Evaluation of in-house and commercial genotyping assays for molecular typing of hepatitis C virus in Hong Kong

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ABSTRACT

This study aims to evaluate genotyping assays for hepatitis C virus (HCV). An in-house nucleic acid sequencing method is performed in parallel with the Roche Linear Array HCV genotyping test on 73 HCV-positive (66 clinical samples and seven proficiency testing quality control samples) and 12 HCV-negative samples (11 clinical samples and one proficiency testing sample). The performance of the in-house method was comparable with that of the Roche assay (concordance rate: 89.4%). Discordant results included four mixed infections missed by the in-house method, two false-negatives with the Roche assay, and three discrepant results. The in-house method exhibited a higher resolution (subtype *vs.* genotype level) at a lower running cost (25% of the commercial assay). The in-house method was also used to genotype 375 HCV clinical isolates to determine the genotypic distribution of HCV in Hong Kong between 2005 and 2008. A total of 441 (52.8%) clinical isolates proved to be genotype 1, which shows a poorer response to interferon therapy. Genotype 6 was the next most common (32.0%). Prevalence of genotypes 2 and 3 was 7.7% and 6.6%, respectively, and prevalence of genotypes 4 and 5 was 0.9% and 0%, respectively. Although the in-house nucleic acid sequencing method failed to detect a few cases of mixed HCV infection, its high resolution and low running cost make it suitable for surveillance and outbreak investigation.

KEY WORDS: Genotype.
Hepatitis C.
Hybridization, genetic.
Polymerase chain reaction.

Introduction

Hepatitis C virus (HCV) infection is a major health issue worldwide. Around three to four million individuals are newly infected by HCV annually, with 80% likely to develop chronic HCV infection.[1] These patients are at risk of developing cirrhosis (10–20%) and hepatocellular carcinoma (1–5%) over the next 30 years and may require prolonged and intensive treatment regimes and, eventually, liver transplantation.[1]

Although the severity of disease may not be predicted by the six HCV genotypes, the response to treatment (commonly a combination of interferon-α and the antiviral drug ribavirin) is genotype-dependent.[2–4] Importantly, genotype 1 is more resistant to interferon therapy and capable of doubling the treatment course from six to 12 months.[4] Determination of HCV genotype is therefore crucial before a treatment decision is made.

Reverse dot blot assays such as the Versant HCV genotype assay (LiPA, Siemens Healthcare Diagnostics) is a common genotyping method for HCV as it is easily performed and interpreted. The Linear Array HCV genotyping test (Roche) is a new hybridisation-based genotyping tool that uses a similar methodology to the Versant HCV assay. After one-step reverse transcriptase-polymerase chain reaction (RT-PCR) of the 5′ untranslated region (UTR) of the HCV genome, denatured amplicons are hybridised with genotype-specific probes immobilised on a nylon strip and are detected by a colorimetric reaction. As systematic evaluation of the Linear Array assay is limited, the current study investigates the application of the Linear Array assay to clinical HCV samples.

Nucleic acid sequence analysis of the HCV genome is another option for HCV genotyping. Various portions of the HCV genome have been suggested as targets for genotyping, including the 5′ UTR, the nucleocapside or core (C) gene, the envelope (E1) region and the RNA polymerase (NS5B) region.[5] In the current investigation, the core gene, which is more variable than the 5′ UTR, is analysed by an in-house nucleic acid sequencing method.[6]

To evaluate their performance, the in-house method is performed in parallel with the Linear Array assay on eight HCV proficiency testing samples and 66 serology-confirmed HCV-positive samples and 11 HCV-negative samples. The in-house method is also used to genotyped 375 HCV-positive samples in an attempt to determine the distribution of HCV genotypes in Hong Kong.

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Materials and methods

Between 2005 and 2008, a total of 441 EDTA-anticoagulated plasma samples were collected from patients before initiation of antiviral therapy in five general hospitals in Hong Kong. Hepatitis C virus infection was diagnosed serologically (AxSYM anti-HCV, version 3.0; Abbott, USA) and the HCV viral load was monitored by the cobas Amplicor HCV monitor test (version 2.0; Roche Diagnostics, USA). Another 11 HCV seronegative plasma samples were included as negative controls for the HCV genotyping assays. Viral load for HCV seropositive samples ranged from $2.96x10^2$ to $6.94x10^6$ iu/mL, whereas none of the seronegative samples indicated a detectable level of HCV RNA.

The 11 HCV-negative samples, together with 66 HCV-positive samples randomly chosen from the 441 positive samples, were analysed by the Linear Array HCV genotyping test (version 2.0; Roche), as described previously.[7,8] Eight HCV genotyping proficiency testing samples, which included one HCV-negative and seven HCV-positive samples of known genotype (Quality Control for Molecular Diagnostics, Scotland), were also included in the evaluation.

In addition to the 73 HCV-positive (66 clinical samples, seven proficiency testing sample) and 12 HCV-negative samples (11 clinical samples, one proficiency testing sample) analysed by the Linear Array assay, another 375 HCV-positive samples were tested using an in-house nucleic acid sequencing method, as described previously.[6]

Hepatitis C virus RNA was extracted by the QIAamp viral RNA mini kit (Qiagen, USA) according to the manufacturer's instructions. Briefly, an amplicon of 441 bp was amplified from 10-µL RNA extract in a 50 µL RT-PCR reaction, which included 1-µL RT/platinum *Thermus aquaticus* (*Taq*) mix (SuperScript one-step RT-PCR with platinum *Taq*; Invitrogen, USA), 1x reaction mix, and 0.2 µmol/L each primer (Sc2: GGGAGGTCTCGTAGACCGTGCACCATG and Ac2: GAGMGGKATRTACCCCATGAGRTCGGC).[6] Cycling profile was as follows: RT at 45˚C for 30 min, heat activation at 94˚C for 5 min, 20 cycles of 94˚C, 45˚C and 72˚C for 1 min each, 20 cycles of 94˚C, 60˚C and 72˚C for 1 min each, followed by a final elongation at 72˚C for 7 min.

Another amplicon of 355 bp was generated in a 50 µL nested PCR containing 2.5 units of AmpliTaq Gold (AmpliTaq Gold with GeneAmp 10x PCR buffer II and $MgCl_2$ solution; Applied Biosystems, USA), 1x PCR buffer II, 1.5 mmol/L $MgCl_2$, 0.4 mmol/L dNTP (Fermentas, USA), 0.8 µmol/L each primer (S7: AGACCGTGCACCATGAGCAC and A5: TACGCCGGGGGTCAKTRGGGCCCCA) and 2 µL RT-PCR product.[6] Cycling profile was as follows: 5 min heat activation at 94˚C, 40 cycles of 94˚C, 58˚C and 72˚C for 1 min each, followed by final elongation at 72˚C for 7 min. The nested product was purified using the QIAquick PCR purification kit (Qiagen).

A 20 µL sequencing reaction was performed containing 2-µL Ready Reaction mix (BigDye Terminator v1.1 cycle sequencing kit; Applied Biosystems), 3 µL 5x sequencing buffer, 0.16 µmol/L sequencing primer (S7: AGACCGTGCACCATGAGCAC or A5: TACGCCGGGGGTCAKTRGGGCCCCA) and 150 ng purified amplicon from the nested PCR.[6] Cycling profile was as follows: heat activation at 96˚C for 1 min followed by 25 cycles of 96˚C for 10 sec, 50˚C for 5 sec and 60˚C for 4 min. After purification using Autoscreen 96-well plates (GE Healthcare, USA), the sequencing product was analysed using the ABI Prism 3700 genetic analyser (Applied Biosystems) according to the manufacturer's instructions.

Genotypes of HCV were determined using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 1. Genotyping of eight HCV proficiency testing samples.

		HCV genotype determined by:		
Sample ID	Viral load (iu/mL)	QCMD	In-house	Linear array
HCVG06-01	4×10^3	3a	3a	3
HCVG06-02	4×10^4	3a	3a	3
HCVG06-03	4×10^4, 1×10^4	1b, 3a	1b	1, 3
HCVG06-04	4×10^4	1a	1a	1
HCVG06-05	3×10^4, 3×10^4	3a, 5a	5a	3, 5
HCVG06-06	–	Negative	Negative	Negative
HCVG06-07	4×10^4	1b	1b	1
HCVG06-08	4×10^4	5a	5a	5

QCMD: quality control for molecular diagnostics.

Results

Performance of the in-house method and the Linear Array assay was comparable when analysing the eight HCV genotyping proficiency testing samples (seven HCV-positive, one HCV-negative) although mixed genotype in two samples was missed by the in-house method (Table 1). Resolution of the in-house method was higher as it differentiated all subtypes. Additional genotyping of 77 clinical samples (66 HCV seropositive, 11 HCV seronegative) demonstrated concordance of 89.4 % (76/85 HCV-positive and HCV-negative samples) between the two methods. Discordant results are summarised in Table 2.

Among the 441 HCV samples, genotype 1, which shows a poorer response to interferon therapy, was the predominant genotype (52.8%), followed by genotype 6 (32%). Prevalence of genotypes 2 and 3 was 7.7% and 6.6%, respectively, and

Table 2. Discordant results between in-house method and Linear Array assay.

		HCV genotype determined by:	
Sample ID	Viral load (iu/mL)	In-house	Linear array
1	2.8×10^5	3a	Negative
2	1.5×10^5	6a	Negative
HCVG06-03	4×10^4, 1×10^4	1b	1, 3
HCVG06-05	3×10^4, 3×10^4	5a	3, 5
3	3.2×10^5	1a	1, 3
4	2.3×10^5	3b	3, 4
5	9.3×10^4	6a	3
6	9.3×10^4	6a	3
7	3.3×10^5	6a	1

prevalence of genotypes 4 and 5 was 0.9% and 0%, respectively. Detail distribution of HCV genotypes in Hong Kong is shown in Table 3.

Table 3. Genotypic distribution of 441 HCV isolates in Hong Kong.

Genotype	Number of isolates (%)	Subtype	Number of isolates (%)
1	233 (52.8)	1a	19 (4.3)
		1b	214 (48.5)
2	34 (7.7)	2a	15 (3.4)
		2b	13 (2.9)
		2c	2 (0.5)
		2d	2 (0.5)
		2f	2 (0.5)
3	29 (6.6)	3a	25 (5.7)
		3b	3 (0.7)
		3g	1 (0.2)
4	4 (0.9)	4a	4 (0.9)
5	0 (0)		
6	141 (32.0)	6a	131 (29.7)
		6d	3 (0.7)
		6g	3 (0.7)
		6i	2 (0.5)
		6k	2 (0.5)
Total	441 (100)		

Discussion

In the current investigation, an in-house genotyping method and the Linear Array assay were shown to be comparable, despite generation of a few discordant results.

Failure to detect mixed infection is not uncommon among PCR sequencing-based genotyping methods.[9,10] However, the number of mixed HCV infections found in the present study was low (2/66 [3%]) and was consistent with previous findings (4–5%).[11,12] Cloning could resolve the problem of mixed infection but it is tedious and therefore impractical for use in diagnostic laboratories.

Genotyping using the more variable core and E1 genes is less sensitive than the highly conserved 5′ UTR.[13] Two samples with high viral load were not detected by the 5′ UTR-based Linear Array assay, but they were detected by the core gene-based in-house method in the current study. The higher sensitivity of the in-house method is probably due to nested PCR, which is not utilised in the Linear Array assay.

Contradictory results were present in three clinical samples analysed by both assays. These HCV isolates are probably a hybrid of two genotypes as two regions of the HCV genome (5′ UTR and core gene) were analysed. Previous studies show that genotype 6 variants isolated from patients in Vietnam and Thailand may have sequences in the 5′ UTR that are similar or identical to those of genotype 1.[14–16] Genotyping based solely on either region of HCV may lead to incorrect interpretation when analysing hybrid strains of HCV.

The resolution of the in-house method was higher than that of the Linear Array assay, as the former differentiated all subtypes. High resolution is a prerequisite in outbreak investigations.[17,18] A sequence-based method is also able to identify potential new subtypes, whereas hybridisation may not.[10]

Nucleic acid sequencing is generally believed to be costly because it involves expensive fluorescence-labelled dideoxynucleotide. The running cost of the in-house method was shown to be three times less than the commercial Linear Array assay and thus is particularly suited to laboratories that perform sequencing as a routine task. Furthermore, the introduction of automation and high-throughput platforms renders nucleic acid sequencing even more robust and accessible.

The in-house method showed that genotypes 1 and 6 (more precisely 1b and 6a) account for the vast majority of HCV isolates in Hong Kong. A retrospective review of the literature reveals that this has not changed over the past 14 years.[11,12,19–21] A strong negative and linear relationship between genotypes 1 and 6 has been observed (correlation coefficient between genotypes 1 and 6 [r1:6: –0.89), which does not exist among the other genotypes (r1:4: –0.77, r1:3: –0.38, r1:2: –0.13 and r1:5: 0.00) when analysing archive data available in the literature.[11,12,19–21] Genotypes 1 and 6 show a descending trend and an ascending trend, respectively, during this period; however, genotype 6 has a more favourable response to interferon treatment.[22] Longitudinal monitoring is imperative in order to confirm the continuity of this trend.

In summary, the current study demonstrates that an in-house nucleic acid sequencing method provides high genotyping resolution at low running cost. It is recommended for laboratories involved in outbreak investigations that experience a low rate of mixed HCV infection in their locality. External quality control (e.g., QCMD) is imperative for these laboratories to monitor the performance of in-house methodologies. However, for routine diagnostic testing, the well-documented US Food and Drug Administration (FDA)-approved Versant HCV genotype assay may be considered suitable.[23] □

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