A hepatitis C avidity test for determining recent and past infections in both plasma and dried blood spots

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ABSTRACT

Background: DBS testing has been used successfully to detect HCV antibody positive individuals. Determining how long someone has been infected is important for surveillance initiatives. Antibody avidity is a method that can be used to calculate recency of infection.

Objectives: A HCV avidity assay was evaluated for both plasma and DBS.

Study design: To measure antibody avidity a commercial HCV ELISA was modified using 7 M urea. The plasma samples were split into: group 1 (recently infected $N = 19$), group 2 (chronic carrier $N = 300$) and group 3 (resolved infection $N = 82$). Mock DBS made from group 1 ($N = 12$), group 2 ($N = 50$), group 3 ($N = 25$) and two seroconverter panels were evaluated. 133 DBS taken from patients known to have a resolved infection or be a chronic carrier were also tested.

Results: The avidity assay cut-off was set at AI ≤ 30 for a recent infection. Using sequential samples the assay could detect a recent infection in the first 4–5 months from the point of infection. Most of the false positive results (AI < 30 among cases known not to have had recent infection) were detected among known resolved infections, in both the plasma and DBS; as a result, a testing algorithm has been designed incorporating both PCR and two dilution factors. The sensitivity and specificity of the assay on plasma was 100% and 99.3%, respectively, while DBS had 100% sensitivity and 98.3% specificity.

Conclusion: The HCV avidity assay can be used to distinguish between chronic and recent infection using either plasma or DBS as the sample type.

1. Background

In Scotland, approximately 1% of the population are hepatitis C virus (HCV) antibody (anti-HCV) positive and a significant proportion acquired infection through injecting drug use.[1] It can be difficult to obtain a conventional blood sample from people who inject drugs (PWID) and dried blood spot (DBS) testing is a non-invasive method that can be used for antibody and PCR testing.[2–4] In specialist drug service settings in Scotland, it has contributed greatly to the rise in the number of HCV diagnoses.[1] DBS testing has also been used successfully for surveillance purposes.[5–7]

Treatment of early HCV infection is associated with higher sustained virological response rates, including among PWID.[8–10] The ability to distinguish between a recently acquired and a past infection is important to help guide the clinical management of patients with HCV and direct prevention initiatives.[6]

A recently acquired HCV infection is often asymptomatic.[10] Traditionally a recent HCV infection is determined by monitoring antibody IgG seroconversion, and/or the detection of viral RNA in the absence of anti-HCV. It can take between 1–4 weeks for RNA and 8–12 months for antibody to become detected after infection with HCV.[11] The presence of anti-HCV and HCV PCR can indicate either an acute or a chronic infection, even in the presence of elevated liver function tests.[11] IgM antibody in HCV can be detected during exacerbation of chronic HCV.[12] An anti-HCV positive and PCR negative result usually indicates a resolved HCV infection, but can also indicate an acute infection with low viraemia.[11]

Antibody avidity is the binding capacity of maturing antibody with antigen, which increases over time. A dissociation agent (DA) can remove weakly bound antibody.[13] The avidity index (AI) can be measured using a modified enzyme linked immunoassay (ELISA) by comparing an untreated sample and one treated with the DA.[14] Antibodies generated early in infection have weak antigen-binding capacity compared to a matured antibody generated against the

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Table 1
Characteristics of the patient groups used in this study.

Patient group	Number of samples	Average age (range)	Average anti-HCV (range)	Average viral load IU/ml (range)	Number of patients with each genotype				
					1	2	3	4	NT
Group 1 (recent) plasma/serum	19	34(20–62)	9.54 (5.65–16.09)	2,436,152[a] (948–16,900,000)	7	3	5	0	4
Group 1 (recent) mock DBS	12	34(20–61)	10.83 (5.65–16.09)	132,671 (948–384,518)	3	3	3	0	3
Group 2 (chronic) plasma/serum	300	42(5–77)	12.93 (6.35–17.97)	1,178,851[b] (1280–9,760,000)	152	14	108	2	24
Group 2 (chronic) mock DBS	50	42(22–70)	12.51 (8.89–15.15)	1,007,687[c] (3686–8,330,000)	25	3	17	2	3
Group 3 (resolved) plasma/serum	82	39(23–60)	10.86 (3.17–17.14)	N/A					
Group 3 (resolved) mock DBS	25	42(25–60)	11.79 (6.13–14.92)	N/A					
Group 4 (chronic) patient DBS	65	39(23–74)	>3[d]	PCR qualitative only					65[e]
Group 5 (resolved) patient DBS	68	37(25–60)	>3 for 36 samples 1.78 (0.89–2.99) for 42 samples	N/A					

[a] 4 samples were tested by a qualitative PCR method, 2 samples tested by Abbott Architect HCV core assay and 2 patients had a viral load < 1000 IU/ml (limit of detection of the HCV PCR was 50 IU/ml).

[b] 33 chronic patients had a viral load < 1000 IU/ml (limit of detection of the HCV PCR was 50 IU/ml).

[c] 6 chronic patients had a viral load < 1000 IU/ml (limit of detection of the HCV PCR was 50 IU/ml).

[d] Anti-HCV assay for DBS is the Ortho manual ELISA (see study design). Any sample with an OD > 0.8 is considered positive. All samples had an OD of >3 with the exception of one sample (OD = 1.980).

[e] HCV genotype is not currently tested on DBS.

same antigen. Several papers have addressed HCV avidity testing but all have been described using plasma/serum samples.[15–19] All the HCV avidity methods reported are modifications of commercial or in house ELISA kits using either guanidine or urea as the DA. The method described in this paper uses a modified commercial assay with an overnight incubation stage for HCV avidity testing. This assay can be used for both plasma/serum and DBS. To the best of our knowledge HCV avidity has not been reported using DBS.

2. Objectives

The aim of this study was to evaluate a HCV avidity test on plasma/serum and DBS for use in a routine laboratory as both a diagnostic and epidemiological surveillance tool.

3. Study design

3.1. Plasma and serum samples

Plasma or serum samples sent to the West of Scotland Specialist Virology Centre (WoSSVC) for routine anti-HCV and HCV PCR testing which had been stored at −80 °C were used in this study. All plasma/serum samples were screened for anti-HCV by the Abbott Architect Anti-HCV assay. The samples to be tested were grouped into recent, chronic and resolved infections (Table 1). Group 1 consisted of 19 patients samples with a recent HCV infection, defined as having either an anti-HCV positive result within (a) 4–6 months of a previous anti-HCV negative result, or (b) within 1 month of a previous anti-HCV negative and PCR positive result. All of group 1 were HCV PCR positive. Two of the patients within group 1 were renal patients involved in a HCV outbreak. These 2 patients had 8 and 5 follow up samples taken sequentially over a 5 and 6 month period, respectively. Three other patients within group 1 had a follow up sample. Group 2 consisted of 300 patients with chronic HCV, defined as those who tested anti-HCV and HCV PCR positive for greater than 1 year. Group 3 consisted of 82 patients with resolved HCV infection, defined as those who had tested anti-HCV positive and HCV PCR negative for an average of 6 years (range 1–14

years). Information on the number of patients treated, the duration of HCV infection or if HCV reinfection occurred was unknown for both groups 2 and 3.

Two seroconvertor panels PHV901 (N = 10) and PHV917 (N = 9) were obtained from Alere™ (Cheshire, UK), the genotypes for the panels were 1a and 2b, respectively.

3.2. Mock DBS

HCV negative whole blood was centrifuged in 200 μl aliquots at 5000 rpm for 5 min, after which 60 μl of plasma was removed and replaced by 60 μl of known HCV positive plasma. The samples were re-suspended and 50 μl was then spotted onto Whatman 903 Protein Saver cards and allowed to dry at room temperature for over 1 h. Twelve of the samples from group 1, 50 samples from group 2, 25 samples from group 3 and seroconvertor panels PHV901 and PHV917 were made into mock DBS samples. The DBS were stored at 4 °C until use.

3.3. Patient DBS samples

DBS sent to the WoSSVC for anti-HCV and HCV PCR testing and subsequently stored at 4 °C were used in this study. The samples came from clinics run by community addition services. Group 4 consisted of 65 DBS taken from known chronic patients (tested anti-HCV and HCV PCR positive for >1 year) and group 5 consisted of 68 DBS from resolved patients (tested anti-HCV and HCV PCR negative for an average of 6 years, range 1–14). The patients chosen for each group had had previous HCV results from plasma samples on record at WoSSVC. Information on the number of patients treated and the duration of HCV infection was unknown for both groups 4 and 5.

3.4. HCV antibody testing of DBS

The ORTHO HCV 3.0 ELISA Test System with Enhanced SAVekit (Ortho Clinical Diagnostics) was used to detect anti-HCV in DBS
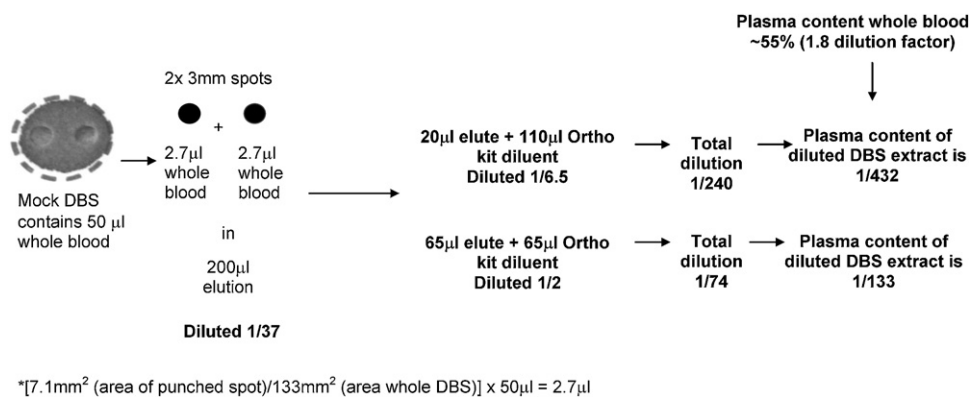
Fig. 1. Diagram indicating how the DBS dilution factors used in the avidity assay was calculated.

using a modified version of the Judd et al[4] method. The modification was two 3 mm discs punched from the DBS and eluted in 200 μl of PBS/0.05% Tween.

3.5. Avidity assay

The avidity assay is a modification of the ORTHO HCV 3.0 ELISA Test System with Enhanced SAVekit (Ortho Clinical Diagnostics). Plasma samples were diluted either 1/100 or 1/400 in kit diluent and 200 μl was added to 2 wells of a 96 well plate. The plate was shaken overnight at room temperature. The samples were washed once to remove unbound material. One sample was washed in 300 μl kit buffer while the duplicate sample was washed with 300 μl 7 M urea (dissolved in kit buffer). This was done three times for 5 min. The microtitre plate was washed seven times. Note that all washes were in 300 μl of kit wash buffer. 200 μl of conjugate was added for 3 h and the plate was then washed six times. The manufacturers' guidelines were then followed for the remaining steps. The AI was calculated by taking the optical density (OD) of the urea treated specimen divided by the OD of the wash buffer treated sample and expressed as a percentage. The OD was measured using the plate reader function on the Siemens BEP III. To calculate the AI, samples treated with wash buffer should have an OD > 1.0.

The avidity assay described above was used for DBS with the following modification 20 μl eluted DBS was added to 110 μl kit diluent or 65 μl eluted DBS was added to 65 μl kit diluent, this resulted in a final dilution factor of either 1/240 or 1/74 which corresponded to a plasma dilution of 1/432 and 1/133, respectively (Fig. 1).

3.6. HCV RNA extraction and amplification

HCV RNA was extracted from plasma and serum samples using the Abbott extraction method, and the 5′NCR region was amplified using an in-house method as described by Daniel et al.[20] The DBS were extracted and amplified as described in Bennett et al.[21]

3.7. Statistical analysis

Exact confidence intervals calculated from the binomial distribution. The difference in AI values between plasma and DBS samples was assessed using the Wilcoxon signed rank test for paired data.

4. Results

4.1. HCV avidity test on plasma

Initially, the plasma samples were all diluted 1/400. The mean AI for groups 1 (relating to acute infection), 2 (chronic) and 3 (resolved) were 8 (range 2–15), 91 (range 25–100), and 48 (AI range 7–100), respectively (Table 2). The low AI generated among the resolved infections indicated that this group could be mistaken for recent infection. The samples from group 3 were then diluted 1/100 which generated an increase in the AI: mean AI increased to 72 (range 21–100). Therefore, by diluting group 3 initially by 1/400 and then re-diluting the samples when the AI < 30 the mean AI became 56 (range 21–100) for this group. Two way comparisons between the 3 distributions (acute, chronic and resolved) were all significant (p < 0.0001). The two lowest avidity indices from group

Table 2
Avidity results on plasma samples from individuals with acute (group 1), chronic (group 2) and resolved (group 3) infection.

Dilution	Group 1 (recent)		Group 2 (chronic)		Group 3 (resolved)	
	1/400	1/100	1/400	1/400 and 1/100[a]	1/400	1/400 and 1/100[a]
Number of samples	19	12	300	300	82	82
Mean avidity index (%) (range)	8 (2–15)	13 (2–28)	91 (25–100)	91 (35–100)	48 (7–100)	56 (21–100)
Avidity index (%): 0–10	17	6	0	0	3	0
11–20	2	4	0	0	12	0
21–30	0	2	2	0	14	6
31–40	0	0	6	6	11	9
41–50	0	0	10	10	7	6
51–60	0	0	13	14	12	6
61–70	0	0	12	12	4	11
71–80	0	0	8	8	4	5
81–90	0	0	26	27	4	9
91–100	0	0	223	223	11	30

[a] Samples with AI values ≤ 30, with dilution 1/400, were retested using 1/100 dilution.

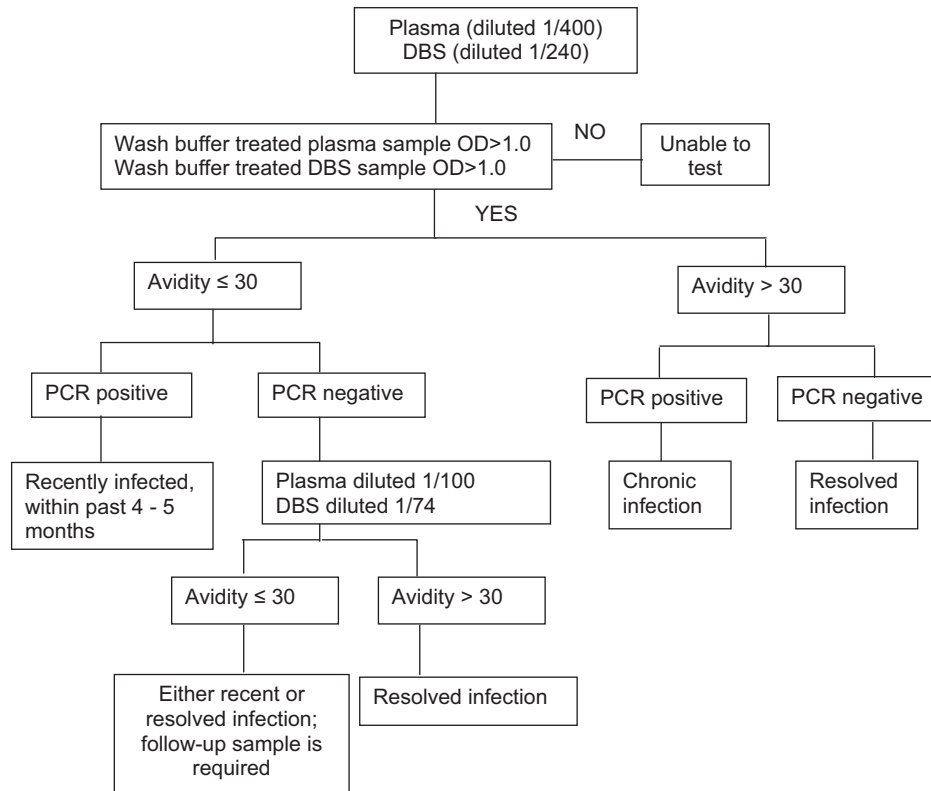*S.J. Shepherd et al. / Journal of Clinical Virology xxx (2013) xxx–xxx*

Fig. 2. HCV avidity assay algorithm for testing plasma and DBS on HCV antibody positive individuals.

2 (chronic) – with AI of 25 and 30 – were retested at a 1/100 dilution and produced an AI of 60 and 85, respectively. The genotypes for these 2 discrepant chronic samples were genotype 2 and 3. When 12 samples from group 1 (insufficient sample was available to test all 19) were re-tested at a dilution of 1/100, the AI remained below 30 (mean AI 13, range 2–28). Based on the above results, the AI cut-off was set at ≤30 and an algorithm for HCV avidity testing was defined (Fig. 2).

Applying the algorithm outlined in Fig. 2, the sensitivity of the avidity test to detect recent infection on plasma was 100% (CI 82.4–100%); based on the 19 (PCR positive) acute infected samples having all tested below the AI cut-off of 30. The specificity of the avidity test was calculated as 99.3% (CI 97.6–99.9%); based on 298 of 300 chronic infected samples having tested above the AI cut-off of 30.

Five patients from group 1 had follow up samples. Patient 1 had an AI = 6, the follow up sample 11 days later was AI = 9. Thirteen days after an initial-anti-HCV positive result, patient 2 had an AI < 10. The AI increased to 15 on day 65 post initial anti-HCV positive test. Patient 3 was AI = 8, 74 days after the initial positive antibody result (insufficient sample to test original antibody positive sample), 13 days later the AI had increased to 81 (total of 87 days post 1st anti-HCV positive result). Patients 4 and 5 were renal patients, infected with HCV during an outbreak in a renal dialysis unit with the AI monitored over sequential samples (Table 4). Patient 4 and 5 had an AI ≤ 30 up to ~124 days (4.1 months) and 96 days (3.2 months), respectively post first HCV PCR positive sample.

Two seroconverter panels were tested with the HCV avidity assay (Table 3). For panel PHV901, the avidity index remained low (≤30) on plasma up to 74 days (2.4 months) post their first HCV PCR positive result, and then increased above 30 at 101 days (3.3 months). For panel PHV917, the avidity index remained low (≤30) on plasma up to 132 days (4.3 months) since their first HCV PCR positive result was detected.

Table 3
Avidity results on 2 seroconverter panels: (a) PHV901 and (b) PHV917. Samples with AI < 10 indicate that the antibody level was detected on the kit wash buffer sample but not detected on the urea treated sample, indicating early infection. There were 3 wash buffer treated mock DBS in PHV917 that had an antibody level < 1.0 (low on table).

(a) PHV901						(b) PHV917					
Sample	Bleed dates	Days since first PCR+	Antibody	AI		Sample	Bleed dates	Days since first PCR+	Antibody	AI	
				Blood	Mock DBS					Blood	Mock DBS
1	23/09/1993	Pre PCR+	N	N	N	1	29/05/1996	Pre PCR+	N	N	N
2	27/11/1993	0	N	N	N	2	18/06/1996	0	N	N	N
3	29/12/1993	32	P	AI < 10	8	3	20/06/1996	2	N	N	N
4	31/12/1993	34	P	AI < 10	7	4	22/08/1996	65	P	AI < 10	AI < 10
5	05/01/1994	39	P	AI < 10	7	5	07/10/1996	111	P	AI < 10	Low
6	07/01/1994	41	P	AI < 10	6	6	11/10/1996	115	P	AI < 10	Low
7	01/02/1994	66	P	9	9	7	14/10/1996	118	P	AI < 10	Low
8	09/02/1994	74	P	8	9	8	22/10/1996	126	P	10	12
9	08/03/1994	101	P	33	27	9	28/10/1996	132	P	9	8
10	14/04/1994	138	P	84	66						

Table 4
Avidity results for 2 renal dialysis unit patients. HCV transmission occurred during September 2010 for patient 4 while transmission for patient 5 occurred prior to June 2010.

(a) Patient 4					(b) Patient 5				
Sample	Date sample collected	Days since first PCR+ result	Anti-HCV (S/Co)[b]	AIBlood	Sample	Bleed dates	Days since first PCR+ result	Anti-HCV (S/Co)[b]	AIBlood
1	25/11/2010	72[a]	2.36	AI < 10	1	03/06/2010	0	0.06	NT
2	30/11/2010	77	2.89	AI < 10	2	06/09/2010	96	11.18	15
3	15/12//2010	92	15.05	5	3	14/09/2010	104	13.31	46
4	05/01/2011	103	16.09	6	4	11/10/2010	120	12.65	62
5	10/01/2011	108	14.30	6	5	19/10/2010	128	12.39	85
6	24/01/2011	124	11.77	21	6	02/11/2010	142	14.41	100
7	31/01/2011	131	13.02	45					
8	04/02/2011	135	15.17	54					
9	08/03/2011	167	15.00	83					

[a] Approximate number of days since the patient was HCV PCR positive-based on date of suspected transmission and 4 weeks to become PCR positive.

[b] Anti-HCV was detected by Abbott Architect with S/Co > 1 positive.

Based on the serial samples shown in Tables 3 and 4, and patient 3 we estimate the window period for detecting a recent infection with this HCV avidity assay (following a PCR positive result) is approximately 3–4 months. Given the time from infection to detection of HCV RNA can be between 1 and 4 weeks, the assay identifies individuals who have become infected within the past 4–5 months.

4.2. HCV avidity test on DBS

DBS made from the plasma of 12 group 1 (recent), 50 group 2 (chronic) and 25 group 3 (resolved) samples were tested for avidity, and results compared with those from the plasma samples (Fig. 3). For the recent cases, there was no significant difference in AI results between plasma and mock DBS (mean/median difference: −0.4/0, $p = 0.7$); and all of the mock DBS samples had an AI ≤ 30. For the chronic cases, the AI results on mock DBS were significantly lower than those found on plasma (mean/median difference: −9.3/−4, $p < 0.0001$); the majority (98%; 49/50) of the mock DBS samples however had an AI > 30. The one chronic sample with AI = 27 on DBS was retested at 1/74 dilution (Fig. 1) and the AI became 42. This mock DBS was made from a plasma sample with AI of 35.

For the resolved group 3, samples for mock DBS were chosen with AI values on plasma ranging from 15 to 100 on initial testing with dilution 1/400; five plasma samples had AI ≤ 30, but all increased above this threshold with dilution 1/100 (Fig. 2). When the mock DBS were diluted 1/240, there were 2 samples that were antibody negative and 2 that had weak reactive antibody (Fig. 1). Of the remaining 21 resolved DBS samples, 43% (9/21) had an AI ≤ 30 with dilution 1/240, but this reduced to 19% (4/21) with dilution 1/74 (Fig. 2).

Samples from the seroconverter panels PHV901 and PHV917 were also made into mock DBS (Table 3). For panel PHV901, at 101 days (3.3 months) after the first PCR positive sample, the mock DBS had an AI = 27 (below the cut-off and thus still indicating recent infection), whereas the plasma sample had an AI = 32 (above the cut-off and thus no longer indicating recent infection).

The majority (98%, 64/65) of group 4 chronic cases had an AI > 30 on DBS, with dilution 1/240. The one chronic case with an initial AI of 27 (below the threshold) on DBS increased to an AI of 37 on re-testing with dilution 1/74. Applying a dilution of 1/74 for initially low AI (≤30) cases as outlined in the algorithm (Fig. 2), 90% (61/68) of DBS samples in the resolved group 5 generated AIs above the 30 cut-off.

Based on the results from the 12 acute mock DBS samples, the sensitivity of the HCV avidity test on DBS was estimated at 100% (95% CI 73.5–100%). Combining the results from the 50 chronic mock DBS samples with the 65 chronic cases in group 4, the
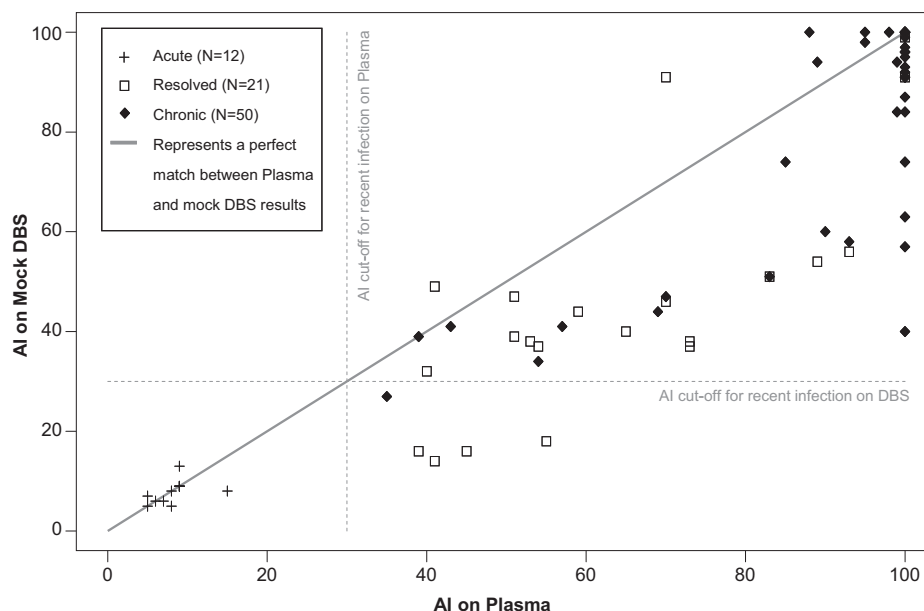

Fig. 3. Comparison of avidity index (AI) results on paired plasma and mock DBS samples from individuals with acute, chronic and resolved HCV infection*. *Dilutions used were 1/400 for plasma and 1/240 for DBS; for samples with low AI (≤30) and PCR negative on initial testing, dilutions used were 1/100 for plasma and 1/74 for DBS.

Table 5
Variability of the HCV avidity assay using both plasma and mock DBS tested on separate runs over a period of time. The mean AI and standard deviation of each sample is shown in table.

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10
Sample type	Plasma	Plasma	Plasma	Plasma	Plasma	Mock DBS	Mock DBS	Mock DBS	Mock DBS	Mock DBS
Original AI	10	15	30	31	100	27	28	29	31	35
Number of times tested	40	6	7	6	40	5	3	3	3	3
Mean AI	10	11	30	30	97	25	29	27	29	30
St Dev	2.0	2.4	3.6	1.4	3.5	1.5	0.5	2.2	1.7	3.7

specificity of the avidity test on DBS was estimated at 98.3% (CI 93.9–99.8%), based on the algorithm given in Fig. 2.

4.3. Inter-test variability

Five plasma and 5 mock DBS samples were repeat tested on different runs to measure the inter-test variablity (Table 5). Two plasma samples were from acutely infected and 1 plasma sample was from a chronic patient. The remaining 7 samples were originally found to be near or on the AI cut-off of 30. Variability was consistent across the range of the test.

5. Discussion

This study has demonstrated that the avidity test in combination with PCR can differentiate between acute, chronic and resolved infection HCV infection. This supports the findings of other papers that have reported avidity testing for HCV. It can be difficult to draw conclusions and comparisons from various avidity tests when different methods or DA are used.[13,22] However our study and those of previous HCV avidity studies indicate that to determine an acute HCV infection a supplemental test is required for accurate diagnosis when a negative anti-HCV sample is not available prior to first anti-HCV positive result.[16,19,23] Previous studies have found that patients with resolved HCV infection can have a lower avidity index compared to chronic patients whose antibodies are continually exposed to the HCV virus.[16–19]

A testing algorithm has been developed for HCV avidity using either plasma or DBS. In our laboratory we found that two dilution factors were required to help differentiate between chronic and resolved infections when the sample had an AI < 30 and was PCR negative. This is because adequate antibody must be present to allow an accurate measurement of OD prior to treatment with a DA.[13,15,16] The dilution factors used may vary in different laboratories depending on the saturation point of the plate reader. Samples with a recent infection retain the low avidity result but samples from resolved infections showed the avidity increased when a lower dilution factor was used.

In the seroconverter panel, one plasma sample had AI = 32 but the mock DBS of this sample had AI = 27. This discrepancy can be explained by the inter-test variability around the AI cut-off. This suggests a grey zone range around the cut-off that would require repeat testing and a follow up sample.

Though the majority of recently acquired HCV infections will be PCR positive a small number may be negative due to fluctuating virus levels or rapid clearance of the virus and so a follow up sample would counter act any negative PCR results in recent infections. This has been incorporated into the algorithm for HCV avidity testing.

Antibody maturation and consequently progression from low avidity to high avidity is not always uniform. Various HCV studies have indicated the variability in individual antibody maturation.[15,16] Many factors have been found to influence the progression from low to high avidity.[23–26] A low avidity result with the assay described in this paper suggested that a recent HCV infection has occurred within ~4–5 months. In this study, group 1 (acute patients) were defined as becoming anti-HCV positive after a previous negative result taken 4–6 months ago. HCV antibodies can take up to 8 weeks to develop and therefore increase the time for 1st positive anti-HCV by at least 2 months. Two of the patients that had been used to estimate the recency were undergoing renal dialysis. Renal dialysis patients can have altered antibody development.[11]

The HCV avidity assay used in this study was a modification of a commercial assay that consisted of a mixture of HCV structural and non structural proteins. With these assays there is the potential to be monitoring antibodies of different avidities to different HCV antigens. A technique to differentiate between acute and chronic infection using individual HCV antigens has recently been described.[27] HIV avidity has been shown to be affected by subtype differences and this may also affect the HCV avidity tests.[28]

The limitation of the current work was the number of samples from recently infected individuals; this was reflected in the confidence intervals. A larger project using more samples and follow up samples from known recently infected cohorts is planned. It would also be of benefit to obtain actual DBS and plasma samples at the same time from patients; this would allow DBS, plasma and mock DBS all to be monitored from an identical time point.

This paper shows that avidity testing can be used on DBS which will not only enhance HCV surveillance but will be a useful tool in identifying individuals who would benefit from early HCV treatment.

Competing interests

None declared.

Ethical approval

Non required.

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