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Published in:
Journal of Medical Virology

DOI:
10.1002/jmv.24919

Publication date:
2018

Document Version
Author accepted manuscript

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ABSTRACT

Accurate detection of incident hepatitis C virus (HCV) infection is required to target and evaluate public health interventions, but acute infection is largely asymptomatic and difficult to detect using traditional methods. Our aim was to evaluate a previously-developed HCV avidity assay to distinguish acute from chronic HCV infection. Plasma samples collected from recent seroconversion subjects in two large Australian cohorts were tested using the avidity assay, and the avidity index (AI) was calculated. Demographic and clinical characteristics of patients with low/high AI were compared via logistic regression. Sensitivity and specificity of the assay for recent infection and the mean duration of recent infection (MDRI) were estimated stratified by HCV genotype. Avidity was assessed in 567 samples (from 215 participants), including 304 with viraemia (defined as ≥250 IU/ml). An inverse relationship between AI and infection duration was found in viraemic samples only. The adjusted odds of a low AI (<30%) decreased with infection duration (odds ratio [OR] per week of 0.93; 95% CI:0.89-0.97), and were lower for G1 compared with G3 samples (OR=0.14; 95% CI:0.05-0.39). Defining recent infection as <26 weeks, sensitivity (at AI cut-off of 20%) was estimated at 48% (95% CI:39-56%), 36% (95% CI:20-52%), and 65% (95% CI:54-75%) and MDRI was 116, 83, and 152 days for all genotypes, G1, and G3, respectively. Specificity (≥52 weeks infection duration, all genotypes) was 96% (95% CI:90-98%). HCV avidity testing has utility for detecting recent HCV infection in patients, and for assessing progress in reaching incidence targets for eliminating transmission, but variation in assay performance across genotype should be recognised.

Keywords: Hepatitis C virus; Recent infection; Avidity assay; genotype; Australia

INTRODUCTION

It is estimated that 130-170 million people worldwide are infected with the hepatitis C virus (HCV), with the prevalence of infection varying between countries (1). The prevalence of HCV antibody (anti-HCV) is frequently used as a marker of infection; however, anti-HCV does not differentiate between acute, chronic, and resolved infection. Knowledge of HCV incidence – the number of new or recent infections within a population over a given period of time – is essential to pinpoint where infection is spreading most rapidly. By identifying populations most at risk of infection, public health interventions can be implemented, and their effectiveness evaluated. However, HCV incidence can be difficult (and expensive) to measure using traditional epidemiological methods, which typically rely on following subjects over time to observe seroconversion. A reliable laboratory test for recent HCV infection would, therefore, be of considerable public health value.

Several HCV assays to measure acute/recent infection have been described (2–8); the majority of these are modified commercial assays measuring antibody avidity. Antibody avidity is the binding capacity of maturing antibody to antigen, which increases over time in primary infection. While HCV avidity assays have been shown to distinguish between acute and chronic infection, they may not differentiate between acute and resolved infection (5–6). Therefore, these assays cannot be used as a stand-alone test and require additional HCV markers, such as PCR or an IgM dilution series (8–9).

We have previously described an HCV avidity assay (8), which is a modified version of the Ortho HCV 3.0 ELISA Test System with Enhanced SAVekit (Ortho Clinical Diagnostics) using the antibody-antigen chaotropic agent, urea. The limitation recognised in this previous

study was the small number of recently infected patients (*N*=19) used to define the assay, as has been the case with many published avidity assay methods (*N*s ≤ 50) (2–7). Recently, Patel et al. measured avidity in 568 PWIDs, of whom only 56 were seroconverters (10). These authors' assay was also based on the Ortho ELISA using the chaotropic agent, diethylamine (DAE). In the current paper, we apply our urea-based avidity assay to two large well-described cohorts involving over 200 recently infected individuals. To our knowledge, this is the first study to examine HCV avidity according to genotype.

METHODS

Study population.

Plasma samples and epidemiological/clinical data from two Australian cohorts were used: (i) the Australian Trial in Acute Hepatitis C (ATAHC), which was a prospective study of recent HCV infection (11), and (ii) the Hepatitis C Incidence and Transmission Study in prison (HITS-p), which is an ongoing study of prison inmates at risk of acute HCV in correctional centres (12). Participants were selected according to the following criteria: subjects had recent HCV infection defined as an initial anti-HCV positive test and either (a) a negative anti-HCV test within two years prior to first testing anti-HCV positive, or (b) acute clinical hepatitis (i.e., evidence of seroconversion illness, including clinically documented jaundice or ALT >400 IU/mL and a history of high-risk exposure within three months of clinical manifestation of acute HCV) at most one year of first testing anti-HCV positive (see Ref. (13) for the further description of the study population). Persons with elevated ALT are very likely to be cases of

early acute HCV infection, given that ALT elevation in the setting of high-risk exposure generally indicates infection within the first 3-4 months [14].

Plasma samples from participants fitting these criteria were identified and HCV avidity was measured (as described below). The epidemiological and clinical data available included demographics (sex, age, ethnicity), interferon lambda 4 (IFNL4) genotype, mode of HCV acquisition, HCV test dates and results (relating to HCV antibody, RNA, and genotype), duration of HCV infection (with time origin defined as the estimated date of HCV infection), clinical presentation (asymptomatic / symptomatic), and HIV co-infection.

The estimated date of HCV infection was calculated as follows: (i) among individuals HCV antibody-negative and HCV RNA-positive at the time of acute HCV detection, the date of HCV infection was defined as four weeks prior to diagnosis date; (ii) among persons with acute symptomatic infection, the date of infection was defined as six weeks prior to the onset of acute clinical hepatitis; (iii) among cases of HCV seroconversion without acute symptomatic infection, the date of infection was set to the mid-point between the last negative HCV antibody and first positive HCV antibody or RNA test [15].

For calculation of the mean duration of recent infection (MDRI) and false recency rate (FRR) measures (see below), the estimated date of infection served as the time origin for quantifying the duration of recent infection.

The *baseline dataset* consisted of the first sample (and associated epidemiological/clinical data) collected from each participant, and the *longitudinal dataset* as the complete set of samples

(and associated epidemiological/clinical data), which includes multiple samples per individual collected over time.

Laboratory testing

Antibody avidity was assessed using a modified version of the Ortho HCV 3.0 ELISA Test System with Enhanced SAVekit (Ortho Clinical Diagnostics). The chaotropic agent used in this study was 7M urea. Two aliquots of each sample were tested simultaneously, one aliquot was treated with 7M urea and the other aliquot was treated with ORTHO kit wash buffer. The AI was calculated by taking the OD of the urea treated sample divided by the OD of the kit wash buffer treated sample and expressed as a percentage. The full method for this assay has previously been described [8]. The following modifications were in place for this study: (i) samples were initially diluted 1/500 with kit diluent; (ii) samples with an optical density (OD) <1.0 were re-tested at a 1/100 dilution; and (iii) samples with an OD ≥4.0 (saturation point on the plate reader) were re-tested at a dilution of 1/1500. AI was calculated and expressed as a percentage. Samples treated with buffer that had an OD <1.0 were excluded from the study due to low antibody levels that could lead to inaccurate AI. All avidity testing was performed at the West of Scotland Specialist Virology Laboratory, and was conducted blinded to any of the epidemiological and HCV information (e.g., duration of infection, HCV RNA level, and genotype). Detection of quantification of HCV RNA for ATAHC samples was performed using the Versant TMA assay (detection <10 IU/mL); HITS-p samples were tested on the COBAS AmpliPrep/COBAS TaqMan HCV assay (detection <15 IU/ml).

For avidity analysis, HCV RNA-positivity is required; therefore, samples were categorised as *non-viraemic* and *viraemic* using HCV RNA definitions of <250 IU/ml and ≥250 IU/ml, respectively. The former category thus includes mainly RNA-negative samples. In sensitivity analysis, two other HCV RNA thresholds are investigated: ≥10 IU/ml (the lower detection limit of the qualitative assays used) and ≥500 IU/ml (as used by Ref. [10]).

Statistical analysis

First, we created boxplots of the distribution of AI values in the baseline samples, according to categorical estimated duration of infection and stratified by the HCV RNA threshold binary variable (<250 IU/ml , ≥250 IU/ml).

Next, logistic regression analysis was used to determine the demographic and clinical characteristics of the patient cohort that were associated with a low AI (<30), following Ref. [8]. This analysis was restricted to baseline samples with level of HCV RNA ≥250 IU/ml, to exclude potential resolved infections (5) from the baseline dataset. The set of potential variables to include in the multivariate logistic regression analysis consisted of factors that were *a priori* anticipated to be associated with a low AI; this set included sex, age, aboriginal ethnicity, duration of infection, type of acute clinical presentation (ALT>400 IU/ml, symptomatic, or asymptomatic), IFNL4 genotype, HCV RNA level, mode of HCV acquisition (injecting drug use, sexual transmission, or other), HIV status, and HCV genotype. The final set of variables included in the multivariate logistic regression analysis was determined from the univariate regression results, using a rejection criterion of $P>0.20$.

Third, the relationship between AI and estimated duration of infection for the baseline samples was plotted and regression lines were fitted separately for G1 and G3 data, as initial investigation indicated genotype 3 was associated with lower AI values.

The sensitivity and specificity of AI (according to specific AI thresholds) as a test for recent HCV infection were estimated via logistic regression models [16–18] fitted to the longitudinal dataset. Although these measures will depend on properties of the population being tested, such as characteristics of the distribution of time since infection, sensitivity and specificity are appropriate metrics for individual patient-level detection of recent infection, and thus have clinical application [19]. Regression models were specified using generalized estimating equations (GEE), which is an appropriate method for handling non-independent (clustered) data. 95% confidence intervals (CIs) for the two indicators were derived using robust standard errors. Sensitivity (for infections with an estimated time since infection of<8 weeks and of<26 weeks) and specificity (for infections with an estimated time since infection ≥52 weeks) were estimated for AI cut-off values ranging from 20% through 40%, in steps of 10%. Estimation of these parameters was carried out for all genotypes, and for G1 and G3 separately.

Finally, to enable the data to be applicable for incidence monitoring at the population-level [17], further measures of assay performance was computed: mean duration of recent infection (MDRI) and false recency rate (MDRI). The former measure was calculated using the binomial regression method [20], using a maximum duration of infection value T of 365 days (where T is the defined threshold of duration of infection beyond which AI values below the cut-off are classified as 'false recent'), for all genotypes and separately for

genotype 1 and 3, for AI cut-off values from 10% to 40%. Bootstrapping was used to determine 95% confidence intervals. FRR was computed with respect to the same value of *T*, and is identical to 100 minus specificity (see above for computation method).

RESULTS

The initial dataset consisted of a total of 635 samples, representing a total of 269 subjects (Fig. 1). To improve the accuracy of the estimation of the date of infection, participants who were recent asymptomatic seroconverters but whose duration of infection was uncertain (≥1 year between last negative and first positive test date; *n*=68) were excluded. Thus, 567 samples (from 215 participants) were included in the avidity analysis (Fig. 1), of which 304 samples had HCV RNA ≥250 IU/ml and 263 samples had HCV RNA <250 IU/ml; the vast majority (97%) of the latter samples were RNA negative.

Characteristics of the study cohort by viraemic category

Demographic and clinical characteristics of the cohort, according to RNA category at baseline are shown in Table 1. The majority of subjects (*n*=215) were male (73%), acquired their HCV infection through injecting drug use (85%), were infected with HCV genotype 1 or 3 (47% and 37% among the viraemic group, respectively), were estimated to have acquired infection ≤26 weeks before the date of baseline sample (68%), and were asymptomatic (60%). The average age at baseline was 31 years. 18% of the study cohort was co-infected with HIV.

Avidity index (AI) by viraemic category

An AI value could be determined for 446 samples (from 180 subjects), including 226 samples (from 127 subjects) with HCV RNA ≥250 IU/ml. AI could not be reliably determined for 30 samples with very low antibody levels (OD<1.0 after 1/100 dilution) and for 91 samples with high antibody levels (OD>4.0 after 1/1500 dilution). The distribution of AI values in the 215 baseline samples as a function of estimated duration of infection differed markedly according to viraemic category (Fig. 2). For the samples with HCV RNA <250 IU/ml, there was no clear relationship between median AI and increasing duration of infection, whereas for viraemic (HCV RNA ≥250 IU/ml) samples, median AI increased monotonically with duration of infection category; subsequent analysis was therefore confined to viraemic samples.

Factors associated with AI among viraemic cases

At baseline, several characteristics differed between high AI (≥30) and low AI (<30) categories (Table 2). After assessment of the characteristics associated with low AI in an initial univariate logistic regression step, the variables remaining for inclusion in the multivariate regression analysis were duration of infection, age, acute clinical presentation category, HIV status, and genotype category (G1, G3, other known genotype, and unknown); mode of HCV acquisition was excluded because of co-linearity between sexual transmission and HIV positive status (16 out of 20 sexual transmission cases were HIV positive). Multivariate logistic regression indicated that, adjusting for the other variables, the odds of a low AI value significantly decreased with duration of infection (odds ratio [OR] per week of 0.93 (95% CI: 0.89-0.97). The odds of AI < 30 were significantly lower for G1 (OR = 0.14; 95% CI: 0.05-0.39) compared with G3.

The relationship between AI and estimated duration of infection for the baseline samples is shown in Fig. 3. Similar linear relationships between these variables were observed for both genotype 1 and genotype 3, with a clear difference in intercept; higher AI values were generally obtained for genotype 1 samples.

Sensitivity and specificity by genotype among viraemic samples

For individual-level detection of recent infection, the diagnostic test metrics sensitivity and specificity were computed via logistic regression. Based on the longitudinal dataset (containing both baseline and follow-up samples: *n*=226), sensitivity (derived from samples with estimated duration of infection <26 weeks) ranged from 34% (95% CI: 26-42%) with an AI cut-off of 10%, to 61% (95% CI: 53-68%) with an AI cut-off of 40%. For the early stage of recent infection (defined as an estimated duration of infection <8 weeks), the overall sensitivity at an AI cut-off value of 20% increased to 91% (95% CI: 75-97%). An AI cut-off value of 20% attained optimal specificity at 96% (95% CI: 90-98%) and sensitivity of 48% (95% CI 39-56%).

Sensitivity also differed across genotype category. At an AI cut-off of 20%, the sensitivity (based on estimated duration of infection <26 weeks) for G1 samples (*n*=86) was lower than for G3 samples (*n*=95): 36% (95% CI: 20-52%) and 65% (95% CI: 54-75%), respectively.

Alternative quantitative RNA thresholds for categorisation of viraemic samples yielded very similar results for both HCV RNA ≥500 IU/ml (all genotypes, AI=20%: sensitivity: 48%, 95% CI: 40-57%; specificity: 96%, 95% CI: 90-98%; see Supporting Information Table S1), and HCV

RNA ≥10 IU/ml (sensitivity: 48%, 95% CI: 39-56%; specificity: 96%, 95% CI: 91-98%), indicating that results were insensitive to the threshold for the main analysis.

Finally, for population-level application of the assay to incidence estimation, two parameters are required: the false recency rate (expressed as a percentage; Table 4) and MDRI; both were derived using estimated duration of infection**.** MDRI varied according to AI cut-off value and genotype (Table 4). MDRI was longer for G3 compared with G1; for instance, at an AI cut-off of 20%, MDRI was 152 days (95% CI: 123-186) for G3, compared with 83 days (58-105) for G1. At the same AI cut-off, MDRI for all genotypes was 116 days (98-135) and the FRR was 4% (95% CI: 2-10%).

DISCUSSION

In this paper, an HCV avidity assay based on a modified version of the Ortho 3rd generation antibody ELISA was evaluated using plasma samples from two well-studied Australian cohorts of recently infected participants. This analysis represents the largest study of HCV avidity in recently infected patients to date, and is also the first study to examine the effect of HCV genotype on HCV avidity. The key findings of our analysis were that: the odds of a low AI decreased with duration of infection; those with genotype 1 were more likely to have a high AI value compared to those with genotype 3; there was a steeper rise in AI with time since infection among those with genotype 1 compared to genotype 3; and avidity test sensitivity and MDRI were dependent on genotype – sensitivity was markedly higher for

genotype 3 than for genotype 1. At a given AI cut-off, MDRI was longer for G3 compared with G1 samples.

Interpretation of our findings in relation to previous studies

Our study population, of whom the majority (85%) acquired HCV through injecting drug use, is representative of patients with newly-acquired HCV in developed countries. Genotypes 1 and 3 were the most commonly occurring HCV genotypes, reflecting the global HCV-infected population as a whole [21–22].

The finding of a decreased likelihood of low AI with increasing duration of infection is consistent with previous studies [2, 3, 6]. The overall sensitivity and specificity of the assay determined here was low compared to what has been previously published by us (100% and 99%, respectively [8]) and by others (98% and 100%, respectively [6]). However, both our previous study and that of Ref. [6] (which used an AI cut-off of 43%) were limited by the small number of recently infected patients (n=19 and n=14, respectively), and both involved samples that had been taken much closer to the time of infection, which likely explains the difference in sensitivity. The study by Patel et al. [10] involved a population approximately one-third the size of the recently infected cohort described here (with 56 seroconverters, 233 samples), but similarly found the assay most sensitive for those samples taken close to seroconversion (i.e., ≤90 days). Their estimates of specificity (99% and 92% among HIV-negatives and HIV-positives, respectively) were based on a much larger cohort (547 individuals, 764 samples) known to have been HCV seropositive for >2 years. Thus, their estimated false positive rate of 0.7% in HIV-negative persons matches what has previously been reported based on 300 chronically HCV-infected persons [8].

Previous studies have suggested that the period of time during which an individual has a low AI – i.e. the duration of recent infection– is four to six months [6, 8]. Two definitions of time since infection were compared (<26 weeks and <8 weeks, where the latter is more likely to be true recent infection), and found that the overall sensitivity of the assay was notably higher for the shorter time since infection, as has been highlighted previously [9]. The improvement in sensitivity with a duration of infection of <8 weeks was greater for genotype 1, indicating that the mean duration of infection for the assay is shortened for this genotype. Genotype differences have been found when viral load was compared suggesting adaptive immune responses may vary across genotype [23]. Similar variation in the duration of recent infection has been reported among different HIV subtypes [24–25].

The present analysis did not identify co-infection with HIV as a unique predictor of low AI value; this was possibly due to a lack of power. It has been demonstrated that HIV infection can cause a delay in anti-HCV production, with some HIV-positive individuals having no detectable anti-HCV up to one year post-HCV infection [26]. The prevalence of HIV among PWID varies by setting [27–28], and given that there was a substantial prevalence of HIV co-infection among our study population (18% overall), some of these individuals may have had delayed HCV seroconversion. This may have affected the sensitivity and specificity of the assay if a low AI is obtained from HIV-positive individuals even if they were infected for more than 26 weeks. HIV infection results in depletion of the CD4+ T cells; with a CD4+ count of <500 cells/mm^3 a marked depletion in anti-HCV response to HCV structural and non-structural antigens occurs, and this in itself may affect the antibody maturation process [29]. HIV co-infection may therefore be an important consideration when measuring HCV avidity in PWID populations.

Subjects with an ALT>400 IU/ml had a two-fold higher likelihood of a low AI, compared with asymptomatic patients, although this was not statistically significant. The ALT level in chronically infected patients is generally lower than in symptomatic, acutely infected HCV patients [30]. Therefore, elevated ALT and low avidity can indicate recent infection. However, in patients with an acute exacerbation of chronic HCV, ALT levels can be >400 IU/ml [31].

Possible clinical and epidemiological applications of the assay

Previously, the major clinical utility in distinguishing recent from chronic HCV infection was to identify patients for early treatment with interferon-based antiviral therapy, which is associated with improved outcomes [32–35]. With the success of direct-acting antivirals (DAAs) in treating chronic infection, the need to identify acute infections for early treatment may no longer be as important [36, 37]. Nevertheless, using the effective new therapies for the treatment of patients with recent infection may have value in preventing the onward transmission of infection [38]. The estimates of the traditional diagnostic test metrics presented here, sensitivity and specificity, are useful for these applications.

Although the sensitivity and specificity of the assay may not be optimal for clinical purposes, the epidemiological application is promising. The assay had a low false positive rate of 4% (aggregated over HCV genotype) at an AI cut-off of 20% and T=365 days (see below for further discussion in relation to false positives); similar false positive rates have been described for HIV avidity assays [39]. Given our relatively small sample size (n=45) for determining the false positive rate, estimation of this parameter could be improved through pooling data from multiple studies. Avidity assays have more use in high- than low-

prevalence populations for detection of recent HCV infection. Due to the higher risk of transmission and thus new infections within a high-prevalence population, an assay with low sensitivity can still be a valuable tool [40]. At the population-level, HCV incidence can be estimated from a single cross-sectional prevalence survey using only the assay-based classification of recent infection and the MDRI and false-positive rate parameters [20]. The WHO global hepatitis strategy includes targets for a 90% reduction in incidence between 2015 and 2030 [41]; however, the vast majority of countries lack HCV surveillance systems to monitor progress towards this target. Avidity testing offers a practical and relative low-cost tool to help monitor HCV incidence and assess progress in meeting global targets.

Another potential epidemiological application for the avidity assay described here is during outbreak investigations and in contact tracing. HCV sequence analysis has demonstrated the relationship between newly-infected PWID and their HCV-positive injecting partners [42]. Antiviral therapy offered to selected contacts may help to prevent the further spread of HCV [43]. Avidity assays can inform the extent of recent /ongoing transmission in certain cohorts. For example, an HIV avidity assay has been used during an ongoing HIV outbreak among a homeless PWID population [44].

Limitations of this study

Although our study population was large enough to yield reasonably precise inferences regarding the sensitivity of the avidity assay, there were certain limitations. Small numbers of participants with HCV genotypes other than 1 or 3 meant that results across all genotypes could not be compared. A limitation of this cohort study is that the exact date of infection was not known: thus, consequent uncertainty in the estimated duration of infection may

have affected the estimates of sensitivity. For example, for the analysis of very recent infection (time since infection of <8 weeks), some individuals may have been infected up to 16 weeks previously. The chaotropic agent used in an avidity assay can also affect the AI cut-off and mean duration of recent infection [10, 45, 46]; therefore, a switch to different chaotropic agents may reduce the genotype effect.

The lower sensitivity observed for genotype 1 is likely explained by faster avidity maturation in genotype 1 antibodies, and is consistent with our finding of a steeper rise in AI among genotype 1 patients in the longitudinal dataset. The Ortho HCV avidity assay used is based on a mixture of G1 recombinant protein antigens, including HCV structural and non-structural proteins. Although the ELISA is well validated in cross-genotypic reactivity in the high avidity sera from patients with chronic infection, the performance of these antigens may differ in acute infection, especially with G3 where cross-genotypic antibody-antigen interactions are likely to lead to lower AI values.

Practical application of AI testing for population-level incidence monitoring means that the ineligibility of samples with very low or high antibody levels – those either falling below the OD threshold or exceeding the saturation point – is a potential drawback.

Finally, the false positive rates reported for AI of 30% were due to only four misclassified samples. From review of serial HCV RNA data for these cases (knowledge unavailable in a real-world setting), one of the samples was taken 46 weeks after a known re-infection (as reported elsewhere, see Ref. [47]); the sample (with a low AI of 22%) had however been counted as a false positive, as it was taken an estimated 71 weeks following initial infection.

For the other three cases, there was also evidence of fluctuating HCV RNA levels recorded over time prior to their 'false positive' sample.

In summary, we have demonstrated that the HCV avidity assay has potential clinical use for detecting patients in the earliest stages of HCV disease, and as a surveillance tool for monitoring incidence trends and evaluating public health prevention measures, but variation in assay performance by genotype needs to be considered.

ACKNOWLEDGEMENTS

The HITS-p investigators include: Andrew Lloyd, Kate Dolan, Paul Haber, William Rawlinson, Carla Treloar, Greg Dore, Lisa Maher, and Fabio Luciani. The HITS-p cohort was supported by grants from NHMRC (Nos. 222887 and 1016351) including NSW Health, the Justice Health & Forensic Mental Health Network, Corrective Services NSW, and Hepatitis NSW as partners. ARL is supported by a NHMRC Practitioner Fellowship (No. 1043067).

DISCLOSURE OF CONFLICTS OF INTERESTS

GJD is an advisory board member and receives honorarium from Gilead, Merck, Abbvie, Bristol-Myers Squibb, and Janssen, and has received research grant funding from Gilead, Merck, Abbvie, Bristol-Myers Squibb, and Janssen, and travel sponsorship from Gilead, Merck, Abbvie, and Bristol-Myers Squibb. DJG has received honoraria from Abbvie, Gilead, Merck and BMS for talks given on non-product related aspects of hepatitis C-associated epidemiology/ public health issues. ARL has served as a speaker for Gilead Sciences, and has received investigator-initiated research funding from Gilead Sciences, Merck, and Bristol-Myers Squibb. JG has served as speaker, consultant and advisory board member for Abbvie, Gilead Sciences, and Merck Sharp & Dohme, and has received research funding from AbbVie, Bristol-Myers Squibb, Cepheid, Gilead Sciences, Merck, and Merck Sharp & Dohme. SJH has received honoraria for presenting at meetings/conferences from Abbvie, Gilead, Janssen, MSD, Roche.

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SUPPLEMENTARY MATERIAL

Table S1: Sensitivity to the HCV RNA threshold used (testing two other thresholds: >10 and >=500). *File:* Table_S1_SuppInfo.pdf

Table 1. Characteristics of study participants at baseline, stratified by HCV RNA category (*n*=215).

	Total (*n*=215)	RNA <250 IU/ml (*n*=58)	RNA ≥250 IU/ml (*n*=157)
Female sex, *n* (%)	59 (27)	26 (45)	33 (21)
Age (yrs), mean [SD]	31.4 [9]	28.8 [7]	32.4 [10]
Aboriginal ethnicity, *n* (%)	30 (14)	15 (26)	15 (10)
Mode of HCV acquisition, *n* (%)			
Injecting drug use	182 (85)	55 (95)	127 (81)
Sexual transmission	20 (9)	1 (2)	19 (12)
Other	13 (6)	2 (3)	11 (7)
IFNL4 genotype (rs12979860)			
CC	117 (54)	38 (66)	79 (50)
CT	71 (33)	14 (24)	57 (36)
TT	15 (7)	1 (2)	14 (9)
Unknown/missing	12 (6)	5 (9)	7 (5)
Estimated duration of infection *			
Weeks, mean [SD]	29 [33]	28 [33]	29 [33]
≤ 8 weeks	24 (11)	10 (17)	14 (9)
9-13 weeks	33 (15)	5 (9)	28 (18)
14-26 weeks	90 (42)	25 (43)	65 (41)
27-52 weeks	45 (21)	11 (19)	34 (22)
>52 weeks	23 (11)	7 (12)	16 (10)
Clinical presentation			
Asymptomatic	128 (60)	47 (81)	81 (52)
Acute clinical (ALT >400 IU/L)	30 (14)	4 (7)	26 (17)
Acute clinical symptomatic	57 (27)	7 (12)	50 (32)
HCV genotype			
1	75 (35)	2 (3)	73 (47)
2	8 (4)	1 (2)	7 (5)
3	65 (30)	7 (12)	58 (37)
4	1 (1)	0 (0)	1 (1)
6	1 (1)	0 (0)	1 (1)
Mixed	7 (3)	2 (3)	5 (3)
Unknown	58 (27)	46 (79)	12 (8)
HCV RNA ($\log_{10}$ IU/mL) *			
Median [IQR]	5 [4–6]	2 [2–2]	5 [4–6]
HIV infection, *n* (%)	39 (18)	1 (2)	38 (24)

Note: IQR = interquartile range. *At time of baseline sample.

Table 2. Factors associated with low avidity index among people with recent HCV infection and HCV RNA ≥250 in the baseline dataset (*n*=121; reduction from *n*=157 as reported in Table 1 was due to exclusion of samples with very low or high antibody levels).

Characteristic	High HCV antibody avidity index (≥30 cut-off) (*n*=67)	Low HCV antibody avidity index (<30 cut-off) (*n*=54)	Unadjusted Odds Ratio for Low HCV antibody avidity index (95% CI)	Adjusted Odds Ratio for low HCV antibody avidity index (95% CI)
Female sex, *n* (%)	18 (27)	9 (17)	0.54 (0.21-1.31)	
Age (years), mean [SD] *	31 [10]	34 [10]	1.03 (0.99-1.07)	1.00 (0.95-1.05)
Aboriginal ethnicity, *n* (%)	5 (8)	7 (13)	1.85 (0.56-6.58)	
Mode of HCV acquisition				
Injecting drug use	58 (87)	40 (74)	Ref.	
Sexual transmission	1 (2)	11 (20)	**16.0 (2.93-297)**	
Other	8 (12)	3 (6)	0.54 (0.11-2.01)	
IFNL4genotype				
CC	27 (40)	29 (54)	Ref.	
CT	28 (42)	18 (33)	0.60 (0.27-1.31)	
TT	8 (12)	5 (9)	0.58 (0.16-1.96)	
Unknown/missing	4 (6)	2 (4)	0.47 (0.06-2.59)	
Estimated duration of infection				
Weeks, mean [SD]*	41 [46]	17 [10]	**0.94 (0.91-0.97)**	**0.93 (0.89-0.97)**
≤ 8 weeks	1 (2)	9 (17)		
9-13 weeks	10 (15)	16 (30)		
14-26 weeks	28 (42)	20 (37)		
27-52 weeks	15 (22)	9 (17)		
>52 weeks	13 (19)	0 (0)		

*Acute clinical presentation**				
Acute (ALT >400 IU/L)	7 (10)	12 (22)	**2.84 (1.01-8.49)**	1.15 (0.28-4.74)
Acute symptomatic	17 (25)	16 (30)	1.56 (0.67-3.62)	1.32 (0.47-3.77)
Asymptomatic	43 (64)	26 (48)	Ref.	Ref.
*HCV genotype **				
1	33 (49)	13 (24)	**0.33 (0.14-0.74)**	**0.14 (0.05-0.39)**
3	24 (36)	29 (54)	Ref.	Ref.
Other (2/4/5/6/mixed)	7 (11)	6 (11)	0.71 (0.20-2.41)	0.23 (0.08-1.32)
Unknown	3 (5)	6 (11)	1.66 (0.39-8.50)	1.09 (0.18-8.87)
HCV RNA (log$_{10}$ IU/mL) †				
Median [IQR]	4.8 [3.5-5.4]	4.8 [3.9-5.9]	1.08 (0.95-1.24)	
<4 log	25 (37)	15 (28)		
>4 log	42 (63)	39 (72)		
HIV infected, *n* (%) *	8 (12)	17 (32)	**3.39 (1.37-9.04)**	2.28 (0.70-7.88)

Note: IQR = interquartile range. * Variable included in multivariate regression: age, estimated duration of infection (as continuous variable), acute clinical presentation, HCV genotype, HIV infected. †At time of baseline sample.**Category 'Other' for multivariate logistic regression is aggregate of G2, G4, G5, G6, and Mixed genotypes.

Table 3. Sensitivity and specificity calculated using logistic regression (with parameters estimated via generalised estimating equations (GEE)), according to selected cut-off avidity index (AI) values and genotype (G1, G3 and All), for the longitudinal dataset at time of follow-up sample. Sensitivity is based on a definition of recent infection duration as either (a) <26 weeks or (b) <8 weeks; specificity is with respect to estimated duration of infection (c) ≥52 weeks.

	Sensitivity/specificity (95% CI)		
AI cut-off (%)	**G1**	**G3***	**All**
*(a) Sensitivity for those with estimated duration of infection : <26 weeks (involving 120 samples for all genotypes; **48** samples for G1 and 48 samples for G3)*			
10	17 (8-26)	53 (40-65)	34 (26-42)
20	36 (20-52)	65 (54-75)	48 (39-56)
30	43 (29-57)	75 (65-83)	53 (45-61)
40	51 (35-65)	88 (80-93)	61 (53-68)
(b) Sensitivity for those with estimated duration of infection: <8 weeks (involving 10 samples for all genotypes; 4 samples for G1 and 4 samples for G3)			
10	77 (53-89)	100 (-)	90 (75-97)
20	77 (53-91)	100 (-)	91 (75-97)
30	77 (52-92)	100 (-)	91 (75-97)
40	78 (52-91)	100 (-)	91 (75-97)
*(c) Specificity for those with estimated duration of infection: ≥52 weeks (involving 45 samples for all genotypes; **18** samples for G1 and **21** samples for G3)*			
10	96 (91-99)	100 (-)	96 (91-98)
20	95 (86-99)	100 (-)	96 (90-98)
30	89 (77-95)	95 (-)	91 (85-95)
40	88 (76-95)	75 (-)	83 (75-98)

* Due to GEE model fitting issues related to small sample sizes, neither specificity (with 95% CIs) nor sensitivity with estimated *duration of infection <8 weeks* could be estimated for genotype 3. The values shown are computed from simple 2 x 2 tables.

Table 4. Mean duration of recent infection (MDRI) according to AI cut-off and genotype in the longitudinal dataset. MDRI is computed based on *T*=365 days, using the binomial regression method with bootstrapped 95% confidence intervals (CI)[17].

	MDRI, in days (95% CI)		
AI cut-off (%)	**G1**	**G3**	**All genotypes**
10	58 (27-72)	123 (95-151)	85 (68-102)
20	83 (58-105)	152 (123-186)	116 (98-135)
30	90 (64-118)	200 (166-238)	143 (122-166)
40	108 (80-144)	247 (208-286)	171 (148-198)

FIGURE LEGENDS

Figure 1. Flowchart describing the definition of the dataset for avidity analysis.

Figure 2. Boxplots of the distribution of AI values in the baseline samples, according to estimated duration of infection. AI distributions for the samples with HCV RNA <250 IU/ml (including RNA-negatives) are shown in the left panel, with the same relationship shown for HCV RNA ≥250 IU/ml samples in the right panel. Sample sizes according to duration of infection category are indicated below axis labels.

Figure 3. Relationship between avidity index (AI) and estimated duration of infection, for baseline study data. Classification of samples according to genotype (G1 and G3 only) is distinguished by colour, and linear regression lines are shown (note the logarithmic x-axis).