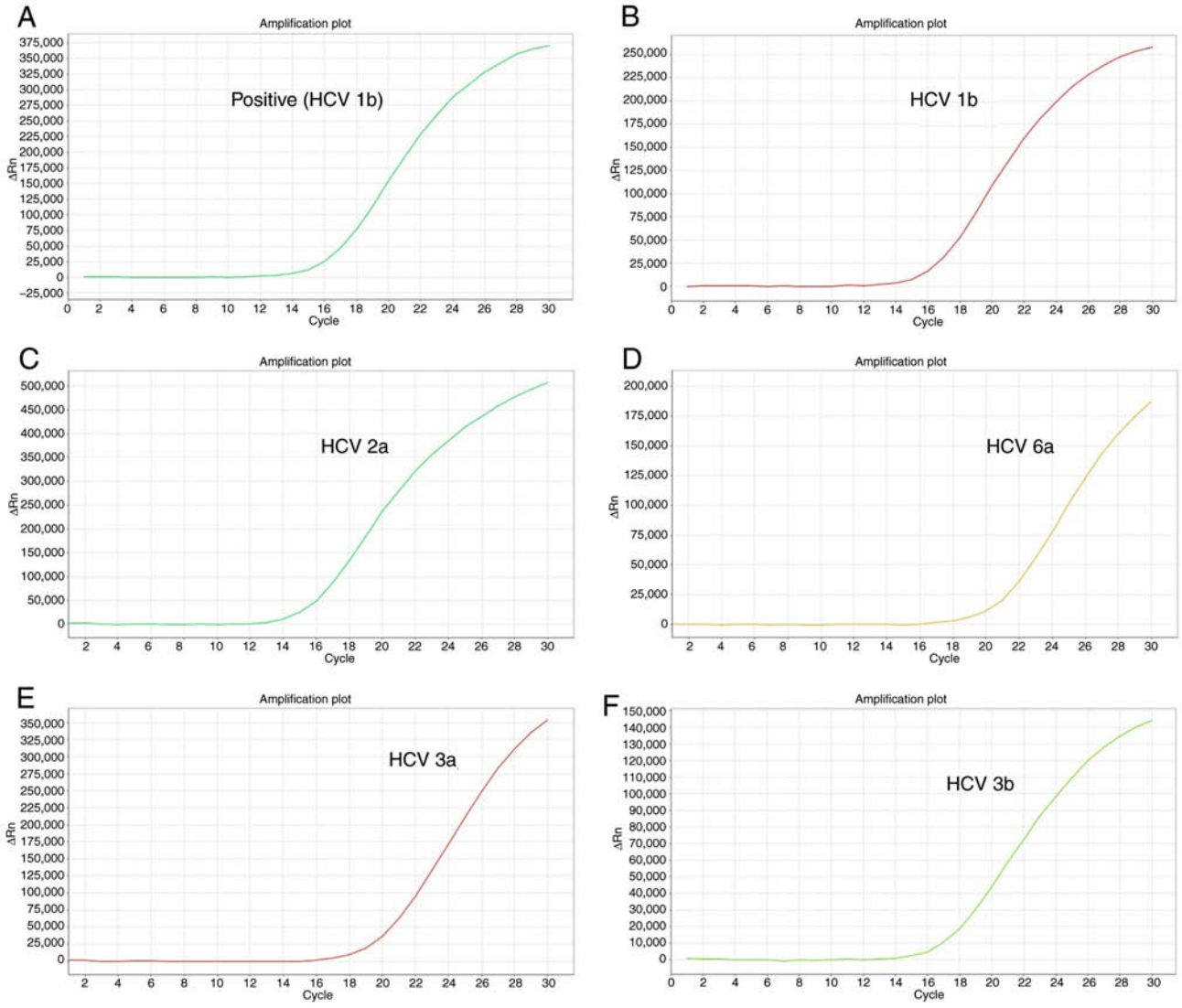

Figure 1. Representative genotyping amplification curve for HCV genotyping. (A) Positive control of HCV 1b genotyping amplification curve. (B) HCV 1b genotyping amplification curve. (C) HCV 2a genotyping amplification curve. (D) HCV 6a genotyping amplification curve. (E) HCV 3a genotyping amplification curve. (F) HCV 3b genotyping amplification curve. HCV, hepatitis C virus.

Results

RT-qPCR detection system and genotyping amplification curve. The HCV RNA genotype was detected with three reaction tubes. The HCV 1b reaction tube confirmed the presence of an HCV 1b subtype infection or a complex infection, the HCV 2a/6a reaction tube confirmed the presence of HCV 2a and 6a subtype infections, and the HCV 3a/3b reaction tube confirmed the presence of HCV 3a and 3b subtype infections. The final genotyping amplification curve of each HCV subtype is shown in Fig. 1. The labeled curves in Fig. 1A-F corresponding to the positive samples were typical S-amplification curves, which show its good performance in amplification efficiency.

Sensitivity of RT-qPCR for HCV genotyping. Regarding the detection rate, five positive HCV samples with different subtypes were diluted to a confirmed concentration of $1x10^3$ IU/ml, which was close to the detection limit, to validate whether the reagent used in RT-qPCR was sensitive enough. The results showed that the detection rate were all at 100%, which shows that the sensitivity was sufficient (Table II).

Specificity of RT-qPCR for HCV genotyping. To verify the specificity of RT-qPCR, two hepatitis B virus (HBV) samples, nucleic acid from two human cytomegalovirus (HCMV) samples, and two *Mycoplasma pulmonis* (MP) samples were extracted for diagnosis via the HCV genotyping kit. The results were all negative, showing that none of these samples cross-reacted with the HCV genotyping reaction system, thereby verifying its specificity.

Accuracy of RT-qPCR for HCV genotyping. RT-qPCR and sequencing were used to test 11 different HCV samples. The sequencing results were compared with data on NCBI to diagnose the subtype of each sample. Among these samples, the Ct values of three samples could not be detected and tested negative by the two methods. The genotyping results of the other samples tested by those two methods were identical

Table II. Sensitivity of quantitative reverse transcription PCR for HCV genotyping.

	Ct value for subtype...				
Test number	1b	2a	3a	3b	6a
1	25.32	24.99	25.08	25.37	23.12
2	25.72	25.63	24.75	25.32	23.66
3	26.50	25.82	25.19	25.70	25.68
4	25.27	25.94	24.63	24.52	25.42
5	25.43	26.27	25.85	25.90	23.66
6	25.58	26.10	26.37	24.91	23.66
7	24.17	24.23	26.42	26.02	24.49
8	24.30	24.28	25.34	26.42	25.93
9	25.08	24.83	25.83	24.48	25.33
10	24.92	24.11	26.21	26.33	24.64

Note that for each subtype, the detection rate was 100%. HCV, hepatitis C virus; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Table III. Comparison reverse transcription-quantitative polymerase chain reaction with sequencing for detection of HCV samples from 11 patients with hepatitis C.

Sample	Ct value	Expected subtype	Detected subtype
HCV-1	17.20	3a	3a
HCV-2	17.15	1b	1b
HCV-3	Undetectable	Negative	Negative
HCV-4	14.16	1b	1b
HCV-5	Undetectable	Negative	Negative
HCV-6	Undetectable	Negative	Negative
HCV-7	12.76	1b	1b
HCV-8	15.88	2a	2a
HCV-9	16.73	6a	6a
HCV-10	11.29	3a	3a
HCV-11	12.38	6b	6b

Note that for all HVC samples, the coincidence rate was 100%. HCV, hepatitis C virus; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

(Table III). The RT-qPCR results for the HCV genotyping method showed concordance with the sequencing results, the gold standard for HCV genotyping.

Anti-interference of RT-qPCR for HCV genotyping. Regarding anti-interference, the RT-qPCR genotyping method also performed well. Compared with the high Ct value HCV 3a subtype samples mixed with saline, the high-value HCV 3a subtype samples mixed with the jaundice sample, lipid sample and hemolysis sample reached 2.9% as the maximum difference percentage in Ct value.

Table IV. Within-run precision of reverse transcription-quantitative polymerase chain reaction for HCV genotyping.

	CV (%)		
Subtype	Test 1	Test 2	Test 3
HCV 1b	1.26	1.48	1.34
HCV 2a	2.28	1.74	1.45
HCV 3a	2.94	2.93	2.60
HCV 3b	2.53	3.19	1.92
HCV 6a	1.09	1.72	2.26

HCV, hepatitis C virus; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

Table V. Between-run precision of reverse transcription-quantitative polymerase chain reaction for HCV genotyping.

HCV subtype	Mean value	SD	CV (%)
1b	25.799	0.181	0.70
2a	21.658	0.177	0.82
3a	22.976	0.067	0.29
3b	22.718	0.191	0.84
6a	24.344	0.102	0.42

HCV, hepatitis C virus; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; CV, coefficient variable.

Table VI. Results of HCV genotyping for 289 patients with hepatitis C.

	No./Percentage (%)		
HCV subtype	Wenzhou	Hangzhou	Total
1b	23 (35)	107 (48)	130 (45)
2a	4 (6)	22 (10)	26 (9)
3a	10 (15)	28 (13)	38 (13)
3b	15 (23)	38 (17)	53 (18)
6a	13 (20)	29 (13)	42 (15)
Total	65	224	289

HCV, hepatitis C virus.

Precision of RT-qPCR for HCV genotyping. According to the precision test results, the CV value of every subtype's Ct value in each one of the three parallel tests of RT-qPCR within-run precision was <5%. Likewise, in the three tests of the RT-qPCR between-run precision, the CV value of each subtype's Ct value was also <5%. This shows that both within-run precision and between-run precision met the requirements of the clinic for diagnosis (Tables IV and V, and Fig. 2).

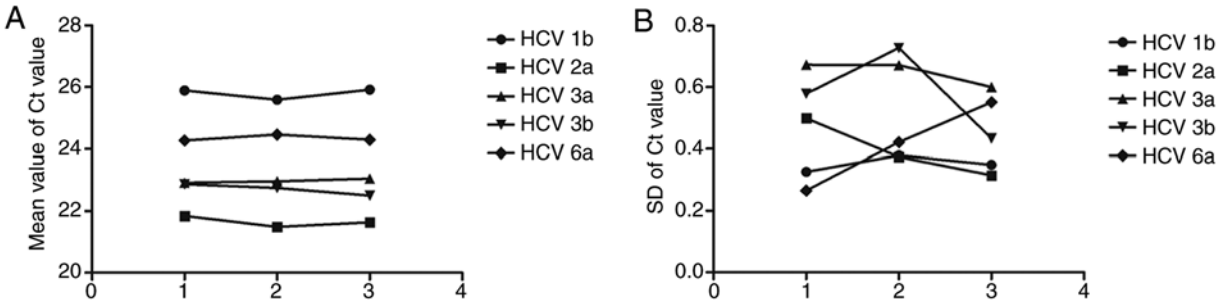

Figure 2. Within-run precision of one-step RT-qPCR for HCV genotyping. (A) The mean value of five HCV subtypes' Ct value in the three within-run precision test. (B) The SD of five HCV subtypes' Ct value in the three within-run precision test. HCV, hepatitis C virus.

Genotyping results for patients with HCV. In this study, genotyping showed that the HCV 1b subtype was the most prevalent subtype, accounting for 45% of the total. This is consistent with other research studying HCV subtype distribution in China (6). In addition, the HCV 3b and HCV 6a subtypes accounted for ~18 and 15%, respectively, while 13% were infected with the HCV 3a subtype. The population of people infected with the HCV 2a subtype was the lowest (Table VI).

Discussion

According to WHO guidelines, ~71 million people were infected with HCV globally in 2015 (2). Moreover, a number of clinical practices have shown that the validation of HCV subtype plays a crucial role in determining the appropriate treatment for HCV. Thus, various methods for HCV genotyping have been developed (19). The present study successfully mitigates some of the problems inherent in HCV genotyping by using RT-qPCR. To some extent, the method presented here solves the problem of the expensive and time-consuming nature of HCV genotyping, and ensures the accuracy of the results.

The method presented here is mainly based on a one-step RT-PCR and Taqman fluorescence probe technique. Firstly, to obtain optimally designed primers that could provide the most accurate genotyping results, the conserved region of five HCV subtypes (1b, 2a, 3a, 3b and 6a) was focused upon. Probes were matched with primers to allow the detection of five HCV subtypes in one step using only three RT-PCR reaction tubes. Nyan and Swinson (20) reported a method for rapid detection and genotype identification of HCV 1-6 by one-step reverse transcription loop-mediated isothermal amplification. However, the method requires electrophoretic analysis of banding patterns and visual interpretation of fluorescence intensity in the reaction tubes, which may result in the contamination of the non-closed tube test and arbitrary interpretation uncertainty. The method presented here has the advantage of closed tube detection and simple analysis. In addition, coupled with the automation of RT-PCR, the whole procedure requires only a few h to complete and is easier to operate. This makes it superior to many other classic approaches for genotyping, and more suitable for widespread use.

Furthermore, when genotyping 11 samples, the results were concordant with sequencing results, showing a high level of accuracy (Table IV). As accuracy is always the first concern for genotyping, this suggests that the method presented here is reliable enough to be used in clinical practice. Moreover, according to the performance validation, the RT-qPCR method has good performance in additional aspects. When samples were diluted to close to the detection limit, their detection rate still reached 100%, demonstrating a high sensitivity. None of the HBV, HCMV or MP samples were positive, showing the high specificity of this method. When HCV samples were mixed with a jaundice sample, lipid sample and haemolysis sample, the maximum difference percentage of the Ct value only reached 2.9%, revealing that it also exhibited good anti-interference qualities. Finally, all CV values of each test for both within-run precision and between-run precision were <5%, thereby demonstrating high reproducibility.

No mixed HCV genotypes or subtypes were encountered in the present study. However, the established method could detect the mixed positive plasmid of the 5 detectable subtypes (data not shown), which suggests that the method used in the present study could detect the 5 detectable subtypes of both single and mixed HCV samples. Due to the difference between the recombinant DNA strains and the clinical samples, more studies are needed to confirm this result.

However, the RT-qPCR method does have limitations. The most evident one is that it can only detect five HCV subtypes, and it cannot detect certain low proportion HCV genotypes in China, such as subtype 1a, Group 5 subtypes and several other Group 6 subtypes. No method for genotyping subtype 1a was used in the present study, due to the low proportion of this subtype in China. For example, Tong *et al* (6) found only 1.59% of samples are subtype 1a. Other studies have provided similar data (21,22). Overall, the proportion of subtype 1a is extremely low in China, and the five subtypes described in the present study (1b, 2a, 3a, 3b, 6a) are the most common HCV RNA genotypes in China (21,22). The 289 samples used here fell into the categories of the 5 detectable types and no other types (including HCV 1a) were detected. The method presented here could be useful in regions such as China, where the most prevalent HCV subtypes are the five described in this study (22). To make up for the deficiency in not detecting all HCV subtypes, when the genotyping data from HCV RNA-positive samples showed negative results via the RT-qPCR detection method, it could be considered as a rare subtype. In this instance, sequencing should then be performed.

Due to the superiority of DAA drugs, they are now the main treatment for HCV. The specific treatment of DAA drugs varies according to the subtype of infecting HCV RNA.

Therefore, HCV genotyping, using methods such as the one presented here, greatly aids the treatment of HCV. According to the genotyping results in the present study, patients with HCV were treated appropriately. However, the small sample size is a limitation of this study, and future studies with a larger number of clinical samples are required to verify its findings.

In conclusion, this study presents an accurate, convenient and cost-effective method for HCV genotyping, which contributes to the novel use of RT-qPCR techniques and the elaborate design of primers and probes. Its application in clinical practice could more accurately, sufficiently and economically enable the treatment of HCV-infected patients.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZGC and JY designed and supervised the project. YX and JY provided the samples, validated them and provided clinical information about the samples. XY, YX and HH performed the experiments. HH provided instructions for the experiments. XY and TD analysed the data and wrote the manuscript. ZC and TD edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of the Second Affiliated Hospital of Wenzhou Medical University (approval no. L-2018-10). All patients provided written informed consent, agreed to the use of their samples in scientific research, and approved the publication of data.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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