Evaluation and comparison of genotyping assays for molecular epidemiological study of HCV in Hong Kong

P.S. Cheng and W.C. Yam

The Hong Kong Society For Molecular Diagnostic Sciences
4th Floor, Duke of Windsor Social Service Building, 15 Hennessy Road, Hong Kong.

Address for correspondence:
Dr. YAM Wing-Cheong, Honorary Treasurer, The Hong Kong Society For Molecular Diagnostic Sciences, 4th Floor, Duke of Windsor Social Service Building, 15 Hennessy Road, Hong Kong.

Abstract

Treatment regimen for hepatitis C virus infection is viral genotype dependent. Serum samples were used to evaluate and compare a commercial and a newly developed *in-house* HCV genotyping protocol. The sequencing data obtained from *in-house* genotyping method were subjected to epidemiology analysis of major HCV genotypes prevalent in Hong Kong. A total of 337 serum samples were included in this study. Among these 337 samples, 85 samples were tested by both assays, and the other 252 were tested only by the *in-house* developed method. HCV viral load detection was performed on all samples. Molecular epidemiology was further elucidated on HCV subtypes 1b and 6a which are highly prevalent in Hong Kong. Results showed an overall concordance rate of 89.4% between the assays. The commercial HCV genotyping test is capable to detect mixed genotypes in the same sample while the *in-house* developed method can be operated in a lower running cost. The local HCV subtypes 1b and 6a showed a low genetic divergence to that of other geographic areas. Our findings highlight the potential application of *in-house* HCV genotyping protocol which is suitable for routine laboratory diagnosis at a lower running cost.

Key words: Genotyping, linear array, sequencing, epidemiology analysis

Introduction

Hepatitis C virus is the leading pathogen causing non-A and non-B hepatitis since its discovery in 1989.[1] According to the estimation of World Health Organization, about 3% of the global population are infected with Hepatitis C virus and about 170 millions of people are HCV carriers.[2] At present, there is a tendency that HCV infection emerges worldwide. Numerous research groups suggested that the distribution of HCV genotypes is geographically and groups related. Subtype 6a is common in Hong Kong and China but it is not the case in other regions.[3] Furthermore, patients co-infected with HCV and HIV exhibited higher mortality rate and lower quality of life than patients infected with either viral agents.[4-7]

Hepatitis C viruses are sub-divided into 6 major genotypes according to their genetic variability.[11] Various methods and technologies are used to genotype or even subtype the HCV RNA in patients.[8-11] Genotyping and viral load monitoring are useful in treatment efficacy evaluation and

prediction of likelihood of response since clinical studies claimed that the efficacy of the interferon alpha 2b plus ribavirin is genotype dependent.[7, 12-14]

In this assay, we compared and discussed the utility of HCV genotyping by using a commercial hybridisation method as well as an *in-house* developed method.

Materials and methods

Serum samples from patients who were suffering from HCV infection in a few general hospitals in Hong Kong were included in this study. All of the HCV patient samples were confirmed to be positive for HCV antibody. A total of 337 serum samples were included in this HCV study. These samples included 303 HCV patient samples, 8 HCV genotyping proficiency program samples (http://www.qcmd.org) and 26 HCV negative control samples. All of these 337 samples were subjected to the *in-house* developed HCV genotyping test. Among the 337 serum samples, 85 were tested by the commercial *linear array* genotyping test.

The *LINEAR ARRAY Hepatitis C Virus Genotyping Test* (Roche Molecular Systems, Inc. USA) was used for HCV genotyping comparison and all procedures were carried out according to the manufacturer's instructions. It is a commercial *in-vitro* diagnostic testing used for determining the genotypes in Hepatitis C virus infected patients in HCV genotypes 1 to 6 (GT1 to GT6).

The *in-house* developed HCV genotyping method is a sequencing based method and samples were analyzed by using ABI PRISM 3700 Genetic Analyzer according to the manufacturer's instructions. The obtained sequence alignments were analyzed by annotated in Genbank and compared with the published sequences.

HCV viral load of samples were done by COBAS® AMPLICOR™ HCV MONITOR Test, v2.0 (Roche Molecular Systems, Inc. USA) and manufacturer's instructions were followed.

Sequences obtained from the *in-house* developed HCV genotyping method were analyzed and categorized. Two HCV subtypes, 1b and 6a, were predominant in the study population. Phylogenetic analysis was performed on them. Sequences of the 2 subtypes were aligned separately by using the ClustalW program.[15] The aligned sequences were edited by BioEdit software version 7.0.4.1 and trees of 1b and 6a were generated with PAUP* 4.0 software package. Trees were constructed by using Neighbor-joining method and used 100 boostrap replicates. Trees were displayed with the TreeView program version 1.6.6.[16] The phylogenetic tree for subtype 1b sequences was rooted using subtype 1a (NCBI accession number: U55281) as the outgroup while the phylogenetic tree for 6a sequences was rooted using subtype 1b (NCBI accession number: U10234) as the outgroup.

Results

Commercial linear array genotyping test

Table 1 summarized the HCV genotyping results of the *linear array* method. A total of 85 samples were tested. Among the 85 samples, 71 samples were successfully genotyped by the method and the rest could not be amplified in the initial PCR step (by COBAS AMPLICOR Analyzer) and were identified as negative by the *linear array* method. Representative schematic results of the *LINEAR ARRAY Hepatitis C Genotyping Test* were revealed in Figure 1a and 1b. Four samples were typed as mixed HCV genotypes by the *linear array* assay. Among the 4 mixed HCV genotypes samples, 2 were HCV patient samples and 2 were HCV genotyping proficiency program samples. For the 2 HCV patient samples, the results could not be concluded since the kit did not include the interpretation procedures or guidelines for multiple genotypes samples. It was confirmed that the *linear array* assay could accurately genotyped the HCV genotyping proficiency program samples by combining the HCV genotype patterns given by the manufacturer. The detection range for the samples that were successfully genotyped by the *linear array* genotyping test was between 5.33×10^3 and 2.00×10^6 IU/mL.

Table 1. HCV genotyping results by *linear array* genotyping test.

	Genotype	Number of Sample
HCV Patient Samples - *linear array* assay tested	1	35
	2	2
	3	9
	4	3
	6	13
	Mixed	2 (GT1 + GT3) & (GT3 + GT4)
	Failed	2
HCV Genotyping Proficiency Program Samples	1	2
	3	2
	5	1
	Mixed	2 (GT1 + GT3) & (GT3 + GT5)
	Failed	1
Negative Controls	Failed	11

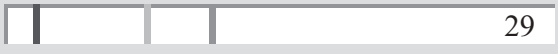
Stripe	Genotype	HCV Genotype Pattern
 06	GT1	1, 4, 9
 72	GT1	1, 4, 7, 9
 09	GT2	2, 4, 9
 67	GT2	2, 4, 9
 02	GT3	3, 4, 9
 46	GT3	4, 9
 03	GT4	6, 9
 29	GT4	6, 9
 76	GT5*	7, 9
 14	GT6	1, 4, 8, 9
 44	GT6	4, 8, 9

Figure 1a. Single genotypes.

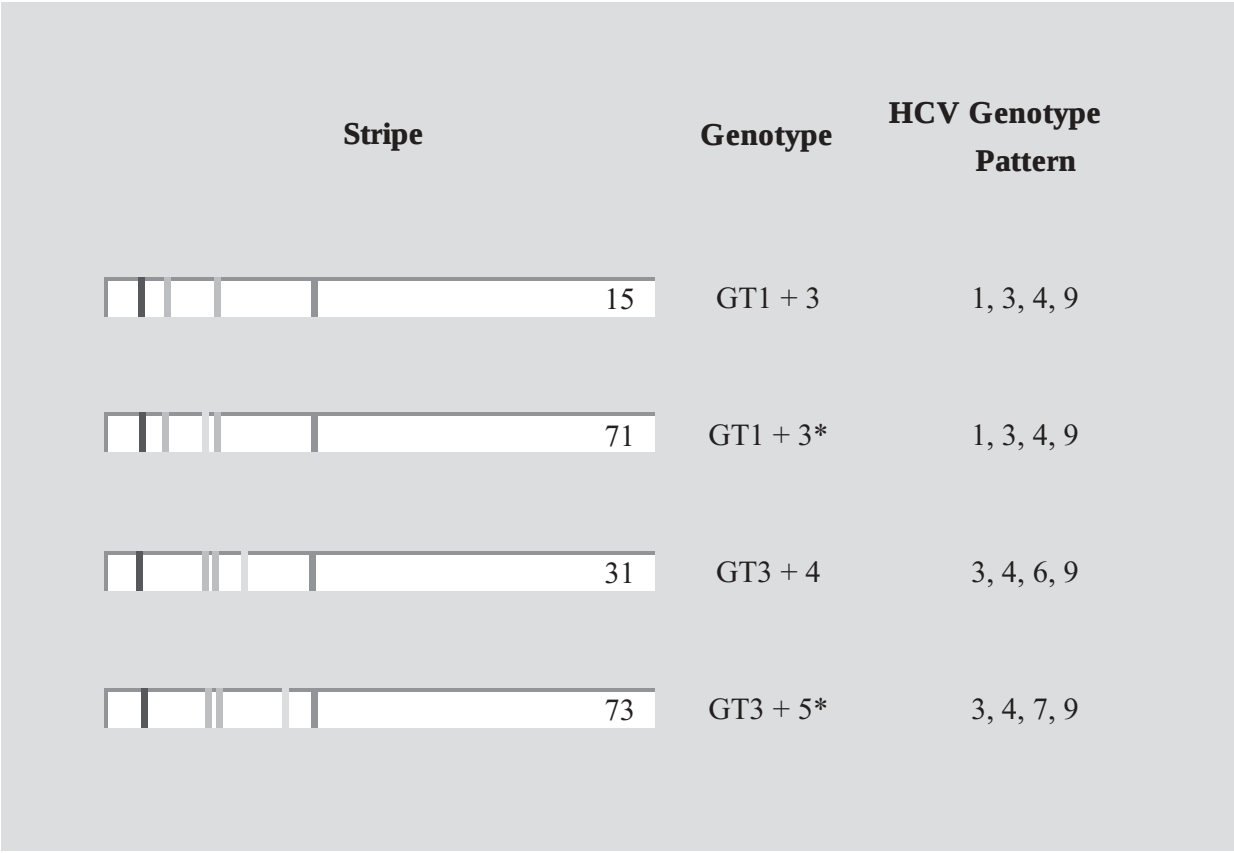


Figure 1b. Mixed genotypes.

Figure 1a and 1b. Schematic results of the *LINEAR ARRAY Hepatitis C Virus Genotyping Test*. Representative strips of different HCV genotypes were selected and presented. The underlined probes were the specific probes for the corresponding genotypes. The strips marked with "*" were HCV genotyping proficiency program samples.

In-house developed method

Table 2 tabulated the results of the HCV genotyping by the *in-house* developed method. Total 337 serum samples, including 303 HCV patient samples, 8 HCV genotyping proficiency program samples and 26 HCV negative control samples, were genotyped by the method. Three hundred and three HCV positive samples, including 295 HCV patient samples (97.4% of HCV patient samples; 295 out of 303), and 7 HCV genotyping proficiency program samples were successfully genotyped. Eight HCV patient samples and 27 negative controls were typed as negative in the test. The detection range of the *in-house* developed method was between 2.96×10^2 and 6.94×10^6 IU/mL.

Table 2. HCV genotyping results by *in-house* developed method.

	Genotype	Number of Sample
HCV Patient Samples	1a	13
	1b	145
	2a	10
	2b	9
	2c	1
	2d	1
	2f	1
	3a	17
	3b	2
	3g	1
	4a	3
	6a	86
	6d	2
	6g	2
	6i	1
	6k	1
	Failed	8
HCV Genotyping Proficiency Program Samples	1a	1
	1b	1
	3a	2
	5a	2
	Failed	1
Negative Controls	Failed	26

Phylogenetic analysis

The phylogenetic trees of HCV 1b and 6a were shown in Figure 2 and 3. Sequences obtained from the *in-house* method were aligned and analysed. Several closely related sequences published in NCBI (National Centre for Biotechnology Information)[17] were included and plotted in the form of phylogenetic trees to investigate the genetic divergence of local HCV subtypes when compared with the other regions. (1b reference sequences NCBI accession numbers: AB086054, AB269351, AB291002, AB291015, AB291019, AB291024, AB291028, AM286057, AM286182, AM286183, AM286184, AM422816, AM422820, AY365213, DQ417430, DQ417431, DQ417432, DQ485285, U10213 and U10234); (6a reference sequences NCBI accession numbers: Y12083, AB162866, AB162867, AB162869, AB301811, AB301812, D88476 and U33435)

The phylogenetic trees share similarities of the HCV sequences published in other areas. The trees do not show apparent and discrete clusters and the results may suggest that the HCV subtypes 1b and 6a found in Hong Kong show low genetic divergence when compared with other regions. It may be due to the result of traffic and economic globalisation.

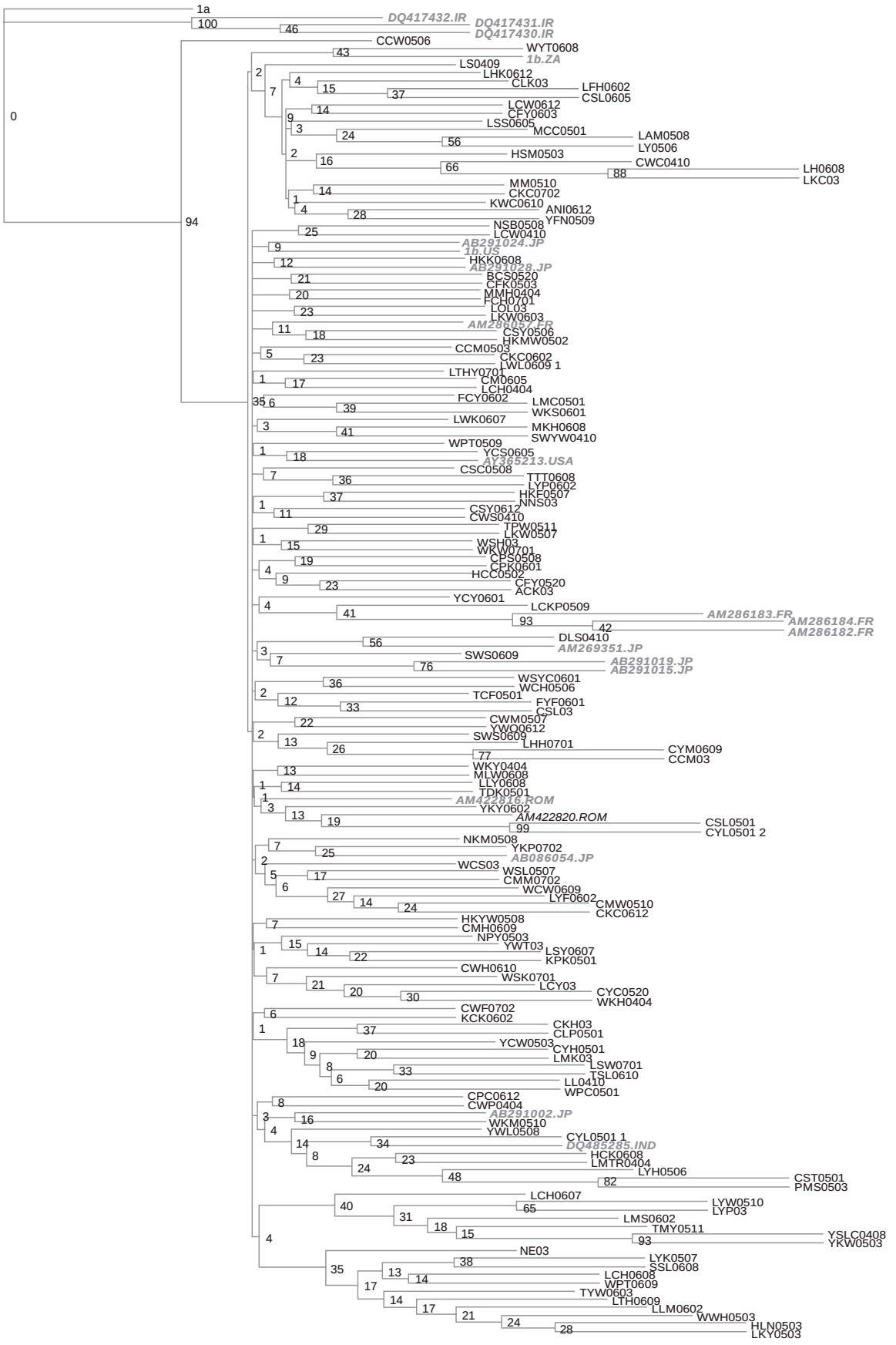


Figure 2. Phylogenetic tree of HCV 1b. The highlighted sequences were HCV 1b reference sequences published in NCBI found in different areas. Abbreviations: FR: France; GER: Germany; IND: India; IR: Ireland; JP: Japan; LAT: Latvia; ROM: Romania; USA: United States and ZA: Zaire.

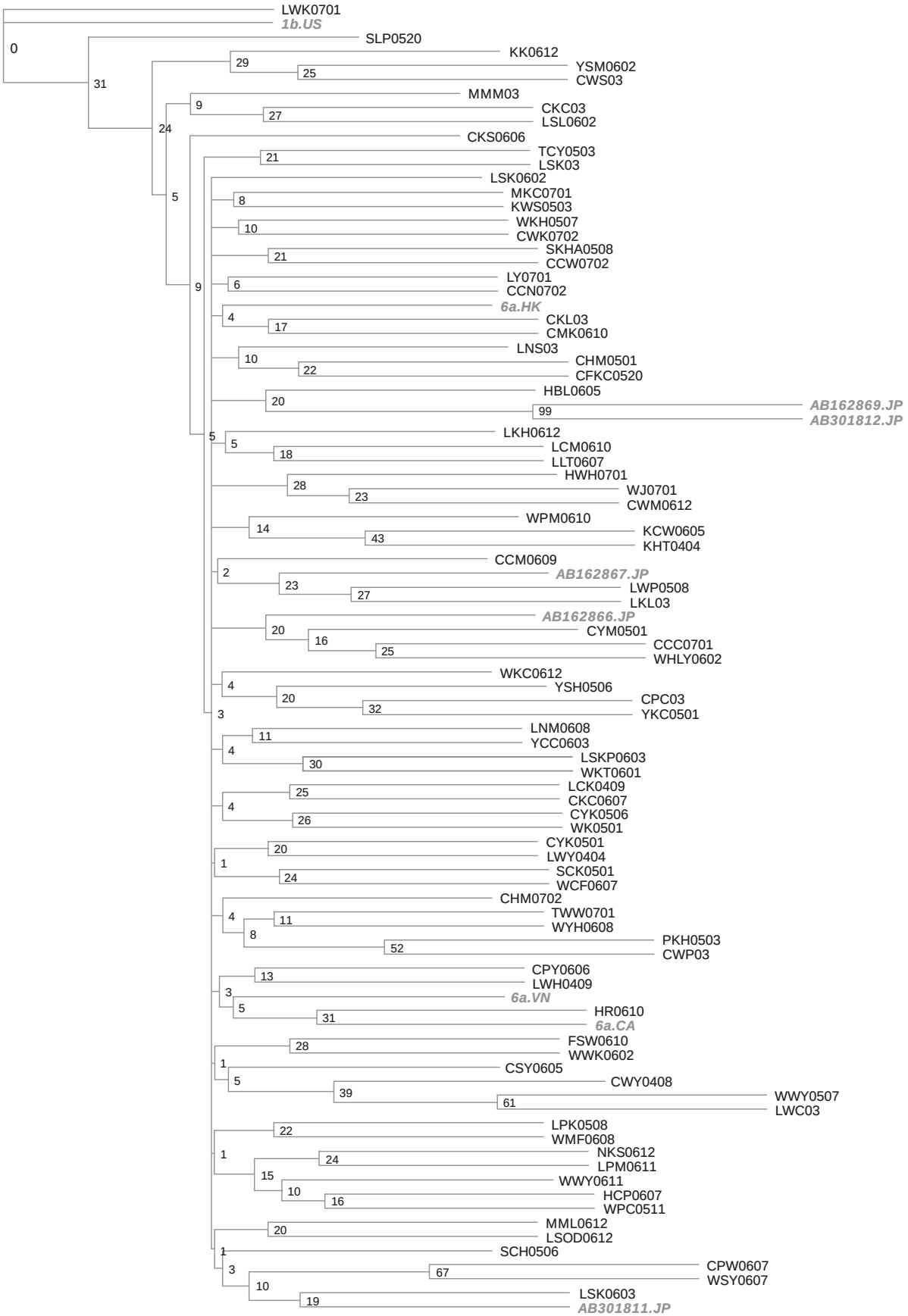


Figure 3. Phylogenetic tree of HCV 6a. The highlighted sequences were HCV 6a reference sequences published in NCBI found in different areas. Abbreviations: CA: Canada; HK: Hong Kong; JP: Japan; US: United States and VN: Vietnam.

Discussions

In this study, the *linear array* assay showed better performance to identify mixed genotypes in tested samples when compared with the *in-house* method. The present *in-house* method does not show a significant advantage as the sequencing technique failed to distinguish mixed HCV genotypes. In this study, the *in-house* method only detected the predominant genotype in the sample.

Discrepant genotyping results were obtained and several reasons may explain it. Firstly, there is a possibility of recombination and formation of hybrid viruses in the samples. Both genotyping assays only target a single region of the HCV genome and it cannot represent the whole sequence of the organism. Secondly, it has been shown that HCV GT3 and GT6 have more genetic diversity considerably and comparatively when compare with other genotypes[18], leading to more common errors in genotyping process for GT3 and GT6 samples than other HCV genotypes. Two samples were discrepantly typed as subtype 6a and GT3 by the *in-house* method and *linear array* assay respectively. Similarly, the high genetic diversity of these 2 genotypes may cause the mistyping of HCV RNA. Studies also showed that some GT6 variants identified in Southeast Asia have 5'UTR sequences identical to those of subtypes 1a and 1b.[19-22] This may account for a single sample typed as subtype 6a and GT1 by the assays used in this study. Furthermore, the instability of RNA may account for the discrepant results obtained in this study. More samples will be needed to resolve the discrepancy.

The detection ranges for the *linear array* assay and *in-house* developed method were $\log_{10}$ 3.73 - 6.47 and 2.47 - 6.84 respectively. It seems that the *in-house* method showed a wider range of detection. However, the inferior performance of the *linear array* assay in the detection range may be the result of the limited sample size. According to the manufacturer's evaluation results, the *linear array* assay claimed that the test can detect HCV RNA from GT1 to GT6 in both EDTA-plasma and serum at concentrations of ≧500 IU/mL ($\log_{10}$ 2.70) with a positivity rate of >95%. Therefore, the results from this study can only project a rough contrast to the detection range between the assays.

The 2 assays also differ in the turn-around-time (TAT) and running cost. The turn-around-time mentioned in this study defines as the time needed for sample preparation through reporting of results. The TAT for both assays is around 2 to 3 days. Both assays can be finished in the predicted TAT. This is beneficial to control the testing TAT and hence the quality of patient care. The running cost of the *linear array* assay and *in-house* developed method were around (HKD $400) and (HKD $100) per each sample respectively. There is a 3/4 reduction of the running cost when *linear array* test is replaced by the *in-house* method. The *in-house* method is more cost effective if it is introduced in routine clinical diagnosis.

Based on the phylogenetic results of this study, 1b and 6a are the 2 predominant HCV subtypes in Hong Kong. They are genetically similar to the corresponding subtypes identified in other geographic areas. It is expected for subtype 1b since it is the most prevalent HCV subtype in many regions.[3, 23] Due to the globalisation effect, the transmission of microorganisms among different geographic areas has become more feasible and easy. However, the phylogenetic result of subtype 6a was quite surprising. Subtype 6a is commonly found in restricted areas such as Hong Kong, China and Vietnam but rarely found in the rest of the world.[3] It is expected that the subtype 6a would be genetically distinct in Hong Kong from other areas. This unanticipated result may have 2 explanations. Firstly, the increased traffic of people and culture interaction between the nearby areas may account for the low genetic divergence of the HCV subtypes among these areas. Another reason may be a technical problem. The sequences analysed in this study were about 280 bp in sizes. Short sequences may limit the efficiency of phylogenetic analysis. In order to confirm the genetic divergence of subtype 6a in Hong Kong, amplification of longer sequences for phylogenetic analysis may be helpful.

Conclusion

The *in-house* developed HCV genotyping method showed comparable performance to the commercial assay in terms of the accuracy, detection range and turn-around time. This method is suitable for routine diagnosis and

commendable to HCV treatment management because of its low running cost.

HCV subtypes 1b and 6a are most prevalent in Hong Kong. In this preliminary study, both subtypes showed low genetic divergence when compared with HCV strains in other geographic regions. Initiation of large scale molecular epidemiological study will provide key information on the spread and transmission of HCV in Hong Kong.

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