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RESEARCH ARTICLE

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# Comparison of double antigen sandwich and indirect enzyme-linked immunosorbent assay for the diagnosis of hepatitis C virus antibodies

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## Abstract

**Background:** The aim of this study is to compare double-antigen sandwich enzyme-linked immunosorbent assay (ELISA) and indirect ELISA in the diagnosis of hepatitis C virus ( HCV ) infection.

**Methods and materials:** A total of 176 samples from the Tumor Hospital Affiliated to Xin Jiang Medical University were utilized to comparison. All serum samples were tested using double-antigen sandwich ELISA and indirect ELISA. Cohen's kappa statistics were used to assess the agreement between the two assays, and multivariate analysis was used to evaluate risk factors for the discordance between the double-antigen ELISA and indirect ELISA.

**Results:** The positivities of indirect ELISA (Beijing Wantai), double-antigen sandwich ELISA (Beijing Wantai), and indirect ELISA (Beijing Jinhao) were 74.43%, 68.75%, and 73.30%, respectively. The agreement between the indirect ELISA (Beijing Wantai) and double-antigen sandwich ELISA (Beijing Wantai) was high ($\kappa = 0.829$;$P < .001$), and the agreement between the double-antigen sandwich ELISA (Beijing Wantai) and indirect ELISA (Beijing Jinhao) was high ($\kappa = 0.847$;$P < .001$). Variables associated with discordant results between the double-antigen sandwich and indirect ELISA in multivariate analysis were as follows: female (OR:1.462; $P < .05$), age (<35 years old; OR:3.667; $P < .05$), and cancer (suffer from malignant tumor; OR:3.621; $P < .05$).

**Conclusion:** In detection of HCV, high agreement was found between the double-antigen sandwich ELISA and indirect ELISA. Female, younger age, and suffer from malignant tumor were significant risk factors for the discordance. Based on double-antigen sandwich ELISA has distinct methodological advantages over indirect ELISA. It is recommended for the diagnosis of HCV infection.

KEYWORDS

double-antigen sandwich ELISA, hepatitis C antibodies, indirect ELISA

## 1 | INTRODUCTION

The virus which is the most important etiologic agent of non-A, non-B hepatitis was officially named HCV, belongs to the family flaviviridae hepatitis virus genus and is a small, enveloped, positive-sense single-stranded RNA virus with a genome length of 9.4-9.6 kb.[1,2] The genome sequence is substantial genetic diversity and can be divided into at least 7 genotypes and 67 subtypes, and there are general susceptibilities regardless of age, gender, and race.[3,4] Related studies have shown that some HCV genotypes have their specific distribution areas, and there were considerable differences in the natural course of disease after people infected with different genotype or subtypes.[5] For example, genotype Ia plays a vital role in North America, genotype Ib is more common in Western Europe and Japan which is closely to invasive liver disease, and genotypes 1b and 2a predominate in China.[6] WHO estimates that HCV infects nearly 3% of the world population as a highly pathogenic virus.[7] People who infected with this virus tend to be mild in symptoms and more progressed to chronic hepatitis, and this character results approximately 400 000 people died in 2016.[8] Vaccination is the most effective method and cost-effective manner in order to control the spread of contagious diseases, but there are still some problems inhibit development of an effective vaccine against HCV at this stage. The application of direct antiviral drugs (DAAs) has brought new hope to triumph over the HCV. The treatment of chronic HCV infection has entered the era of pan-genotype with the intensive research and development of DAAs, but the high cost of DAAs threatens the affordability of patients with HCV infection.[9,10]

Depending on the above, the principal contradiction of HCV by the treatment into screening. The diagnosis and monitoring of HCV infection are based on 2 types of methods: One is detecting HCV antigen-specific antibodies and the other is detecting viral RNA or HCV core antigens. Making a clinical diagnosis was based on historical epidemiology, clinical manifestations, and HCV antibody screening results, and then making a definite diagnosis by viral nucleic acid testing. Thus, the screening of HCV antibody occupies an important position at this stage. Anti-HCV was detected by indirect ELISA was always used as a screening tool.[11] In recent years, double-antigen sandwich ELISA has received wide attention because of its key advantages in methodology. So the aim of this work was to compare three commercial ELISA kits in the diagnosis of HCV infection.

## 2 | MATERIAL AND METHOD

### 2.1 | Subjects

A total of 176 samples from outpatient and inpatient were used for comparison, 80 males and 96 females (mean age of 55.41 years, SD = 13.52 years, and range = 18-86 years old). We are collecting specimens from the Tumor Hospital Affiliated to Xin Jiang Medical University from January 2018 to October 2018. Venous blood samples were collected from studying subjects for serum separation. The same protocol of sample collection was used in both outpatients and inpatients. Study subjects' whole blood, obtained by venipuncture, was collected in 4 mL BD Vacutainer Plus Plastic Serum Tubes. Samples were allowed to clot for 30 min at room temperature, followed by centrifugation at 1728*g* for 10 minutes, and then aliquots of 1 mL serum were transferred to 1.5mL sterile microcentrifuge tubes using a disposable transfer pipet. The serum samples of the patients were withdrawn from the storage refrigerator and mixed thoroughly. All serum samples were tested using double-antigen sandwich ELISA (Beijing Wantai bio Pharmaceutical Co., Ltd), indirect ELISA (Beijing Wantai bio Pharmaceutical Co., Ltd) and indirect ELISA (Beijing Jinhao bio Pharmaceutical Co., Ltd), respectively. Quality control materials, and positive and negative controlled substance were used in parallel. All assays were performed according to the manufacturers' instructions.

### 2.2 | Commercial assays

Double-antigen sandwich ELISA kit (Beijing Wantai bio Pharmaceutical Co., Ltd) is a qualitative test intended for the detection of HCV infection in human serum or plasma samples. This kit using horseradish peroxidase (HRP) tagged biotinylated recombinant antigen and solid-phase multi-epitope gene recombination antigens (HCV core, NS3, NS4, NS5, et al) to specifically bind to anti-HCV with twice. Meanwhile, this commercial assay kit used the biotin-streptavidin binding system to amplify the detection signal and it can be used for detecting both IgM and IgG antibodies. The cutoff value was calculated according to kit instructions (Cutoff value = Positive control mean + 0.12). The anti-HCV results of sample are negative when absorbance values are less than the cutoff value, and there is positive result when absorbance value is greater than or equal to cutoff value.

Indirect ELISA (Beijing Wantai bio Pharmaceutical Co., Ltd) also is qualitative test. Indirect ELISA kit was the most widely used method for early screening and auxiliary tool in the diagnosis of HCV infection. Both kits used HRP-labeled nonspecific anti-human antibody as secondary antibody, meanwhile, recombinant antigens binding to solid support. There are a lot of factors that can influence the absorbance measurement. To see whether the sample was positive or not, we need to calculate cutoff value according to the reagent kit instructions (Cutoff value = Negative control mean + 0.12, Beijing Wantai; Cutoff value = 0.13 × Positive control mean + Negative control mean, Beijing Jinhao). Samples giving an absorbance less than the cutoff were scored as negative, while those giving an absorbance equal to or greater than the cutoff were scored as positive.

In addition, a few parameters like total bilirubin (TBil), direct bilirubin (TBil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), and a-L-Fucosidase (AFU) related to serological indicators of liver function were recorded. And above-mentioned biochemical indexes were tested by Roche modular fully automatic biochemical analyzer.

### 2.3 | Statistical analysis

Statistical analysis was performed using the SPSS statistical software package (SPSS version 18). Qualitative variables are presented as frequency (percentage), and quantitative statistics are presented as mean ± standard deviation (SD). We used the Cohen kappa statistics to evaluate concordance of test results by double-antigen sandwich and indirect ELISA, and multiple factor analysis was assessed by multivariate logistic regression analysis to calculate the odds ratio (OR), and a *P* value of less than 0.05 was taken to indicate a statistically significant difference.

## 3 | RESULTS

### 3.1 | Comparison of consistency between double-antigen sandwich ELISA (Beijing Wantai) and indirect ELISA (Beijing Wantai)

Among the 176 subjects, the positivities of double-antigen sandwich ELISA (Beijing Wantai) and indirect ELISA (Beijing Wantai) were 68.75% and 74.43%, respectively. The agreement between the two approaches was high ($\kappa = 0.829$;$P < .001$) (Table 1).

### 3.2 | Comparison of consistency between double-antigen sandwich ELISA (Beijing Wantai) and indirect ELISA (Beijing Jinhao)

Among the 176 subjects, the positivities of double-antigen sandwich ELISA(Beijing Wantai) and indirect ELISA (Beijing Jinhao) were 68.75% and 73.30%, respectively. The agreement between the two approaches was high ($\kappa = 0.847$; $P < .001$) (Table 2).

### 3.3 | Discordance among the test results

Variables associated with discordant results between the double-antigen sandwich ELISA and indirect ELISA in multivariate analysis were as follows: female (OR:1.462; $P < .05$), age (<35 years old; OR:3.667; $P < .05$), and cancer (suffer from malignant tumor; OR:3.621; $P < .05$) (Table 3).

## 4 | DISCUSSION

There are no symptoms or only mild symptoms such as right upper abdominal discomfort and oil aversion in the incubation period after HCV infection, and it is likely to take 20-40 years to developed into serious clinical complication such as liver fibrosis or even hepatocellular carcinoma.[12,13] The exact time of HCV infection is difficult to determine the symptoms are mild or absent, easily transform into chronic, and higher chronic rate was 55% to 85%. Additionally, the understanding degree of the disease at a lower level, autonomous screening rate is low and public lacks attention with this disease. It caused tremendous burden on both China and the world economy. Along with a deeper understanding of the HCV life cycle, direct antiviral drugs (DAAs) have become widely used since its introduction, which is highly effective, less adverse reactions and a short course of treatment available to fight HCV.[14,15] DAAs are working directly on the non-structural protein (NS) 3/4A, 5A, and 5B to achieve virus clearance, so the deadlock of clinical treatment of viral hepatitis C was broken even promising to get complete cure; thus, WHO sets targets to eliminate hepatitis C as a public health threat by 2030.[16,17] However, related research suggests that DAAs are still at a high price, at the same time, reinfection cannot be avoided, and the cured patients still have the risk of hepatocellular carcinoma.[18-20] Vaccine is the most cost-effective and effective measure to prevent and combat the spread of disease. However, there is not any effective vaccine to prevent HCV infection.[21] The successful development and clinical application of HCV vaccine are unpredictable.[22] In summary, the focal problems for hepatitis C are shifting from the therapeutic strategies to screening program launched in high-risk groups.

To date, early screening and diagnosis of HCV infection are based on three different methods, which may be utilized alone or in combination. These are (a) detection of HCV core antigen (HCV-Ag); (b) nucleic acid testing (NAT) to detect HCV-RNA by PCR; and (c) detection of an IgG antibody by ELISA (anti-HCV).[23] Related literature reports that there is a good correlation between the contents of HCV-Ag and the quantity of HCV-RNA as viral load exceeds 3000 IU/mL in the serum samples of HCV patients, so HCV core antigen can be used as early diagnostic indicators of HCV infection.[24] Meanwhile, detection of HCV-Ag has the advantages of relatively low cost, handiness, and simplicity and not need to add special instruments. However, the structure of the HCV-cAg is very special and the preparation of antibody is more difficult, and detection

**TABLE 1** HCV antibody detection by double-antigen sandwich ELISA (Beijing Wantai) and indirect ELISA (Beijing Wantai)

| Indirect ELISA (Wantai), number(%) S/CO | Double-antigen Sandwich ELISA (Wantai), number(%) S/CO | | | |
|---|---|---|---|---|
| | 0-1 | 1-4 | >4 | Total |
| 0-1 | 44 (25.0) | 1 (0.6) | 0 | 45 (25.6) |
| 1-4 | 11 (6.2) | 22 (12.5) | 3 (1.7) | 36 (20.4) |
| >4 | 0 | 3 (1.7) | 92 (52.3) | 95 (54.0) |
| Total | 55 (31.2) | 26 (14.8) | 95 (54.0) | 176 |

**TABLE 2** HCV antibody detection by double-antigen sandwich

| Indirect ELISA (Jinhao), number(%) S/CO | Double-Antigen Sandwich ELISA (Wantai), number(%) S/CO | | | |
|---|---|---|---|---|
| | 0-1 | 1-4 | >4 | Total |
| 0-1 | 45 (25.6) | 2 (1.1) | 0 | 47 (26.7) |
| 1-4 | 9 (5.1) | 22 (12.5) | 2 (1.1) | 33 (18.7) |
| >4 | 1 (0.6) | 2 (1.1) | 93 (52.9) | 96 (54.6) |
| Total | 55 (31.3) | 26 (14.7) | 95 (54.0) | 176 |

*Note:* ELISA(Beijing Wantai) and indirect ELISA (Beijing Jinhao).

**TABLE 3** Univariate analysis of risk factors associated with discordance between the double-antigen sandwich and indirect ELIS in detection of HCV antibody

| | Discordance group | Concordance group | Total | *P* value | 0R |
|---|---|---|---|---|---|
| Number | 24 | 152 | 176 | | |
| Sex | | | | | |
| Male | 5 (20.8%) | 70 (46.1%) | 75 (42.6%) | .020 | 1.462 |
| Female | 19 (79.2%) | 82 (53.9%) | 101 (57.4%) | | |
| Age | | | | | |
| <35 y old | 15 (62.5%) | 40 (26.3%) | 55 (31.3%) | <.001 | 3.667 |
| >35 y old | 9 (37.5%) | 112 (73.7%) | 121 (68.7%) | | |
| Liver function indicator | | | | | |
| TBil | 16.95 ± 6.00 | 15.3 ± 4.40 | 16.3 ± 5.65 | .895 | |
| DBil | 4.79 ± 2.32 | 6.28 ± 1.70 | 5.89 ± 1.757 | .867 | |
| AST | 27.94 ± 35.51 | 29.94 ± 36.79 | 28.9 ± 35.93 | .823 | |
| ALT | 26.14 ± 33.37 | 29.84 ± 31.91 | 28.1 ± 31.91 | .765 | |
| r-GGT | 45.45 ± 59.51 | 49.85 ± 62.01 | 47.4 ± 60.11 | .630 | |
| AFU | 22.93 ± 10.68 | 26.93 ± 11.67 | 25.9 ± 11.08 | .505 | |
| Underlying diseases | | | | | |
| Malignant tumor | 21 (87.5%) | 95 (62.5%) | 116 (65.9%) | .016 | 3.621 |
| Non-malignant tumor | 3 (12.5%) | 57 (37.5%) | 60 (34.1%) | | |

Abbreviations: AFU, a-L-Fucosidase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBil, direct bilirubin; r- GGT, gamma-glutamyl transpeptidase; TBil, total bilirubin.

sensitivity of HCV-cAg is lower than that of anti-HCV by using ELISA. It can be used as a supplement to anti-HCV detection but cannot replace the determination of antibody. The samples were tested HCV-RNA positive are the reliable index of viral replication activity, and the HCV-RNA methodology can be subdivided into qualitative and quantitative methods.[25,26] Qualitative analysis has played a key role in blood screening of blood banks, it as a means of indicating the presence or absence of viremia but not measure the viral load. In recent years, the real-time PCR (RT-PCR) is gradually replacing qualitative analysis as a common method used in detecting HCV-RNA, as it features high sensitivity and specificity. However, it cannot be used as a routine method because of its complicated operation and higher experimental conditions needed. In particular, most hospitals, especially primary healthcare facilities, do not have the capacity to detect HCV-RNA and provide accurate results.[27]

According to guidelines for the prevention and treatment of hepatitis C (2019 version) revised by the Chinese Medical Association, along with WS 213—2018 Diagnosis for hepatitis C (National Health and Family Planning Commission of the People's Republic of China). Compared with (HCV-Ag) and nucleic acid testing (NAT), the anti-HCV immunoassays are the most frequently used laboratory tests for detection of HCV infection. And now it has the following advantages in the detection of HCV antibodies with the rapid development of genetic engineering technology: high sensitivity and specificity, better stability, automation, low cost, and convenience. Indirect ELISA had already gone through three reformation, the first generation anti-HCV assay using recombinant c100-3 epitope from the NS4 protein, which had limited sensitivity and specificity. Second generation assay was developed using epitopes from the core, NS3, and NS4 proteins, a multi-antigen format that can reduce the time

required and increase the sensitivity. More recently, an highly conserved NS5 antigen have adopted in third generation assays. If the sample contains anti-HCV, incubate for a certain period of time, solid-phase antigen will fully bind to the anti-HCV in the serum, and then react with the added IgG-HRP. Tetramethylbenzidine (TMB) color development or not to indicate present or absent of anti-HCV in human serum or plasma.[28] However, indirect ELISA can always result in false positives due to interfering factors, including high gamma globulin levels, nephritic syndrome, pregnancy, sample hemolysis, the splashing, and pollution of the blood sample.[29]

Double-antigen sandwich ELISA is a developing technology with great potential.[30] At the beginning of development, it faces two major obstacles, the HCV antigen labeled with HRP directly is easy to cause the space steric hindrance and ratio of enzyme-labeled antigens is difficult to control. Subsequently, preparation of growth arm biotinylated HCV antigen as a sandwich antigen using genetic engineering, with horseradish peroxidase mildew affinity tag chain element as enzymes, so anti-HCV could perform twice specific binding in the sample and amplification of the signal was achieved using biotin–streptavidin system. Related research shows that compared to traditional indirect ELISA, double-antigen sandwich ELISA displays higher specificity and better sensitivity, and it can utilize to detect IgM.[31] Double-antigen sandwich ELISA could make up for the methodological deficiencies inherent in indirect ELISA and has clinical utility in both screening and diagnosis of HCV infection.

Objective of our study is to investigate the consistency of three commercially available anti-HCV assays, and analysis of influencing factors to disagreement between the double-antigen sandwich and indirect ELISA. So as to provide pertinent evidence for the selection of methods of detecting anti-HCV in clinical laboratories. In our study, among the 176 subjects, the positivities of double-antigen sandwich ELISA (Beijing Wantai), indirect ELISA (Beijing Wantai), and indirect ELISA (Beijing Jinhao) were 68.75%, 74.43%, and 73.30%, respectively. The agreement between the double-antigen sandwich ELISA (Beijing Wantai) and indirect ELISA (Beijing Wantai) was high ($\kappa = 0.829$; $P < .001$). The agreement between the double-antigen sandwich ELISA (Beijing Wantai) and indirect ELISA (Beijing Jinhao) was high ($\kappa = 0.847$; $P < .001$). But there are still inconsistent results in the analysis of anti-HCV by using double-antigen sandwich and indirect ELISA. It was found that among the fifty-five specimens that were tested negative using by double-antigen sandwich ELISA (Beijing Wantai), eleven were positive using by indirect ELISA (Beijing Wantai) and ten were positive using by indirect ELISA (Beijing Jinhao), and the possible cause of this problem perhaps is that nonspecific immunoglobulin, rheumatoid factor, hydrophilic antibodies, and other interfering substances in the specimen, which can be nonspecifically combined with the enzyme-labeled secondary antibody, lead to false-positive results. Among one hundred and twenty-one samples tested positive using by double-antigen sandwich ELISA (Beijing Wantai), one was negative using by indirect ELISA (Beijing Wantai) and two were negative by using indirect ELISA (Beijing Jinhao). The probable reason is that the patients are in the window period of infection and the body has not yet produced IgG. Variables associated with discordant results between the double-antigen sandwich ELISA and indirect ELISA in multivariate analysis were as follows: female (OR: 1.462; $P < .05$), age (<35 years old; OR:3.667; $P < .05$), and cancer (suffer from malignant tumor; OR:3.621; $P < .05$). Analysis of possible reasons were as follows: (a) More women predisposed to autoimmune diseases and autoantibody positivity was higher than men; (b) positive antinuclear antibodies in malignancies than benign tumor; and (c) pregnant woman in people younger than 35 years old was high and the presence of some interfering substances may be affect the results significantly. These discrepancies emphasize the need for further research, and we will follow up in subsequent studies.

## 5 | CONCLUSION

In detection of HCV infection, high agreement was found between the double-antigen sandwich ELISA and indirect ELISA. Based on double-antigen sandwich ELISA has distinct methodological advantages over indirect ELISA, considering double-antigen sandwich ELISA has high clinical popularization value in the screening of HCV infection. Female, younger age, and malignancy were significant risk factors for the discordance. It is recommended that relevant laboratories should pay more attention to the existence of the above significant risk factors by using indirect ELISA and suggest to detect anti-HCV by using double-antigen sandwich ELISA if condition permits.

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