INTRODUCTION

Infection with hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide, and it has been estimated that approximately 70 million people were chronically infected in 2016.1 In recent years, direct-acting antiviral (DAA) drugs for chronic HCV infection have been welcomed as an effective intervention in reducing HCV-related liver disease burden.2-4 However, a major barrier to HCV treatment and elimination 5 is low rates of testing and diagnosis, with large variations across different regions, countries and at-risk populations. Therefore, improved HCV screening is required to identify people with HCV, to allow improved linkage to care and treatment,6 with

Abstract

Following positive serology, the gold standard confirmatory test of hepatitis C virus (HCV) infection is detection of HCV RNA by PCR. We assessed the utility of HCV core antigen testing to identify active infection among those positive for anti-HCV antibodies, when introduced to routine testing. We identified serum samples that were tested at a single laboratory in Scotland from June 2011 to December 2017. Serum samples testing positive for HCV antibodies (HCV Ab positive) followed by reflex HCV core antigen (Ag) testing during the study period were identified. Those patients for whom a PCR test was requested on the baseline sample were also identified. For this group, the sensitivity and specificity of HCV Ag as a diagnostic tool were assessed using HCV PCR as gold standard. In our cohort of 744 patients, we demonstrated a sensitivity of 82.1% (95% CI 77.1%-86.2%) and a specificity of 99.8% (95% CI 98.6%-100%). Genotype 3 was associated with increased odds of a false-negative result (OR = 3.59, 95% CI: 1.32-9.71), and reduced odds of a false negative were associated with older age (odds ratio (OR)=0.92, 95% CI: 0.88-0.97 per year) and viral load (OR = 0.10, 95% CI: 0.05-0.21 per log10 IU/ml). While the implementation of HCV core antigen testing for diagnosis could lead to significant cost savings in national screening programmes, our data suggest that a significant proportion of HCV-infected individuals may be missed. These findings have implications for HCV diagnosis and determination of viral clearance after treatment, particularly in low- and middle-income regions, where genotype 3 is prevalent.

KEYWORDS
core antigen test, genotype, hepatitis C virus, Scotland, sensitivity, specificity

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a view to meeting the World Health Organisation (WHO) goals of eliminating HCV as a public health concern by 2030.7

The gold standard algorithm for diagnosis of HCV infection requires serologic testing for antibodies, and in those who are tested as being antibody-positive, further confirmation of viral replication by HCV RNA testing.8 HCV core antigen testing, as an alternative to RNA testing, has the potential to reduce diagnostic costs, if it is both sensitive and specific enough without the need for supplementary PCR testing.9 For cost reasons, HCV core antigen testing has been recommended for use in low- to middle-income countries (LMICs).6 However, there are limited data on the performance of core antigen testing when introduced as routine testing and how this concomitantly links patients to appropriate clinical management.

The sensitivity of HCV core antigen detection has been demonstrated to be lower than that of HCV RNA detection by PCR-based methods. Most studies have reported a lower limit of detection for HCV core antigen at an equivalent of 3000-10 000 IU/ml of HCV RNA compared with 12-15 IU/ml HCV RNA for detection by PCR.10-12 Scotland is well placed to evaluate real-world diagnostic performance as has well-established surveillance of HCV covering testing, diagnosis and treatment.13,14 In addition, HCV diagnostic testing is provided in a small number of National Health Services (NHS) laboratories only, with no commercial laboratories offering HCV testing within Scotland. The West of Scotland Specialist Virology Centre (WSSVC) provides HCV RNA testing to NHS Greater Glasgow and Clyde Health Board (NHS GG&C), where 25% and 35% of Scotland’s general and HCV diagnosed populations resides, respectively.15

The WSSVC introduced reflex antigen testing as the standard diagnostic test to detect ongoing infection among HCV antibody-positive patients in NHS GG&C in 2011. Antigen-positive patients were diagnosed with active infection, and a report issued recommending referral to treatment services (where a PCR test would be performed as part of pre-treatment assessment).16 For antigen-negative patients, reports indicated the absence of ongoing infection but recommended a further sample was requested for PCR testing to confirm. Alternatively, a PCR test could be requested on the same sample by contacting the laboratory. We aimed to assess the utility of HCV core antigen testing when introduced into standard practice by comparing the accuracy of antigen compared with PCR when both tests were performed on the same sample. Our principal objective was therefore to estimate the sensitivity and specificity of HCV Ag using HCV RNA status from PCR testing as the gold standard.

2 | MATERIALS AND METHODS

2.1 | Data sources

Record linkage was performed for the Scottish Hepatitis C Test Database (a sentinel surveillance database of all hepatitis C diagnostic tests performed in four NHS Boards Scotland) and HCV Clinical Databases (a comprehensive record of all HCV patients attending specialist care, covering 17/18 specialist liver clinics across Scotland.17,18). Permission to link databases was obtained from the Public Benefit and Privacy Panel, National Services Scotland, and resulting linkage was anonymized before analysis.

2.2 | Inclusion/exclusion criteria

We identified serum samples that were tested at WSSVC from June 2011, when HCV core antigen (HCV Ag) testing was implemented, to December 2017. Serum samples testing positive for HCV antibodies (HCV Ab positive) followed by reflex HCV Ag testing during the study period were identified. From this group, samples that also underwent on-request PCR testing were selected.

For samples sent from some locations (Renal Dialysis Units, Sexual Health Clinics) and in some clinical circumstances (as part of an acute hepatitis order set), HCV Ag was performed as the first line test, in place of HCV antibody. These samples were excluded due to the heterogeneous nature of these settings. In addition, a large volume of HCV testing (particularly in prisons and community addictions settings) was carried out using dried blood spot testing.12 HCV Ab-positive samples on dried blood spot testing continued to have PCR testing performed during the study period and were therefore not eligible for inclusion.

2.3 | Virological testing

HCV Ab testing was performed using the Abbott Architect Anti-HCV assay, and the HCV Ag was tested using the Abbott Architect HCV Antigen assay. Samples with HCV Ag < 3 fmol/L were reported as negative, and on-request PCR testing was performed using the Abbott real-time HCV PCR assay. Samples with intermediate HCV Ag values (3-50 fmol/L) were reported as equivocal and routinely underwent PCR testing. Samples > 50 fmol/L were reported as having ongoing HCV infection.

For all statistical analyses except the multifactorial logistic regression (below), in the case of multiple eligible samples for an individual only the earliest test result was retained.

2.4 | Statistical analysis

Comparison of database linkage proportion and characteristics between the PCR-tested cohort and the samples for which PCR was not requested (only the earliest tested sample was retained in the case of multiple samples for a patient) was carried out using Student's t and chi-squared tests, as appropriate.

The odds of a false-negative HCV Ag result were modelled using multifactorial logistic regression using generalized estimating equations (GEE; appropriate for nonindependent data), with sex, age (in years), viral load (in log_{10} IU/ml units; approximately centred by subtracting 4.0) and genotype category (GT3, other) entered as covariates. The presence of an interaction between
genotype and viral load was hypothesized, and tested by fitting an appropriate interaction term. Regression models were fitted using R statistical software.\textsuperscript{19} Multiple imputation using chained equations\textsuperscript{20} was carried out using the R package mice\textsuperscript{21} to handle missing/unknown values for sex (n = 1), viral load (n = 57) and genotype (n = 85).

### 3 RESULTS

#### 3.1 Patient cohort description

4693 HCV Ab-positive samples underwent reflex HCV Ag testing during the study period. Of these, 877 (18.7\%) had on-request
PCR testing performed, of which 58 samples were excluded due to insufficient material (leaving 819). Thirty-four samples with equivocal (ie not definitively positive or negative) HCV Ag results were excluded from analysis; all of these were subsequently determined to be HCV RNA-positive. Ten samples were classified as HCV RNA-positive yet had a viral load < 12 IU/ml; these were excluded. The remaining 775 samples related to 744 unique patients. Of these, 290 (39.0%) were HCV RNA-positive and 454 (61.0%) were HCV RNA-negative.

Comparison of these data with the samples that did not undergo PCR testing (n = 3816 samples relating to 3017 unique individuals) indicated a difference in the proportion linking to the HCV Clinical Database (57.9% vs. 73.8%) and in proportion HCV Ag positive (32.1% vs. 62.1%; χ² = 217.4, P < .001), but not in age distribution (mean of 43.9 vs. 44.4 years; t = −1.05, P = .29). Among the linked samples only, the proportion genotype 3 (excluding samples with unknown genotype) was higher in the on-request PCR samples (57.7% vs. 46.8%; χ² = 10.5, P < .001), but the proportion with cirrhosis was comparable (23.0% vs. 24.7%; χ² = 0.52, P = .47), and the proportion with PWID risk (excluding unknown risk) was also similar (85.3% vs. 87.3%; χ² = 0.66, P = .42).

Baseline demographic and clinical factors relating to the cohort are shown in Table 1. For those with known route of HCV transmission, injecting drug use was the most frequent risk factor, and consequently, the vast majority of the cohort with known risk factor (233/273, 85.3%) consisted of people who inject drugs (PWID) (ie currently inject or had ever injected drugs). The majority of PWID were on opiate substitution therapy such as methadone (161/233, 69%). Among those with known genotype, the majority had either GT3 (58%; 159/276) or GT1 (38%; 106/276) infection. Hepatitis B and HIV co-infection were observed in 0.5% and 5.0% of patients, respectively.

### 3.2 Sensitivity and specificity of HCV core antigen

In this cohort of 744 patients, 238 out of 290 HCV RNA-positive samples were HCV Ag positive (Table 1; sensitivity was therefore estimated at 82.1%; 95% CI: 77.1-86.2%) while 453 of 454 HCV RNA-negative samples were HCV Ag negative (specificity of 99.8%; 95% CI: 98.6-100%). Sensitivity and specificity for GT3 samples only were 79% (95% CI: 70-86%) and 100% (95% CI: 91-100%), respectively. Sensitivity and specificity for GT1/2/4 samples only were 87% (95% CI: 77-93%) and 100% (95% CI: 91-100%), respectively. The transmission risk factor distribution among HCV RNA-positives (with known risk factor) who were falsely negative (ie HCV Ag negative) or true positive was not significantly different (χ² = 0.29, P = .86).

### 3.3 Multivariate regression analysis

Table 2 shows the results of the GEE logistic regression analysis. As the regression model containing a genotype category by viral load interaction term did not indicate evidence for effect modification, we report the results of the model without this interaction term. GT3 was associated with increased odds of a false-negative result (OR = 3.59, 95% CI: 1.32-9.71), and reduced odds of a false-negative result were associated with older age (odds ratio = 0.92, 95% CI: 0.88-0.97 per year) and with viral load (OR = 0.10, 95% CI: 0.05-0.21 per log₁₀ IU/ml).

In Table 3, the percentage of false negatives observed per cross-tabulated genotype category (GT1/2/4 vs. GT3) and viral load (categorized as < 3000 vs. ≥3000 IU/ml; this threshold was selected based on the meta-analysis of Freiman et al (2016)) is shown for complete case data. Figure 1 shows the individual data points stratified by genotype category. All of the false negatives in the higher viral load category were observed for GT3 (11/68 (16%) of RNA-positive GT3 samples with measured viral load higher than 3000 IU/ml tested negative for HCV Ag). The median log₁₀ viral load among the false negatives for all genotypes combined (among samples with ≥ 3000 IU/ml viral load) was 3.93 (range 3.48-5.19), significantly lower than the log₁₀ viral load among the true positives (median of 4.54; range 3.50-6.48) (Mann-Whitney test, P < .01).

### Table 2 Results of multifactorial logistic regression analysis using GEE on all HCV RNA-positive samples, with dependent variable false-negative rate (total of 302 samples)

<table>
<thead>
<tr>
<th>Covariate</th>
<th>n</th>
<th>N</th>
<th>Univariate Odds Ratio</th>
<th>95% CI</th>
<th>Adjusted Odds ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex Male</td>
<td>40</td>
<td>207</td>
<td>1.01</td>
<td>0.54-1.90</td>
<td>1.15</td>
<td>0.43-3.07</td>
</tr>
<tr>
<td>Female</td>
<td>18</td>
<td>94</td>
<td>Ref.</td>
<td></td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>Age (per year)</td>
<td>-</td>
<td>-</td>
<td>0.96</td>
<td>0.93-0.99</td>
<td>0.92</td>
<td>0.88-0.97</td>
</tr>
<tr>
<td>Genotype GT3</td>
<td>25</td>
<td>110</td>
<td>1.93</td>
<td>0.91-4.09</td>
<td>3.59</td>
<td>1.32-9.71</td>
</tr>
<tr>
<td>Other</td>
<td>11</td>
<td>85</td>
<td>Ref.</td>
<td></td>
<td>Ref.</td>
<td></td>
</tr>
<tr>
<td>Viral load (log₁₀ IU/ml)</td>
<td>-</td>
<td>-</td>
<td>0.14</td>
<td>0.08-0.24</td>
<td>0.10</td>
<td>0.05-0.21</td>
</tr>
</tbody>
</table>

Note: N indicates number of HCV RNA-positive samples per covariate category; n indicates number of false negatives (ie number HCV Ag-negative samples among N). 1 sample was missing Sex (n = 0 false negative); 1 sample was missing age (n = 0 false negative); 107 samples were missing genotype (of which 22 were false negative); and 66 samples were missing viral load (of which 1 was false negative).
DISCUSSION

The utility of HCV Ag in place of PCR within LMICs is financially attractive, with testing estimated at US$8-22.22 The 2016 EASL guidelines and the WHO Global Hepatitis Report 2017 included HCV Ag testing as an alternative to HCV RNA testing for confirmation of HCV diagnosis.6,16 Furthermore, the WHO has provisionally accepted the ARCHITECT HCV Ag assay as a pre-qualified in vitro diagnostic modality for the detection of newly acquired HCV infection.23 Despite these recommendations, WHO recognizes several research gaps relating to HCV Ag. Of relevance to our study, they note that it is unclear how many patients are missed by assays insensitive at low-level viremia, and highlight the need for more information on patients with high viral loads and negative HCV Ag to inform optimization of antigen detection.

Previous groups examining the utility of HCV Ag have retrospectively tested selected samples previously tested by other methods8,10,24 or have described small prospective studies.9 Numbers of RNA-positive samples included have been small8-10 or contained few patients with GT3 infection.24 It is therefore important to confirm the utility of HCV Ag when introduced as routine, in particular in locations where non-GT1 infection is common. GT3 infection accounts for around half of HCV infections in Scotland.25 To our knowledge, ours is the first data addressing the utility of HCV Ag in a high volume laboratory, as a routine (nonresearch) test.

While the HCV core Ag test had a specificity of 99.8% in our clinical cohort of 744 cases within Scotland, the sensitivity of 82.1% was unacceptably low. In addition to the expected finding of reduced sensitivity with a low level of viraemia, we also found that the odds of a false negative being detected were associated with genotype. HCV core Ag was falsely negative in one of six patients with GT3 infection and a viral load > 3000 IU/ml, compared with no observed false negatives among GT1/2/4 patients with similar viral loads.

The WHO guidelines on hepatitis C testing26 reference a systematic review of 20 studies assessing the sensitivity and specificity of the ARCHITECT HCV antigen assay. Sixteen of the studies included HCV Ab-positive individuals only, with a sensitivity of 92.5% (95% CI 86.9-95.8) and specificity 97.8% (95% CI 94.7-99.1). However, most of these studies did not appraise the circulating genotypes or were comparatively small in sample size. In our study, approximately 60% of those known genotypes were infected with HCV genotype 3. Previously, Nguyen et al27 described a significant association between genetic mutations (A48T and T49A/P) in HCV genotype 3–infected patients and failure to detect HCV Ag in these individuals. These mutations have previously been observed in other studies evaluating the correlation of HCV RNA and Ag diagnostic tests, also confirming under-quantification of HCV-infected patients using HCV Ag assays.28 Finally, Garbuglia and colleagues29 also assessed the correlation between PCR and antigen-based technologies in 355 HIV/HCV co-infected patients and showed a greater discordance in detecting a true positive in HCV genotype 3–infected patients and failure to detect HCV Ag in these individuals. These mutations have previously been observed in other studies evaluating the correlation of HCV RNA and Ag diagnostic tests, also confirming under-quantification of HCV-infected patients using HCV Ag assays.28 Finally, Garbuglia and colleagues29 also assessed the correlation between PCR and antigen-based technologies in 355 HIV/HCV co-infected patients and showed a greater discordance in detecting a true positive in HCV genotype 3–infected patients. A combination of low-level viraemia and a high proportion of genotype 3 infection would appear to explain the poorer performance of HCV Ag in our cohort. It would seem that those patients infected with genotype 3 tend to have lower viral load than other circulating genotypes.30

In a more recent study which evaluated HCV core antigen prospectively in drug treatment centres, the sensitivity of HCV core

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Viral load &lt; 3000 IU/ml</th>
<th>Viral load ≥ 3000 IU/ml</th>
<th>All</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FN (%) (n/N)</td>
<td>FN (%) (n/N)</td>
<td>FN (%) (n/N)</td>
</tr>
<tr>
<td>Genotype 1/2/4</td>
<td>58% (11/19)</td>
<td>0% (0/40)</td>
<td>11/59</td>
</tr>
<tr>
<td>Genotype 3</td>
<td>61% (14/23)</td>
<td>16% (11/68)</td>
<td>25/91</td>
</tr>
<tr>
<td>All known genotypes</td>
<td>60% (25/42)</td>
<td>10% (11/108)</td>
<td>36/150</td>
</tr>
</tbody>
</table>

Note: N indicates number of HCV RNA-positive samples; n indicates number of false negatives (ie number HCV Ag-negative samples among N). Total number of samples is 150 due to exclusion of samples with missing data on genotype and viral load.
Ag for detection of active infection in plasma was 98.1% (95% CI: 90-100) and 100% (95% CI: 93-100) when compared to HCV RNA thresholds of ≥ 12 and ≥ 1000 IU/mL, respectively. However, the relatively small sample size with low active viraemia detected, in addition to lack of genotyping, was noted as limitations.

This study has several limitations and strengths. This was a single-centre retrospective study, where a significant proportion of cases had missing clinical data in relation to genotype and transmission route. The retrospective nature of the study precluded sequencing of false-negative samples to identify mutations associated with failure to detect core Ag. However, we included samples from a large clinical cohort, which included patients infected with all genotypes. Although we used a single commercial HCV core antigen assay, other studies have shown similar diagnostic outcomes with other HCV core antigen assays.11,31

As it was at the requester’s discretion to request a PCR on HCV Ab positive samples, it is impossible to exclude that selection bias affects our findings. Almost one in five (18.7%) samples had PCR testing requested, suggesting that if present, the characteristics selected for are common. To investigate such an effect, we compared characteristics of those undergoing on-request PCR testing and those who did not. As a positive Ag test was reported as indicating active infection, and referral indicated, it was unsurprising that those without on-request PCR testing were more often HCV Ag positive and more often linked to the HCV Clinical Database. There was a small, but statistically significant (46.8% vs. 57.7%, P < .001) difference in genotype 3 prevalence. Given our other findings, this may have made some contribution to the lower than expected sensitivity observed. However other patient characteristics were similar, with no demonstrable difference in age, proportion with PWID as risk factor for infection, or in rates of cirrhosis.

While the implementation of HCV core antigen testing for diagnosis has the potential to lead to significant economic savings,32 it is beyond the scope of our study to make such an evaluation. However, our data do suggest that approximately 20% of HCV-infected individuals could be missed. This is a significant proportion and these findings have implications for HCV screening and diagnosis, particularly in LMICs where HCV genotype 3 is the most prevalent HCV genotype. HCV Ag testing has the potential to be cost-effective, but must be used with care, especially in settings with high GT3 prevalence. We recommend that HCV PCR is performed on all HCV Ab-positive and Ag-negative samples, as per the UK Standards for Microbiological Investigation of hepatitis C.16 Further evaluation of HCV Ag should be undertaken in different regions, particularly those with prevalent genotype 3, to better understand its utility. Further investigation of core mutations associated with false-negative results is required, and understanding of the geographic prevalence of these may aid decision-making on the appropriateness of introducing this test in different settings.

REFERENCES


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jvh.13337