Research Article

The utility of enzyme-linked immunosorbent assays to test core antigen in the diagnosis and antiviral therapy management of hepatitis C virus infections[†]

Running title: The utility of ELISAs to test HCV Ag

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ABSTRACT

In this study, we evaluate the performance of the enzyme-linked immunosorbent assays (ELISAs) for HCV Ag detection in the diagnosis and antiviral therapy management of HCV infections. For the diagnosis **of** an active HCV infection, the limit of detection of HCV Ag corresponding to HCV RNA level was approximately 7300 IU/ml; the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of HCV Ag were 88.96%, **100%, 100%, and 91.33%, respectively**. The Pearson's correlation coefficient between HCV-Ag and HCV RNA was 0.891. All patients with negative HCV Ag at interferon-α2α/ribavirin therapy week 1 achieved **a** sustained viral response (SVR), **and** the PPV was 100%; whereas in patients with positive HCV Ag at therapy weeks 12, the NPV for achieving non-response (NR) **was** 100%. The results showed that ELISAs for HCV Ag detection could be cost effectively applied to diagnose and evaluate the response **to** antiviral therapy for HCV **infections**.

KEY WORDS hepatitis C virus; core antigen ; Enzyme-linked immunosorbent assays

INTRODUCTION

HCV **infections are** a serious public health issue, with approximately 160-170 million people infected worldwide (1), **and early** detection and treatment **are** key for preventing its transmission and providing effective treatment. **Currently**, anti-HCV testing **remains** the preferred screening tool for HCV infections (3,4,8, 25), but it is not sensitive **during** the window period of **infections**(8-10) and hemodialysis (8)**;** false positives are often **observed** in pregnancy and **patients with** autoimmune diseases(16), and it also cannot distinguish between active and past **HCV infections** (2, 9, 19). HCV RNA detection is used to confirm and manage antiviral therapy for HCV infections. However, the assay for HCV RNA is expensive and requires highly trained personnel and separate facilities. The limitations of anti-HCV and HCV RNA detection have restricted their applications in clinical practice.

HCV Ag **has been** proposed as an indirect marker of viral replication(6, 11), and ELISAs **have** been introduced to test serum HCV Ag since 1999 (12). In recent years, assays for HCV Ag have been developed, such as enzyme immunoassays (EIAs) (2,13,14) or chemiluminescent immunoassays (CMIA) (2, 3, 9, 11). **Previous** studies showed that the limit of detection of HCV Ag using CMIA is approximately 0.06 pg/ml (or 3 fmol/L) (2, 3, 9, 11), **which** corresponds to **a** viral load range of 428 to 2700 IU/ml (9, 15-21); **in addition**, there is **an** excellent correlation between HCV Ag concentrations and HCV RNA levels (6, 15-19). With high analytical sensitivity and a short turn-around time, **the quantification** of HCV Ag by means of CMIA has been suggested as an alternative to HCV RNA testing in **the** diagnosis and treatment monitoring **of** HCV **infections** (16,18, 20, 21, 23**,30**). **Compared to** ELISAs, CMIA is rather expensive and requires costly equipment**; therefore**, it is rarely used to detect HCV Ag in developing countries. **One** report demonstrated that the limit of detection of HCV Ag can reach up to 1 pg/ml when ELISAs **were** utilized with **a** domestic kit (29), and the ELISAs **were** approved to be used to detect HCV

Ag in clinical practice by **the** China Food and Drug Administration (CFDA) in 2011. The aim of **this** study was to evaluate the performance of ELISAs for HCV Ag detection in **the** diagnosis **of** HCV **infections** and assess **their** value in **the** management **of** antiviral therapy.

MATERIALS AND METHODS

Patients

The study was performed in 333 patients with suspected HCV infections. Among **these patients**, 154 were HCV RNA positive, and 179 were HCV RNA negative. **A total of** 49 patients **were** treated with interferon-α2α 180 μg once a week and ribavirin (≤65 kg, 800 mg daily and >65 kg, 1000 mg daily) for 24 weeks. **The patients provided written informed consent before the treatment, and the protocol was approved by the Ethics Committee of the First Affiliated Hospital of Xi'an JiaoTong University.** The characteristics of the included patients **are** shown in Table 1. HCV Ag and HCV RNA were determined at baseline **and** then at therapy weeks 1, 2, 4, **and** 12, and HCV RNA was also determined 24 weeks after therapy completion. The patients **who were** both HCV RNA and HCV Ag positive were included for the treatment, and patients **who were pregnant, who had** cirrhosis **or** antiviral therapy **in the past** 6 months, **or were** co-infected with hepatitis A, B, **or** E virus were excluded **from** the treatment.

Definition

In **this** study, HCV RNA **levels during the therapy** >2 $\log_{10}$ and <2 $\log_{10}$ decline, **corresponding to baseline**, were defined as partial response **and** no response, respectively; whereas undetectable HCV RNA was defined as **a** complete response, and HCV RNA >2 $\log_{10}$ decline or undetectable HCV RNA was defined as **a** response. **Twenty-four** weeks after the therapy completion, undetectable HCV RNA and

detectable HCV RNA were defined as sustained viral response (SVR) **and** non-response (NR), **respectively**.

Hepatitis C virus core antigen (HCV Ag) detection

The ELISAs for HCV Ag detection **are** based on the double antibody sandwich test (LaiBo Biotechnology, Co. Ltd, JiNan, ShanDong, China). **A total of** 100 μl of each sample and controls were added **to** the wells with 100 μl of dilution, **and** the plate was incubated at 37°C for 90 min. After washing five times, 200 μl of Horseradish Peroxidase (HRP)-labeled antibody **against** HCV **were** added **to** each well, and the plate was incubated at 37℃ for 30 min. Then, **the plates were washed** five times again **and** incubated for 30 min at 37℃ with 200 μl of **the** substrate. **The** reaction was stopped by the addition of 200 μl **of** a 2N sulfuric acid solution in each well. The optical densities (ODs) were read at **450 nm** using **630 nm** reference wavelength. The **cut-off** value was 0.12. **A** positive result was defined as **a** S/CO ≥1 (S was the optical density of **the** sample, and CO was the **cut-off** value), and **a** negative result was a S/CO <1.

Detection of hepatitis C virus antibody (anti-HCV)

The ELISAs for **the** anti-HCV detection **are** based on the indirect test (InTec Products, INC, XiaMen, FuJian, China). **A total of** 10 μl of each sample and controls **was** added **to** the wells with 100 μl of dilution, **and** the plate was incubated at 37°C for 60 min. After washing five times, the plate was incubated at 37℃ for 30 min with 100 μl of HRP-labeled antibody **against** IgG. **Then, the plates were washed** five times again, 100 μl of **the** substrate **were** added **to** each well, and the plate was incubated for 30 min at 37℃. **Then**, 50 μl of a 2N sulfuric acid solution **were** added **to** each well to stop the reaction. The ODs were read at **450 nm** using a **630 nm** reference wavelength. The **cut-off** value was 0.11, **and** S/CO ≥1 and S/CO <1 were defined as **a** positive result **and a** negative result, **respectively**.

HCV RNA detection

The quantification of serum HCV RNA was performed **using** the Abbott RealTi*m*e platform (Abbott Molecular Inc., Des Plaines, IL, USA) that includes the m2000sp for **the** automated extraction and m2000® rt for **the** amplification with real time fluorescent polymerase chain reaction (RT-PCR) detection. The lower limit of detection is **approximately** 12 IU/mL.

Statistical analysis

Statistical analyses were performed using SPSS 13.0 (serial number 5026743; SPSS Inc., Chicago, Illinois, USA). HCV RNA testing was used as the gold standard in the study, **and** the median and range **were used** for **the** descriptive statistics. Mann–Whitney *U*-test and chi-square tests were used for the comparison of the medians and rates, **respectively**. Pearson's correlation coefficient was calculated for the correlation between HCV Ag, anti-HCV and HCV RNA. All p-values are two-tailed, and a p-value < 0.05 was considered statistically significant.

RESULTS

Analytical performance of HCV Ag and anti-HCV

A total of 154 patients **had** active HCV **infections**; the viral loads ranged **from** 2.08 to 8.92 $\log_{10}$ IU/mL (median 5.82), **and** the positive rates of HCV Ag and anti-HCV were 88.96% (137/154) and 98.1% (151/154). In 179 patients with undetectable HCV RNA, the negative rates of HCV Ag and anti-HCV were 100% (179/179) **and** 81.01% (145/179), **respectively**. **Comparing** the analytical **performances of** HCV Ag and anti-HCV testing for **the** diagnosis active HCV **infections**, the specificity and positive predictive value (PPV) of HCV Ag were significantly higher than those of anti-HCV (100% vs 81%, p=0.000; 100% vs 81.6%, p=0.000), but the sensitivity and negative predictive value (NPV) of anti-HCV were significantly

higher than those of HCV Ag (98.1% vs 88.96%, p=0.005; 98% vs 91.3%, p=0.007). The anti-HCV was undetected in 2 patients with positive HCV Ag, and they were confirmed as **being in** the window period of **an** acute infection by RT-PCR (HCV RNA were 11800 and 38600 IU/mL). Anti-HCV was positive in 34/179 **patients**, whereas both HCV RNA and HCV Ag were **undetected**. **However**, the recombinant immunoblot assay (RIBA) was not performed to confirm whether the anti-HCV was true or a false positive in the 34 patients (Table 1).

Analyzing the performance of HCV Ag and anti-HCV according to the viral loads, the positive rate of HCV Ag (30.77%, 8/26) was significantly lower than that of anti-HCV (96.2%, 25/26) in patients with HCV RNA <4 $\log_{10}$ IU/mL, p=0.000. **Particularly**, patients with HCV RNA < 7300 IU/ml (range 121 to 6900 IU/ml), anti-HCV **was** positive in 19/20 **patients**, but the HCV Ag was clearly negative in 18/20 **patients**, and 2 patients were in the grey zone (S/CO values: 0.82, 0.87) with HCV RNA 6600 **and** 6900 IU/ml. In patients with HCV RNA ≥7300 IU/ml, HCV Ag and anti-HCV were detected in 127/128 **patients** and 126/128 **patients**, **respectively**. The results showed that when using ELISAs, the limit of detection of HCV Ag corresponding to **the** viral load was approximately 7300 IU/ml.

Correlation between HCV Ag, anti-HCV and HCV RNA

A correlation analysis in 154 patients with **an** active HCV infection showed that the S/CO values of HCV Ag and anti-HCV had significant correlations with the logarithmic values of HCV-RNA concentrations **($p<0.05$)**. The Pearson's correlation coefficients of HCV Ag and anti-HCV were 0.891 **and** 0.351, **respectively**. **However**, HCV Ag had a better correlation with HCV RNA than that **observed** with anti-HCV (Fig. 1).

Predictive values of HCV Ag and HCV RNA for IFN/RBV treatment

After 24 weeks of IFN/RBV therapy, 28 of 49 patients (57.14%) achieved SVR, and 21 of 49 patients (42.86%) achieved NR. The medians of **the** HCV RNA levels and HCV Ag S/CO **values** at baseline were significantly lower in patients with SVR than those in patients with NR (4.98 vs 5.83 Log_{10} IU/ml and **11.14 vs 15.67 S/CO**, $p < 0.0001$, Fig. 2). The proportion of males **among the** patients with SVR was significantly higher than that in patients with NR (20/28 vs 7/21, p=0.007), but there **were** no significant differences in age between patients with SVR and NR (p=0.679) (Table 1).

At week 1 of therapy, 8 patients (8/8) with negative HCV Ag and 2 patients (2/2) with **a** response of HCV RNA achieved SVR, **and** 21 patients (21/41) with positive HCV Ag and 21 patients (21/47) with no response achieved NR. At week 2 of therapy, SVR was achieved in 18/20 patients with negative HCV Ag and 19/21 patients with **a** response, whereas 19/29 patients with positive HCV Ag and 19/28 patients with no response achieved NR. At week 4, SVR was **achieved** in 27/33 patients with negative HCV Ag and 26/30 patients with **a** response, and 15/16 patients with positive HCV Ag and 17/19 patients with no response achieved NR. At week 12, **neither** positive HCV Ag **nor** no response were observed in patients **who achieved** SVR, **and** 28/36 patients with negative HCV Ag and 28/38 patients with response achieved SVR. From week 2 to week 12 of the therapy, all patients with **a** complete response achieved SVR, but, for patients with a partial response, at weeks 1, 2, 4, **and** 12 of the therapy, 2/2, 14/16, 12/16 and 4/14 patients achieved SVR, respectively (Table 2).

Therefore, within 12 weeks of treatment, the PPV of achieving SVR in patients with **a** complete response was 100%, whereas in patients with **a** partial response, the PPVs at weeks 1, 2, 4, and 12 of therapy were 100%, 87.5%, 75%, **and** 28.6%, **respectively**. In patients with negative HCV Ag at weeks 1,

2, 4, and 12 of therapy, the PPVs of achieving SVR were 100%, 90%,81.8% and 77.8%, **respectively**. The NPVs of achieving NR at weeks 1, 2, 4, and 12 of therapy were 51.2%, 65.5%, 93.8%, 100% for positive HCV Ag and 45.7%, 67.9%, 89.5%, 100% for HCV RNA no response, **respectively**.

DISCUSSION

ELISAs **were** utilized to detect HCV Ag for **the** diagnosis **of** active HCV **infections** in **this** study. The limit of detection corresponding to HCV RNA was approximately 7300 IU/ml, **which was** similar to the result **by** Bouvier (6), **and** the sensitivity (88.96%) was significantly lower than that **previously reported for** anti-HCV (98.1%) and CMIA (15, 18, 20, 22). Among patients with low viral loads (HCV RNA range 121 to 9780 IU/ml), the sensitivity of HCV Ag was only 30.77%, and it was significantly lower than that of anti-HCV (96.2%). During the window period of infection, anti-HCV **levels** were undetected in 2 patients with positive HCV Ag, **which** confirmed that anti-HCV detection is not sensitive in the window period of HCV infection (8-10). HCV Ag levels had a strong correlation with HCV RNA concentrations in patients with **an** active HCV infection, **which** was **consistent** with the previous reports (11,15-19), **and** Pearson's correlation coefficient was 0.891. The results of HCV Ag in 2 patients were **in the** grey zone (S/CO value: 0.82, 0.87) with HCV RNA level of 6600 **and** 6900 IU/ml. We suggest that if **a** grey zone is observed for HCV Ag testing, **HCV RNA detection should be performed**. Anti-HCV was positive in 34/179 patients with **undetectable levels of** both HCV RNA and HCV Ag, although the RIBA was not performed to confirm whether the anti-HCV was **a** true positive or **a** false positive; however, this may be the consequence **of** seroconversion.

IFN/RBV is the standard therapy for HCV **infections** (1, 27), **but** cost-efficiency and side effects should be considered for the therapy. The treatment regimens for HCV **infections are** usually based on the

baseline characteristics, such as genotype (1, 11, 26-28), HCV RNA level (11, 15, 26) or liver disease (26, 27). **Whether there is a rapid** decline or **inability to detect** HCV RNA and HCV Ag during **the** therapy (1, 11,15, 26-28) were also used to manage the IFN/RBV therapy. In our study, the median levels of HCV Ag and HCV RNA at baseline were significantly lower in patients with SVR than those with NR, **which** was **consistent** with previous reports (11, 26). As predictors of SVR and NR in our study, all patients who **showed a** complete response within 12 weeks of therapy or **a** partial response at therapy week 1 achieved SVR, **and** the PPV was 100%. In patients with negative HCV Ag at therapy weeks 1, 2, 4, **and** 12, the PPVs were 100%, 90%, 81.8% and 77.8%, **respectively**. At weeks 4 **and** 12 of therapy, there were high NPVs of achieving NR in patients with either positive HCV Ag or no response in the study, **and** the NPVs were 94.11% for HCV Ag and 95% for HCV RNA at week 4; for **both** HCV Ag and HCV RNA at week 12, the NPVs were 100%. **Therefore, a** complete response **was achieved** within 12 weeks of the therapy; a partial response or negative HCV Ag at therapy week 1 were reliable predictors for **the** shortening treatment, whereas no response, **a** partial response or positive HCV Ag at therapy weeks 12 were predictors for **the** stopping rule or extended treatment.

Overall, ELISAs for HCV Ag could be cost effectively applied to diagnose and manage the antiviral therapy **of** HCV **infections**. When screening HCV infections, anti-HCV testing is still the preferred tool, but ELISAs for HCV Ag can be implemented as a supplemental test to avoid missing or **to confirm** positive anti-HCV. We also found in the study that the results of HCV Ag or HCV RNA testing at different therapy time points could be used for treatment response monitoring (complete response within 12 weeks of the therapy; partial response or negative HCV Ag at therapy week 1 for SVR; and no response, partial response or positive HCV Ag at therapy weeks 12 for NR).

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Table 1: Characteristics of patients

Characteristics	Values
HCV-RNA positive patients(n=154)	
Age, years (median)	23~84 (56)
Male/Female (n/n)	78/76
HCV-RNA, Log $_{10}$ IU/mL (median)	2.08~8.92（5.82）
HCV-Ag Positive/Negative (n/n)	137/17
Anti-HCV Positive/Negative (n/n)	151/3
HCV-RNA negative patients(n=179)	
Age, years (median)	9-89（46）
Male/Female (n/n)	100/79
HCV-Ag Positive/Negative (n/n)	0/179
Anti-HCV Positive/Negative (n/n)	34/145
Baseline values in Patients with SVR(n=28)	
Age, years (median)	25~72（59）
Male/Female (n/n)	20/8
HCV-RNA, Log $_{10}$IU/mL (median)	4.29~6.12(4.98)
HCV-Ag S/CO (median)	5.64~18.45(11.14)
Baseline values in Patients with NR(n=21)	
Age, years (median)	23~80（59）
Male/Female (n/n)	7/14
HCV-RNA, Log $_{10}$IU/mL (median)	4.97~8.92(5.83)
HCV-Ag S/CO (median)	9.87~35.12(15.67)

Note: SVR was sustained viral response and NR was non-response.

Table 2: Results of therapy response for hepatitis C virus infection patients

HCV-Ag or RNA level	No.achieved SVR (n=28)				No.achieved NR (n=21)			
	week 1	week 2	week 4	week12	week 1	week 2	week 4	week12
HCV-RNA								
Response (Undetect)	2(0)	19(5)	26(14)	28 (24)	0	2(0)	4(0)	10(0)
<2-Log10 Decline	26	9	2	0	21	19	17	11
HCV-Ag								
Negative	8	18	27	28	0	2	6	8
Positive	20	10	1	0	21	19	15	16

Note: SVR was sustained viral response and NR was non-response.

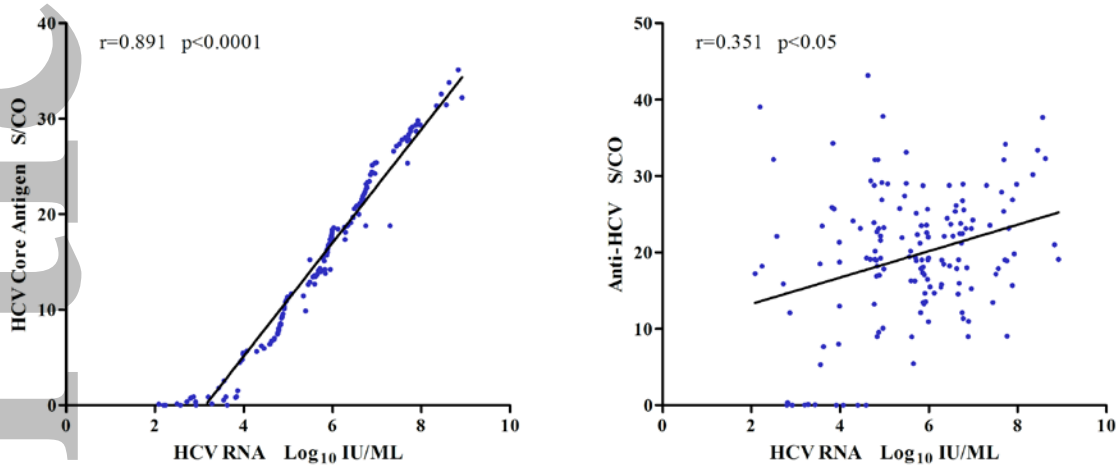

Fig. 1. Correlation analysis in 154 patients with HCV active infection between S/CO values of HCV Ag, anti-HCV and the logarithmic values of HCV-RNA concentrations.

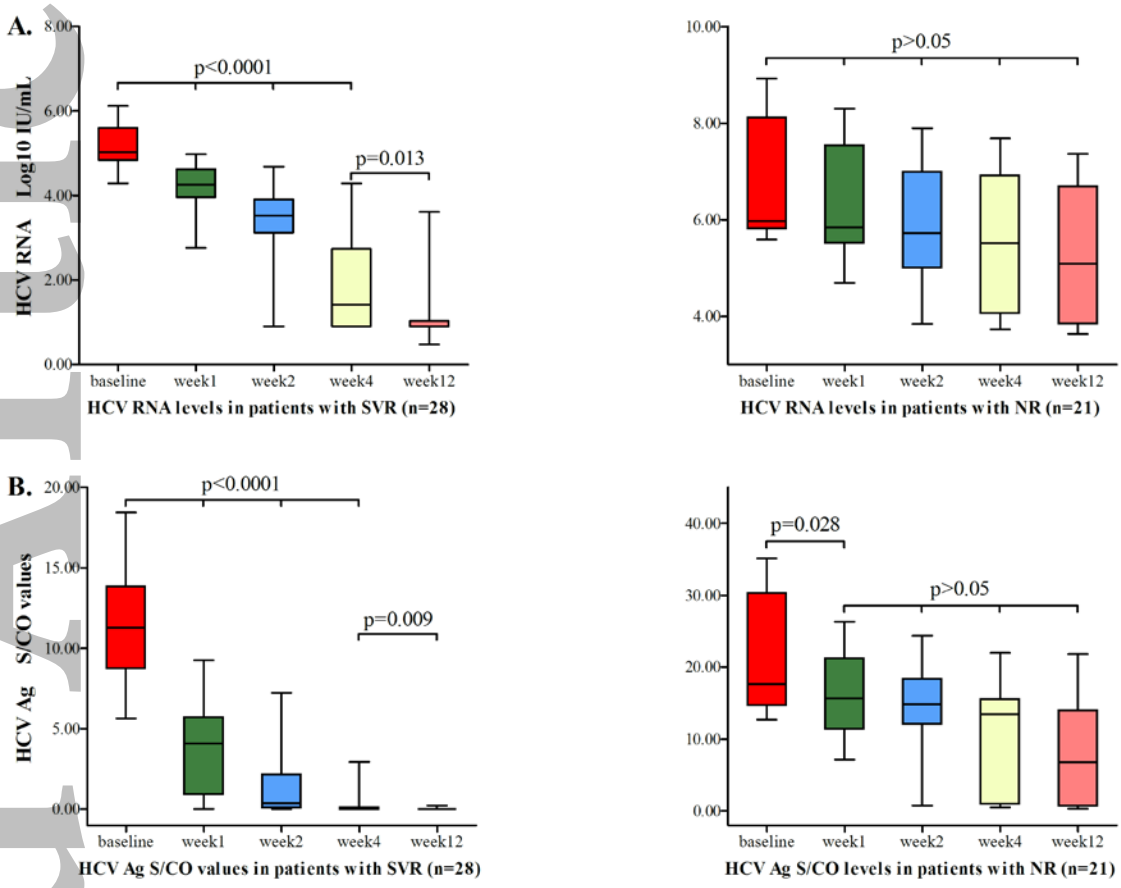

Fig. 2. The comparison of testing results in patients with sustained virological response (SVR)‼ ┼ and non-response (NR) for HCV RNA (Fig. 2.A.) and HCV-Ag (Fig. 2.B.).