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Specific detection of Hepatitis C virus in clinical samples using a novel simplification strategy

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic hepatitis, cirrhosis and liver cancer, with over 170 million individuals infected worldwide. Conventional lab diagnosis is based on serological tests that detect the presence of antibodies to HCV. However, one of the most sensitive methods for the detection of HCV is reverse transcriptase PCR (RT-PCR), which, in addition to improved sensitivity, can also be used to monitor disease activity in response to anti-viral drugs. We aimed to produce a RT-PCR assay using 'simplified' HCV RNA as template that was as sensitive and specific as current HCV RT-PCR assays and which can simultaneously detect all strains of HCV.

Treatment of DNA with sodium bisulphite results in the chemical conversion of all unmethylated cytosines to thymine via a uracil intermediate, effectively converting a 4 base genome into a 3 base genome as depicted in Figure 1.

Figure 1: The effect of sodium bisulfite on cytosine

This technology can be utilised to reduce the complexity of the genome of different organisms or strains so that they are more similar to each other, thus facilitating the detection of multiple organisms/ strains in a single PCR reaction without the need for multiplexing. However, treatment of RNA with sodium bisulphite under the same conditions results in complete degradation of the RNA and is of no clinical utility. We have developed a completely new method for the simplification of RNA using sodium bisulphite. An example of how simplification can be applied to the detection of different HCV strains is shown in Figure 2. Our assay can effectively reduce the consensus sequence heterogeneity from 64 combinations to just 4 combinations and increase the sequence homology from 66% to 89%. This represents a 94 % simplification of the original divergent sequences.

Detection of different HCV strains by DNA simplification

HCV Strain	Before Simplification	After Simplification
1a	CAAGTTCCCGGGTGGCGG	TAAGTTTTTGGGTGGTGG
1b	CAAGTTCCCGGGCGGTGG	TAAGTTTTTGGGTGGTGG
1c	TAAGTTCCCGGGTGGCGG	TAAGTTTTTGGGTGGTGG
2a	TAAGTTTCCGGGCGGCGG	TAAGTTTTTGGGTGGTGG
2b	CAAGTTCCCGGGTGGCGG	TAAGTTTTTGGGTGGTGG
2c	TAAGTTCCCGGGCGGTGG	TAAGTTTTTGGGTGGTGG
2k	CAAGTTCCCGGGCGGTGG	TAAGTTTTTGGGTGGTGG
3a	TAAGTTCCCGGGTGGCGG	TAAGTTTTTGGGTGGTGG
3b	TAAGTTCCCGGCTGGCGG	TAAGTTTTTGGTTGGTGG
3k	TAAGTTCCCAGGCGGCGG	TAAGTTTTTAGGTGGTGG
4a	TAAGTTCCCGGGTGGTGG	TAAGTTTTTGGGTGGTGG
5a	CAAGTTCCCGGGCGGTGG	TAAGTTTTTGGGTGGTGG
6a	CAAGTTCCCGGGTGGCGG	TAAGTTTTTGGGTGGTGG
6b	CAAGTTCCCGGGCGGCGG	TAAGTTTTTGGGTGGTGG
6g	CAAGTTCCCGGGCGGTGG	TAAGTTTTTGGGTGGTGG
6h	CAAGTTCCCGGGCGGCGG	TAAGTTTTTGGGTGGTGG
6k	TAAGTTCCCGGGTGGCGG	TAAGTTTTTGGGTGGTGG
Consensus	YAAGTTYCCRGSYGGYGG	TAAGTTTTTRGKTGGTGG
	66% homology over 18 bases **64** possible primer combinations	89% homology over 18 bases **4** possible primer combinations

Figure 2: Consensus sequence of HCV genotypes before and after RNA simplification. The simplification has resulted in an increased homology from 66% to 89%.

To demonstrate the performance of our assay, we have validated it using a series of commercially available HCV performance, linearity and genotyping panels. Finally the assay was assessed on a blinded panel of 138 clinical samples which demonstrated that this multi-strain single assay design shows similar sensitivity and specificity to conventional approaches for the simultaneous detection of all strains of HCV.

Methods

HCV RNA samples were obtained from Acrometrix (OptiQual HCV high positive control), or BBI diagnostics (HCV RNA linearity panel PHW804, and Worldwide HCV genotype panel WWHV302) and purified with the QiaAmp Ultrasens Viral purification kit according to the manufacturer's instructions (Qiagen). Blinded clinical samples were obtained from a local hospital and purified as above. Samples were treated with sodium bisulphite and 11µl of the converted HCV RNA samples were reverse transcribed with Superscript III reverse transcriptase (Invitrogen) or iScript reverse transcriptase (Biorad) using random primers. One tenth of the cDNA was then subjected to either end-point or real-time PCR amplification with primers (and probe) specific for converted HCV RNA. End-point PCR was performed in 50µl reactions using 1.5X Promega master mix and 100ng each of forward and reverse primers and cycled at 95oC, 3 mins; [95oC, 10 secs; 53oC, 1 min; 68oC, 1 min] x40 in a Hybaid PX2 thermal cycler. Real-time PCR was performed in 25µl reactions using 1x Sigma Jumpstart master mix, 50ng each of forward and reverse primers, 5mM MgCl2, and 400nM FAM-labelled probe and cycled at 95oC, 10 mins; [95oC, 10 secs; 53oC, 90 secs; 60oC, 30 secs] x50 in a Corbett 6000 Rotor Gene. One third of the PCR product was electrophoresed on a 2% precast agarose e-gel (Invitrogen).

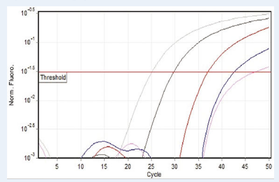
Results

An example of the results obtained when using our RNA bisulphite conversion RT-PCR assay is shown in Figure 3. Using this assay we are able to detect HCV from 1 562 500 IU down to 39IU/mL.

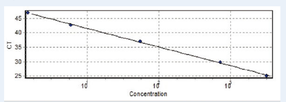
Figure 3: A dynamic range of concentrations from 39 IU to 1 562 500 IU/mL were purified from Acrometrix Optiqual HCV high positive control, bisulphite converted, reverse transcribed and 1/10th of the cDNA subjected to PCR. One third of the PCR product was electrophoresed. The legend indicates the amount, in IU, of HCV RNA equivalence in the PCR. The 0 indicates a serum negative control which has been subjected to identical treatment and handling.

Figure 4 shows the real-time PCR results obtained from the linearity panel. A series of known concentrations of viral RNA, over 3 orders of magnitude, were purified, bisulphite converted, reverse transcribed and amplified by real-time PCR. The standard curves generated show that the reaction efficiencies are constant and linear over the range of concentrations examined, as exemplified by the R2 value being close to 1. The results of this linearity panel and that of the dynamic range demonstrate that there is good sensitivity and specificity for the detection of HCV viral RNA, using a viral-specific probe, ranging from 1 562 500 IU down to 15IU, illustrating that the assay can detect the presence of viral RNA over a very broad range of concentrations.

a) Linearity panel



b) Standard curve

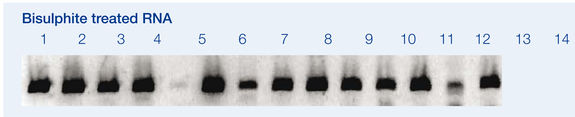


R Value	0.99947
R^2 Value	0.99894

c) Quantitation data

No.	Colour	Ct	Given Conc (IU/PCR)	Calc Conc (IU/PCR)	% Var
1		25.06	3,250.0	3,591.8	10.5%
2		29.75	732.5	670.0	8.5%
3		37.03	55.0	49.5	10.0%
4		42.60	6.0	6.8	12.6%
5		46.87	1.5	1.5	2.4%

The HCV RNA present in the Acrometrix Optiqual control and the BBI linearity panel is of genotype 1. In order to ensure that our assay was able to detect all common HCV genotypes, we evaluated it's ability to detect a range of HCV genotypes present in the BBI Worldwide performance/genotyping panel. This panel consists of patient plasma specimens from diverse geographical locations with differing viral loads. The results are given in Figure 5 and clearly show that all the major genotypes present in this panel are detected both in wild-type (non-bisulphite converted) and bisulphite converted samples using our assay. However, genotyping can still be performed by sequencing as there is sufficient heterogeneity remaining in the samples post simplification.

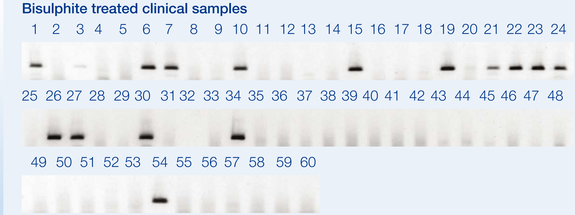


		HCV genotyping		HCV quantitation		Anti-HCV EIA		
Sample	Origin	Bayer Trugene	INNO-LiPA	COBAS AMPLICOR PCR (IU/ml)	Bayer Versant HCV 3 (IU/ml)	Abbott HCV 2.0	Abbott	Ortho HCV 3.0
1	China	1b	1b	1.3x105	1.2x106	4.4	60.6	4.9
2	Thailand	1	1a	4.3x105	4.3x105	4.4	97.0	4.9
3	S. Africa	1b	1b	1.4x105	7.0x104	4.4	59.0	4.9
4	China	2a	2a/c	9.1x105	5.3x105	4.4	76.4	4.9
5	USA	2a	2a/c	1.1x103	<615	0.6	4.6	2.4
6	China	3b	3b	2.4x106	1.7x106	4.4	75.7	4.9
7	USA	3a	3a	3.0x105	9.0x104	4.4	65.2	4.9
8	Thailand	3a	3a	7.2x105	4.9x105	4.4	58.8	4.9
9	Egypt	4a	4	3.8x105	1.7x105	4.4	83.9	4.9
10	Egypt	4	4	3.2x104	2.4x104	4.4	48.5	4.9
11	Egypt	4a	4	1.4x105	8.6x104	4.4	45.8	4.9
12	Unknown	5a	5a	2.4x105	8.4x103	4.4	85.5	4.9
13	S. Africa	5a	5a	2.0x104	8.4x103	4.4	69.6	4.9
14	Unknown	6a	6a	6.1x105	1.4x105	4.4	74.4	4.9

Figure 5: Performance evaluation of HGS HCV assay on a World Wide Performance/ Genotype panel. 0.1 mL of each sample, made up to 1mL with human serum negative for HCV (Sigma #H4522), were purified, bisulphite converted (where indicated), reverse transcribed and 1/10th of the cDNA subjected to end-point PCR. Information provided with the samples by the manufacturer pertaining to geographical location, quantitation etc is also given.

After establishing the sensitivity of our assay and the ability to detect various genotypes, we assessed 138 blinded clinical samples and compared the results to those obtained from a range of commercially available HCV assays. A selection of the results are given in Figure 6 and these clearly show that the HGS HCV assay reliably detects HCV RNA in patient samples and in some cases is more sensitive than the Bayer Versant HCV RNA 3.0 assay – for example see sample 3. Overall, the HGS assay performed well relative to the Bayer Versant HCV RNA 3.0 test and the Roche COBAS AMPLICOR 2.0 test with 132/138 (96%) in agreement. Of the 6 that were not in agreement with one or other of these tests 3 were positive by our test and had been positive by one of the other tests at an earlier testing but was subsequently assessed to be negative or below the limit of detection of the assay. On repeated testing, these samples were confirmed to be positive by our test and were shown to contain HCV by sequencing. Thus, we believe this illustrates that our test is more sensitive than the other assays. Two of the other three samples tested negative by our test but had been previously shown to be positive by the Roche or Bayer test at least one year prior to our testing. However, we had very limited amounts of these samples and so it is likely that we had too little for detection and/or that the samples had deteriorated over the storage period. The final sample was positive by our test but negative by the Roche or Bayer test, but there was insufficient sample to confirm the finding, but this may represent another case of improved sensitivity of our assay.

Bisulphite treated clinical samples



Human Genetic Signatures

Convert Simplify Understand

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Sample #	Hospital result	HGS result	Sample #	Hospital result	HGS result	Sample #	Hospital result	HGS result
1	Positive	Positive	21	Positive	Positive	41	ND	ND
2	ND	ND	22	Positive	Positive	42	ND	ND
3	ND	Positive	23	Positive	Positive	43	ND	ND
4	ND	ND	24	Positive	Positive	44	Positive	ND
5	ND	ND	25	ND	ND	45	ND	ND
6	Positive	Positive	26	Positive	Positive	46	ND	ND
7	Positive	Positive	27	Positive	Positive	47	ND	ND
8	ND	ND	28	<615	ND	48	ND	ND
9	ND	ND	29	ND	ND	49	ND	ND
10	Positive	Positive	30	Positive	Positive	50	ND	ND
11	ND	ND	31	ND	ND	51	ND	ND
12	ND	ND	32	ND	ND	52	<615	ND
13	ND	ND	33	ND	ND	53	ND	ND
14	ND	ND	34	Positive	Positive	54	Positive	Positive
15	Positive	Positive	35	ND	ND	55	ND	ND
16	ND	ND	36	<615	ND	56	ND	ND
17	ND	ND	37	ND	ND	57	ND	ND
18	<615	ND	38	ND	ND	58	<615	ND
19	Positive	Positive	39	ND	ND	59	ND	ND
20	Positive	Positive	40	ND	ND	60	ND	ND

Figure 6: Selection of the results from the blinded clinical samples assayed for HCV RNA by either the Bayer Versant HCV RNA 3.0 or Roche AMPLICOR HCV RNA 2.0 test and the HGS HCV assay.

Discussion & Conclusions

We have developed a completely novel assay for detecting all strains of HCV based on simplification of the RNA by sodium bisulphite. Bisulphite modification of RNA has not been achieved previously due to the complete degradation of the RNA but our protocol has overcome this hurdle. This assay is quick and easy to perform and results in exquisite sensitivity over a broad range of HCV concentrations ranging from 1 562 500 IU/mL down to 15 IU HCV/mL.

Detection is currently based on end-point or real-time PCR using primers specific for bisulphite modified template, but can be adapted to any nucleic acid detection method. Furthermore, our assay can simultaneously detect all major genotypes of HCV including 1a, 1b, 2a/c, 3a, 3b, 4a, 5a and 6a from a wide range of geographical locations. Importantly, genotyping can still be performed by sequencing as there is sufficient heterogeneity remaining in the samples post simplification.

The HGS HCV assay was used to assess a blinded panel of 138 clinical samples and was shown to perform well relative to the Bayer Versant HCV RNA 3.0 test and the Roche COBAS AMPLICOR 2.0 test with 132/138 (96%) samples in agreement. Of the six discordant samples, four were positive by the HGS HCV assay, and the presence of HCV in the samples was confirmed by sequencing, indicating an increased level of sensitivity of our assay in comparison to the other tests.