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Hepatitis C Virus (HCV) Genotyping by Annealing Reverse Transcription-PCR Products with Genotype-Specific Capture Probes

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The genotype of the hepatitis C virus (HCV) strain infecting a given patient is an important predictive factor for the clinical outcome of chronic liver disease and its response to anti-viral therapeutic agents. We herein sought to develop a new easy, sensitive and accurate HCV genotyping method using annealing genotype-specific capture probes (AGSCP) in an automation-friendly 96-well plate format. The validation of our new AGSCP was performed using the Standard HCV Genotype Panel. We then used both our AGSCP and the commercially available INNO-LiPA assay to analyze the HCV genotypes from 111 Korean patients. Discordant results were analyzed by direct sequencing. AGSCP successfully genotyped the standard panel. The genotypes of 111 patient samples were also obtained successfully by AGSCP and INNO-LiPA. We observed a high concordance rate (93 matched samples, 83.8%) between the two assays. Sequencing analysis of the 18 discordant results revealed that the AGSCP had correctly identified 12 samples, whereas the INNO-LiPA had correctly identified only 6. These results collectively indicate that AGSCP assay is a convenient and sensitive method for large-scale genotyping, and it may be a promising tool for the determination of HCV and other genotypes in clinical settings.

***Keywords*:** hepatitis C virus, genotype, reverse-hybridization

Hepatitis C virus (HCV) is an enveloped positive sense single-stranded RNA virus containing a single open reading frame (ORF), which encodes a polypeptide of ~3,010 amino acids (Choo *et al*., 1991; Ogata *et al*., 1991). HCV infection is a major public health problem worldwide, in that it causes liver disease that can progress to liver cirrhosis and hepatocellular carcinoma (Di Bisceglie *et al*., 1991).

Comparative analysis of HCV genomic sequences from different geographic areas has allowed HCV to be classified into at least six major genotypes and over 50 subtypes (Smith *et al*., 1995; Simmonds *et al*., 1996). HCV genotyping has helped improve our understanding of the pathogenicity, evolution and molecular epidemiology of the HCV virus (Brechot, 1994; Pozzato *et al*., 1994), and has also been recognized as an important factor for predicting the clinical outcome of chronic HCV infection and its response to antiviral therapy (Zein, 2000; Zeuzem, 2001).

To date, HCV genotypes have been primarily determined by sequence-based PCR products analysis techniques, including direct sequencing (Ansaldi *et al*., 2001; Arens, 2001), restriction endonuclease digestion (Mellor *et al*., 1996), and reverse hybridization against genotype-specific probes (Le Pogam *et al*., 1998). The indirect analysis via genotype-specific antibodies is also applied in some cases (Zhang *et al*., 1995). Although direct sequencing is considered most accurate among these genotyping methods, its complicated, time consuming genotyping procedure is often impractical in the clinical setting. Thus, the development of a simpler, more efficient and cost-effective method for HCV genotyping would be highly beneficial.

Here, we report the development of a convenient and quantitative method for HCV genotyping of patient samples (Fig. 1). This assay is based on annealing genotype-specific capture probes (AGSCP) with reverse transcription (RT)-PCR viral RNA products amplified from patient serum samples.

Materials and Methods

Clinical samples

The standard HCV Genotype Panel was purchased from Boston Biomedica Inc.. The serum samples used in this study were obtained from 111 Korean patients chronically infected with HCV. The collection of clinical samples was accompanied by informed consent from each patient and was approved by the Institutional Review Board (IRB).

Synthesis and immobilization of HCV genotype-specific probes for AGSCP

The HCV genotype-specific capture probes contained an

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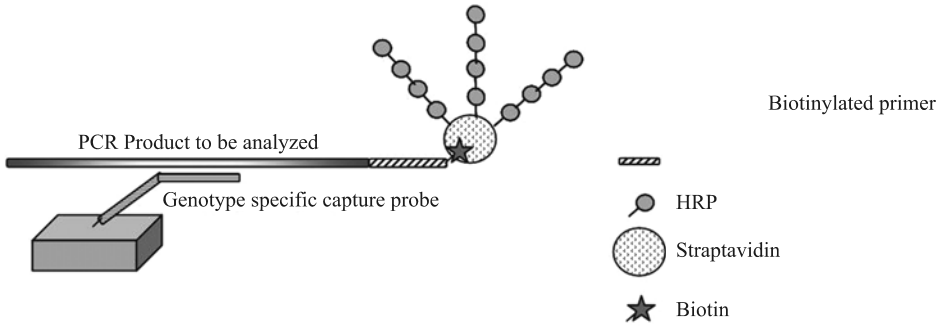

Fig. 1. Schematic diagram of our new AGSCP-based HCV genotyping method. A capture probe bound to the bottom of each well interacts with the corresponding RT-PCR product, which may be visualized via streptavidin conjugation of its bound biotin moiety.

Table 1. Sequences of capture probes

Genotype	Sequence
Universal	5'-TTTTTTTTTTTTGGGCGYGCCCCCGC-3'
1/6	5'-TTTTTTTTTTTCTCCAGGCATTGAGC-3'
1a/2	5'-TTTTTTTTTTCCCCGCAAGACTGCTA-3'
1b	5'-TTTTTTTTTTGCTCAGTGCCTGGAGA-3'
1b/3c/5	5'-TTTTTTTTTTCCGCGAGACYGCTAGC-3'
2	5'-TTTTTTTTTTTAGCGTTGGGTTGCGA-3'
2a/2b/2c/4f/4g/6	5'-TTTTTTTTTGAGTGTCGTACAGCCT-3'
2a/b	5'-TTTTTTTTTTTTRCCGGRAAGACTGG-3'
3	5'-TTTTTTTTTAATCGCTGGGGTGACC-3'
3	5'-TTTTTTTTTTTTCTGGGTATTGAGC-3'
3a/b	5'-TTTTTTTTTTTCTTGGARCAACCCGC-3'
4	5'-TTTTTTTTTTTTCTTGGAACTAACCC-3'
4a/b/c/d/e/g/h	5'-TTTTTTTTTGAGTGTTGTRCAGCCT-3'
5A/5	5'-TTTTTTTTTGAGTGTCGAACAGCCT-3'
5A	5'-TTTTTTTTTTCTCCGGGCATTGAGC-3'
6a/6	5'-TTTTTTTTTGGGTCCTTTCCATTGG-3'

amine group (-NH_2) at the 5' end, a 10 nucleotide (nt) oligo-(dT) spacer sequence, and a 16 nt complementary sequence designed to hybridize with genotype-specific sequences in the 5'-UTR of the HCV genome. Different probes were designed to anneal with single genotypes or groups of genotypes (Stuyver *et al*., 1996) (Table 1). Among the different regions of the HCV genome, the 5'-untranslated region (UTR) is highly conserved, yet contains genotype-specific sequences (Bukh *et al*., 1992; Furione *et al*., 1999), making the 5' UTR ideal for HCV detection and genotyping.

For immobilization of capture probes on the well of a NucleoLink^{TM} 96-well plate (Nunc, USA), 100 μl of coating solution [0.1 mM capture probe, 10 mM N-(3-dimethylaminopropyl) carbodiimide hydrochloride and 10 mM 1-methylimidazole] was dispensed to each well, and the plate was incubated at 50°C for 5 h. Each well was then washed thrice with 200 μl of wash buffer (100 mM Tris-Cl; pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and blocked with 200 μl of PBS buffer containing 1% BSA at room temperature for 4 h. The plate was then air dried and stored at 4°C until use.

Plate configuration

The genotyping plate was configured as shown in Fig. 2. Each plate could be used for up to the 6 test samples, with 16 wells being used to genotype each sample.

Viral RNA purification and RT-PCR amplification

The viral RNAs from the 111 clinical samples were extracted by using Ultra Sense Virus Kit (QIAGEN, USA) as described by manufacturer. A part of the HCV 5'-UTR was amplified from reverse-transcribed RNAs using one-step RT-PCR (One-step RT-PCR kit, QIAGEN) and 5' biotinylated primers: Primer 1; 5'-CCCTGTGAGGAACTWCT GTCTTCACGC-3', Primer 2; 5'-GGTGCACGGTCTACGA GACCT-3', W represents A or T. The amplification conditions consisted of 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and at 72°C for 1 min.

Hybridization of PCR products with capture probes

Each PCR reaction product (20 μl) was incubated at room temperature for 10 min with 80 μl of denaturation solution (400 mM sodium hydroxide and 10 mM EDTA), and then vortexed with 1,700 μl of hybridization buffer (3 M tetramethylammonium chloride, 50 mM Tris-HCl; pH 6.8, 1 mM EDTA, 5× Denhardt's solution, 0.6% SDS, and 100 μl/ml sheared salmon sperm DNA). An aliquot of this solution (100 μl/well) was dispensed into 16 probe-immobilized wells, such that 6 samples could be genotyped per plate (Fig. 2). Each plate was incubated in a shaking incubator at 55°C for 2 h. The hybridization buffer was then discarded, the plate were washed twice with 120 μl of stringent washing buffer per well (3 M tetramethylammonium chloride, 50 mM Tris-HCl; pH 8.0, and 0.2% SDS), and the each well was incubated with 120 μl stringent washing buffer at 55°C for 30 min. Finally, each well was washed three times with rinse buffer (100 mM Tris-HCl; pH 7.5, 150 mM sodium chloride, and 0.1% Tween 20).

Colorimetric detection of HRP activity and data analysis

Streptavidin-HRP polymer (Sigma, USA) was diluted 1:4000 in HRP assay buffer (100 mM Tris-HCl; pH 7.5, 150 mM sodium chloride, 0.1% Tween 20, and 0.5% BSA), 100 μl of

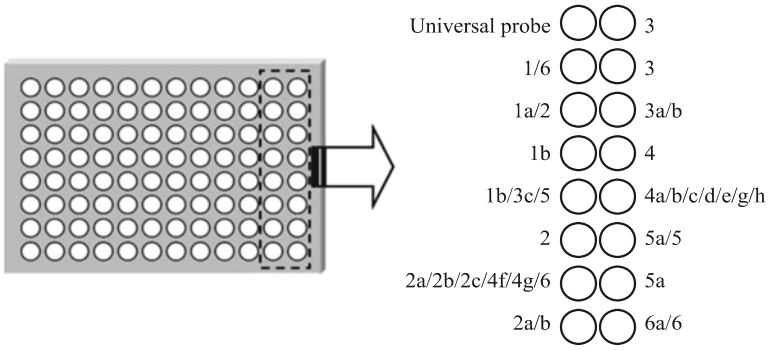

Fig. 2. Configuration of HCV genotyping plate. Captures probes specific for one or more genotype(s) are arrayed as indicated.

Table 2. Theoretical pairs of capture probes and HCV genotypes

Well No.	Genotype specificity	1a		1b	2		3		4			5		6	
1	Universal	○	○	○	○	○	○	○	○	○	○	○	○	○	○
2	1/6	○	○	○										○	○
3	1a/2/4	○			○				○						
4	1b		○												
5	1b/3c/5			○			○					○			
6	2				○	○									
7	2a/2b/2c/4f/4g/6				○	○			○	○				○	
8	2a/b				○	○									
9	3						○	○							
10	3						○	○							
11	3a/b						○	○							
12	4								○	○	○				
13	4a/b/c/d/e/g/h								○	○	○				
14	5A/5											○	○		
15	5A											○	○		
16	6a/6													○	○

○, pairs showing theoretical positivity using the described method. A genotype specific positive signal of used probes could be detected in combination.

the diluted polymer was dispensed into each well, and the plates were incubated at 37°C for 30 min. Each plate was then washed 5 times with 200 μl/well of rinse buffer, and the biotin moieties of the probe-bound PCR products were detected by the addition of 100 μl/well of Ultra TMB chromogen substrate (Pierce, USA). Color development was allowed to proceed at room temperature for 10 min, and then stopped with 50 μl of 2 M H_2SO_4. The color intensity in each well measured by spectrophotometry at 450 nm, and the genotype of each sample was determined by comparing the absorbance pattern with the theoretical absorbance patterns expected for the various genotypes (Table 2).

Direct sequencing

HCV samples showing discordant results were further examined by sequence analysis of the HCV core region, as previously described (Ohno *et al*., 1997). Briefly, cDNAs were reverse transcribed from the samples and amplified using the Sc2 [sense; 5'-GGGAGGTCTCGTAGACCGTGC ACCATG-3'] and Ac2 [antisense; 5'-GAG(A/C)GG(G/T)AT (A/G)TACCCCATGAG(A/G)TCGGC-3'] primers, followed by a second round of PCR using primer mix 1 (Ohno *et al*., 1997). The resulting PCR products were excised from the 2% agarose gel and purified for sequencing using an agarose gel elution kit and a PCR purification kit (both from Intron). Direct sequencing was performed using the ABI PRISM® BigDye™ Terminator sequencing kit V 3.0 and an automatic DNA sequencer (both from Applied Biosystems). The sequences were identified by comparison to genotype-specific sequences retrieved from National Center for Biotechnology Information/National Institute of Health GenBank nucleotide sequence database.

Results

We first examined the efficiency and accuracy of the new

AGSCP assay by applying this technique to a standard HCV Genotype Performance Panel (Boston Biomedica). As shown in Fig. 3 and Table 3, all positive signals had absorbance values equal to or higher than 0.439, and all negative signals had absorbance values lower than 0.288. Thus, the samples could be assigned to the appropriate genotypes using 0.4 and 0.3 as the positive and negative cut-off values, respectively. We did not detect any positive signals from capture probes 1b, 2a/b, 4, and 6a/6, suggesting that the RNAs used in the standard set did not contain the sequences used to design these capture probes. Nonetheless, we were able to unambiguously assign the correct genotypes to each sample in the standard serum panel.

We then applied the AGSCP method to serum samples from 111 Korean patients with chronic HCV infection, and compared the genotyping results with those obtained from a commercially available INNO-LiPA HCV II kit (Innogenetics). The IINO-LiPA is a line probe assay (LiPA) based on reverse transcription-PCR and hybridization with probes, which was developed by Innogenetics (Stuyver *et al*., 1993). Both AGSCP and INNO-LiPA assays use RT-PCR technologies and detect sequence polymorphism by hybridization at highly stringent conditions. However, there are fundamental differences of the assays in preparation of amplified DNAs and in annealing processes using different matrixes as described in Table 4.

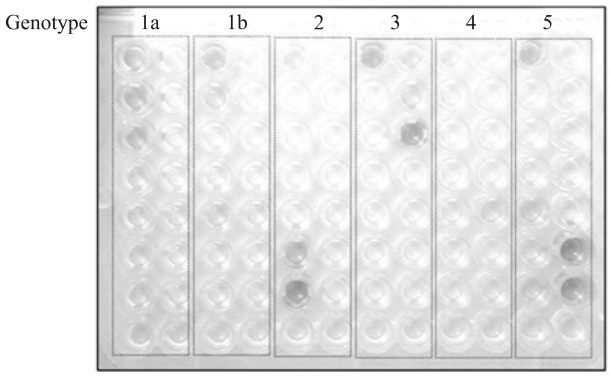

Fig. 3. Genotyping results of a standard panel of known HCV genotypes. Six different known HCV genotypes were analyzed as described in 'Materials and Methods'.

Table 5 shows the distribution of HCV genotypes identified by the two genotyping assays. The INNO-LiPA assay revealed that out of 111 patient samples, 58 (52.3%) were infected with genotype 1b, 38 (34.2%) with genotype 2 subtypes a or c (2a/c), 3 (2.7%) with genotype 1, 7 (6.3%) with genotype 2, 1 (0.9%) with mixed infection of genotypes 1b and 2, 2 (1.8%) with mixed infection of genotypes 1 and 2a, and 2 (1.8%) with mixed infection of genotypes 2 and 4. In contrast, our new AGSCP assay indicated that of the 111 patient serum samples, 68 (61.3%) were infected with genotype 1b and 43 (38.7%) with genotype 2. Thus, the results from the two genotyping systems were concordant in 93 of 111 samples (83.8%) and discordant in 18 samples (16.2%) (Table 5). Since the AGSCP can only discriminate the subtypes 1a and 1b of the genotype 1, we interpreted that specific subtypes belongs to the same genotype as the matched result, except subtype 1a and 1b.

To further examine these discordant results, we sequenced the HCV core regions and unambiguously identified the genotypes by BLAST search (Fig. 4), which revealed that 8 of the discordant samples belonged to genotype 1b and the

Table 3. Quantitative presentation of genotyping results

	1a	1b	2	3	4	5
Universal	**0.566***	**0.773**	**0.471**	**1.218**	**0.412**	**0.891**
1/6	**0.660**	**0.588**	0.175	0.144	0.281	0.174
1a/2	**0.659**	0.187	0.140	0.132	0.135	0.160
1b	0.188	0.197	0.163	0.157	0.150	0.179
1b/3c/5	0.150	**0.474**	0.148	0.141	0.254	**0.553**
2	0.181	0.233	**0.969**	0.162	0.182	0.316
4f/4g/6/2a/2b/2c	0.182	0.147	**1.451**	0.166	0.151	0.331
2a/b	0.188	0.169	0.215	0.186	0.170	0.172
3	0.144	0.164	0.133	**0.567**	0.122	0.132
3	0.173	0.139	0.142	**0.583**	0.121	0.154
3a/b	0.163	0.180	0.134	**1.423**	0.140	0.157
4	0.131	0.124	0.126	0.132	0.126	0.123
4a/b/c/d/e/g/h	0.288	0.279	0.184	0.154	**0.439**	0.142
5a/5	0.136	0.142	0.224	0.137	0.140	**1.400**
5a	0.154	0.161	0.164	0.157	0.185	**1.197**
6a/6	0.173	0.170	0.176	0.169	0.164	0.161

* The degree of color development in each well was quantified by measurement of the absorbance at 450 nm (Cutoff 0.4, Absorbance over 0.4 was marked with bold).

Table 4. Comparison between AGSCP and INNO-LiPA assays

	AGSCP	INNO-LiPA
Assay instrument	96 well plate	Strip (nitrocellulose coated)
Detection method	Colorimetric (quantifiable)	Colorimetric (not quantifiable)
Automation	Possible	Possible but costly
RNA amplification	One-Step RT-PCR (lower cross-contamination)	Multi-steps (Independent reverse transcription and 1st and 2nd round PCR)

Table 5. Comparison of the genotyping results from the INNO-LiPA and AGSCP assays (The genotype of AGSCP was determined using the Table 2)

INNO-LiPA		AGSCP							Discordant result
Genotype	No. of samples	1a	1b	2	3	4	5	6	
1	3		3						3
1b	58		56	2					2
1b+2	1		1						1
1+2a	2			2					2
2	7		1	6					1
2a/c	38		7	31					7
2+4	2		0	2					2
Total	111		68	43					18

Sample No.	Genotype	Sequence of amino acids							
HCV 1	2a	VKFPGGGQIV	GGVYLLPRRG	PRLGVRATRK	ASERSQPRGR	RQPIPKDRRS	TGKSWGKPGY	PWPLYGNEGL	GWAGWLLSPR
HCV 4	2a	----------	----------	---------T	----------	---------T	----------	----------	----------
HCV 5	2a	----------	----------	----------	T---------	----------	----------	----------	----------
HCV 11	2a	----------	----------	----------	T---------	----------	----------	----------	----------
HCV 12	2a	----------	----------	----------	T---------	----------	----------	----------	----------
HCV 13	2a	----------	----------	----------	----------	----------	----------	----------	----------
HCV 15	2a	----------	----------	----------	----------	----------	----------	----------	----------
HCV 16	2a	----------	----------	------T---	T---------	----------	----------	----------	----------
HCV 17	2a	----------	----------	----------	----------	----------	----------	----------	----------
HCV 18	2a	----------	----------	----------	----------	----------	----------	----------	----------
HCV 2	1b	VKFPGGGQIV	GGVYLLPRRG	PRLGVRATRK	TSERSQPRGR	RQPIPKARRP	EGRTWAQPGY	PWPLYGNEGM	GWAGWLLSPR
HCV 3	1b	----------	----------	T---------	--------G-	----------	----------	----------	----------
HCV 6	1b	----------	----------	----------	--------Q-	----------	----------	----------	----------
HCV 7	1b	----------	----------	----------	----------	--------S-	----------	----------	----------
HCV 8	1b	----------	----------	----------	----------	----------	----------	----------	----------
HCV 9	1b	----------	----------	----------	--------Q-	----------	----------	----------	----------
HCV 10	1b	----------	----------	----------	----------	----------	----------	----------	----------
HCV 14	1b	----------	----------	T---------	--------Q-	---A------	---------M	----------	----------

Fig. 4. Comparison of HCV core nucleotide sequences obtained from samples yielding discordant results in the INNO-LiPA and AGSCP assays. Of 18 sequenced samples, ten were identified as genotype 2a and eight samples as genotype 1b.

remaining 10 belonged to genotype 2a. Among the 18 discordant samples, the sequence analysis confirmed the AGSCP-based genotyping in 12 cases and confirmed the INNO-LiPA results in 6 cases (Table 6). These results indicate that INNO-LiPA and AGSCP showed error rates of 11% (12 out of 111 samples) and 5.4% (6 out of 111 samples), respectively.

Table 6. Sequence-based genotyping of samples showing discordant results by INNO-LiPA and AGSCP

Samples	INNO-LiPA	AGSCP	Sequencing
HCV 1	1b	**2**	**2a**
HCV 2	2a	**1b**	**1b**
HCV 3	2a/c	**1b**	**1b**
HCV 4	1+2a	**2**	**2a**
HCV 5	1+2a	**2**	**2a**
HCV 6	2a/c	**1b**	**1b**
HCV 7	1	**1b**	**1b**
HCV 8	1	**1b**	**1b**
HCV 9	1	**1b**	**1b**
HCV 10	1b+2	**1b**	**1b**
HCV 11	2+4	**2**	**2a**
HCV 12	2+4	**2**	**2a**
HCV 13	**2a/c**	1b	**2a**
HCV 14	**1b**	2	**1b**
HCV 15	**2a/c**	1b	**2a**
HCV 16	**2a/c**	1b	**2a**
HCV 17	**2a/c**	1b	**2a**
HCV 18	**2**	1b	**2a**

Discussion

Our analysis of the known HCV genotype panel (Fig. 3) revealed that the AGSCP assay could successfully identify HCV genotypes and subtype 1a and 1b. Since the positive and negative signals are evaluated according to absorbance values, this new method reduces possible errors introduced by the visual inspection of banding patterns required by the currently available genotyping system, INNO-LiPA HCV II assay. Moreover, the utilized protocol allows the HCV RNA to be reverse transcribed and PCR amplified in a single tube. The use of single-tube RT-PCR not only simplifies amplification, it also reduces contamination risks. And finally, this new method is compatible with commercially available ELISA automation systems, bringing the hope that our HCV genotyping process may be partially or fully automated in the future, thus facilitating its use in clinical or research settings.

To assess the specificity and effectiveness of our new AGSCP-based method, we applied it to genotype samples from 111 Korean patients, and compared the results to those obtained using the commercially available INNO-LiPA assay. The results suggest that the majority of Korean patients were infected with HCV genotype 1b, followed by genotype 2 or its subtypes. This finding is consistent with our previous report (Kim *et al*., 1995), wherein we found that genotypes 1b and 2a were the major causative agents of HCV in Korea, accounting for 60% and 33% of infections, respectively.

Our results further indicated that there was a high concordance rate (83.8%) between the two assays. Direct sequencing of the rest 18 discordant samples revealed that the AGSCP had correctly identified 12 samples, whereas the INNO-LiPA had correctly identified only 6. Three samples identified as genotype 1 by INNO-LiPA were correctly identified as genotype 1b by AGSCP, indicating that the latter method may be more specific for identifying this subtype. Interestingly, the AGSCP-based assay correctly identified two cases of single genotype HCV infection that had been identified as mixed genotype infections by INNO-LiPA.

Although direct sequence analysis is regarded as the most accurate and specific method for HCV genotyping, its clinical applicability is limited by its complex protocols, high cost and inability to detect mixed genotypes. However, in clinical practice, it is only truly necessary to distinguish between genotype 1 and non-genotype 1 infections to make informed therapeutic decisions (Mondelli and Silini, 1999). In this regard, genotyping with our new AGSCP assay may be more clinically relevant, in that it is simple and less expensive than sequencing, but is still able to clearly discriminate between genotype 1 and non-genotype 1 infections.

In conclusion, we herein introduce a new AGSCP assay that is a rapid, convenient, and accurate method suitable for large-scale genotyping. This new genotyping system may be a promising tool for the clinical determination of HCV genotypes, and may prove applicable to other assays in the future.

Acknowledgements

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