Double-antigen sandwich time-resolved immunofluorometric assay for the detection of anti-hepatitis C virus total antibodies with improved specificity and sensitivity

Feng-Bo Wu,^{1†} Hai-Qiao Ouyan,¹ Xiao-Yan Tang¹ and Zhen-Xian Zhou^{2†}

Correspondence

Feng-Bo Wu

gaoweiwuli@21cn.com

¹Research & Development Department, SYM-BIO Life-Science Co. Ltd, Tai-Cang City, Jiang-Su Province 215400, PR China

²Clinical Laboratory, Nanjing Second Hospital, Nanjing City 210003, PR China

Current anti-hepatitis C virus (HCV) antibody screening immunoassays are routinely based on an indirect format. Although their use for anti-HCV antibody detection has achieved a very high specificity and sensitivity, false-positive results are still a problem especially among populations with a low prevalence of HCV infection. One strategy to obviate this problem is to adapt the assay from an indirect format to a double-antigen sandwich one to further improve its specificity. In this study, a double-antigen sandwich time-resolved immunofluorometric assay (DAS-TRIFMA) has been developed to detect total anti-HCV antibodies based on biotin–streptavidin interaction. For comparison, 1025 samples were analysed by the DAS-TRIFMA and three indirect anti-HCV antibody detection methods. For samples with discordant results, PCR-ELISA and Inno-LIA were employed as supplementary assays to analyse the presence of HCV antibodies. With regard to the 1025 clinical samples, the overall concordance between the DAS-TRIFMA and the three indirect methods was 99.41, 98.93 and 98.93 % for Ortho ELISA 3.0, WAT ELISA and I-TRIFMA, respectively. The specificity/sensitivity of the DAS-TRIFMA, Ortho HCV ELISA 3.0, WAT HCV ELISA and I-TRIFMA were 100/99.09, 99.34/98.18, 99.23/97.27 and 99.01 %/98.18 %, respectively. The DAS-TRIFMA was able to detect HCV antibodies at a concentration about 1/10 of that detectable by indirect methods. From the obtained results and their comparison, it is concluded that the DAS-TRIFMA is a more specific and reliable method for screening anti-HCV antibodies, and weakly positive S/Co values by the DAS-TRIFMA were more predictive of HCV infection than those by indirect methods.

Received 20 December 2007

Accepted 24 March 2008

INTRODUCTION

The detection of hepatitis C virus (HCV) RNA has become an increasingly useful tool in the diagnosis of HCV infection and in the management of patients during therapy (Richter, 2002; Lok & Gunaratnam, 1997; Chevaliez & Pawlotsky, 2006). Compared to HCV RNA testing, anti-HCV antibody immunoassays are thought to be more practicable as an initial screening test because of the ease of use, relative cost-effectiveness and low variability. Accordingly, up to the present time anti-HCV antibody immunoassays are still the most commonly used tests to determine past or present exposure to HCV. In addition, studies in recent years have revealed that the detection of anti-HCV antibodies still has a role in the diagnosis of HCV infection, since the possibility of active

HCV infection can not be ruled out in patients who test positive for HCV antibodies but negative for HCV RNA in serum due to low-level undetectable viraemia and intermittent viraemia (Carreño *et al.*, 2006; Radkowski *et al.*, 2005; Carreño, 2006).

Anti-HCV antibody immunoassays have now progressed to the third generation. Although these assays have better sensitivity and specificity than their predecessors (Colin *et al.*, 2001; Abdel-Hamid *et al.*, 2002), there is still a high prevalence of false-positive results, especially among immunocompromised patients or populations without liver-related diseases, leading to unnecessary health-care costs and diagnosis puzzles (Ansari & Omrani, 2006; Zylberberg & Pol, 1996; Hyams *et al.*, 2001; CDC, 2000).

Immunoassays for detection of viral-specific antibodies have been developed for various viruses. ELISAs for detection of anti-human immunodeficiency virus or anti-*Treponema pallidum* antibodies have validated that

†These authors contributed equally to this work.

Abbreviations: DAS-TRIFMA, double-antigen sandwich time-resolved immunofluorometric assay; I-TRIFMA, indirect TRIFMA.

employing a double-antigen sandwich (DAS) format instead of the original indirect format can substantially improve the assay's specificity (Bürgisser *et al.*, 1996; Schmidt *et al.*, 2000). However, up to now there has been little investigation into the development and usage of a DAS assay for anti-HCV antibody detection. In this study, we developed a DAS time-resolved immunofluorometric assay (DAS-TRIFMA) for detecting total anti-HCV antibodies by using biotin as indirect label. With the benefits of the sandwich assay format and the biotin-streptavidin interaction, the DAS-TRIFMA showed obviously improved specificity and at least the same good sensitivity compared to that of two widely used commercial indirect ELISAs. This study indicates that most of the false-positive results in the indirect anti-HCV immunoassays are associated with their indirect assay format, and the DAS immunoassay, as exemplified by the present DAS-TRIFMA, is more reliable for screening for the presence of anti-HCV antibodies.

METHODS

Samples. A total of 1025 samples from domestic hospitals and blood centres were used for comparison. Seventeen of the 1025 samples were from asymptomatic blood donors with S/Co values (S/Co, the ratio of the assay signal divided by the cut-off value of the assay) of 0.8–4.7 by indirect anti-HCV ELISAs (Wu-Han Blood Center, Wu-Han City, China). The remaining 1008 samples were from patients in the routine evaluation of the cause of their liver disease.

For hook effect evaluation, a further 56 sera screened as highly anti-HCV antibody reactive by the Abbott AxSYM HCV (version 3.0) were obtained from the First Affiliated Hospital of Guang-Xi Medical University. Eight hundred and seventy negative samples used for cut-off determination were from outpatients and blood centres.

Reagents and instruments. C185 (molecular mass 55 kDa) was a recombinant chimeric antigen condensed with the major HCV epitopes located in the HCV core, NS3, NS4 and NS5 regions (JKE-L Biotechnologies). C188 was a mixture of recombinant peptides composed of HCV core, NS3, NS4 and NS5 fragments (Bite Biological). The low background microtitre plate (8 × 12 wells) was from Nunc. Goat anti-hIgG and monoclonal anti-human IgM (μ -chain specific) antibodies were from Genetimes Technology. Streptavidin, biotin-cap-NHS, BSA, casein and other chemicals were from Sigma-Aldrich. N^1 -Benzyl-DTTA-Eu³⁺ [N^1 -(*p*-isothiocyanato-benzyl)-diethylene-triamine- N^1, N^2, N^3, N^4 -tetraacetate-Eu³⁺], the VICTOR² fluorometer, Plateshake (1296-003) and Platewash (1296-026) were from Perkin-Elmer. The ELISA reader was Multiskan MK3 (Thermo Labsystems). The CP-70MX preparative ultracentrifuge was from Hitachi.

Comparison methods. Two indirect anti-HCV ELISAs used were Ortho HCV ELISA 3.0 (Ortho-Clinical Diagnostic) and WAT HCV ELISA (Beijing, China). Inno-LIA HCV Score (Innogenetics) and quantitative PCR-ELISA (Hao-Yuan Biotechnologies) were used as a supplementary assay to confirm the anti-HCV antibody detection. PCR-ELISA was a colorimetric microtitre plate based assay for detection of HCV RNA, in which RT-PCR was performed in the first step for amplification of HCV RNA and ELISA was used for amplicon identification. Inno-LIA was performed based on the 16 h sample incubation procedure. The above assays were carried out strictly according to the manufacturer's instructions.

In order to obtain direct comparison, an indirect anti-HCV TRIFMA (I-TRIFMA) based on the same solid-phase antigen, C188, as that

used in the DAS-TRIFMA was designed as described in the 'I-TRIFMA of anti-HCV antibodies' section. The schematic diagram of the DAS-TRIFMA and indirect methods for anti-HCV antibody detection is shown in Fig. 1.

Biotinylation and Eu³⁺ labelling. One millilitre of the recombinant C185 was dialysed for 12 h at room temperature (RT) against PBS buffer (0.1 M, pH 7.0) containing 5 mol urea l⁻¹, 2% (w/v) ethylene glycol and 0.01 mol dithiothreitol l⁻¹. The solution was transferred to a glass bottle and 10 μ l biotin-cap-NHS at 50 mg ml⁻¹ in *N,N*-dimethylformamide was added with continuous stirring. After 2 h reaction, the mixture was dialysed against the same PBS buffer to remove the unconjugated biotin molecules. The biotinylated antigen (biotin-C185) was centrifuged at 4 °C for 1.5 h at 35 000 g in the P40ST rotor of an Ultracentrifuge CP-70MX; the clear supernatant containing the biotinylated C185 was stored at 4 °C.

Streptavidin, goat anti-hIgG and monoclonal anti- μ -chain of human IgM were labelled with Eu³⁺ using the same protocol as previously described (Wu *et al.*, 1999).

Microwell coating. One hundred microlitres of C188 at 2 μ g ml⁻¹ in phosphate buffer (0.1 M, pH 7.0, containing 6 M urea) was incubated in microwells for 12 h at RT. The microwells were then washed twice with washing solution (10 mM Tris/HCl buffer, containing 0.9% NaCl, 0.05% NaN₃ and 0.05% Tween 20). One hundred and fifty microlitres of 0.1 M phosphate buffer (pH 7.0) containing 0.5% casein and 10% calf serum was added and incubated for 3 h to block the coated wells.

DAS-TRIFMA of the total anti-HCV antibodies. In the DAS-TRIFMA (Fig. 1), 25 μ l undiluted samples and 100 μ l assay buffer (50 mM Tris/HCl, pH 7.5, containing 0.9% NaCl, 0.05% NaN₃, 0.05% Tween 20, 0.5% casein and 10% calf serum) containing 300 ng biotin-C185 ml⁻¹ were added in microwells successively. The anti-HCV antibodies in the sample were allowed to react with the surface antigen and biotin-C185 for 30 min at RT with slow stirring. The plate was washed four times with washing buffer, then 100 μ l assay buffer containing 1 μ g Eu³⁺-labelled streptavidin ml⁻¹ was added and stirred for 15 min. The wells were washed six times. One hundred microlitres of fluorescence enhancement solution was added and stirred for 5 min to dissociate Eu³⁺ from the surface complex into the solution, where a highly fluorescent complex was formed. The results of the DAS-TRIFMA were interpreted as positive or negative based on the S/Co values: an S/Co $\geq$ 1 represented anti-HCV

Fig. 1. Mechanism of the DAS-TRIFMA (a) and typical indirect immunoassays (b) for antibody detection.

antibody positive; otherwise, negative. This criterion was the same for the other immunoassays in this study. Each sample was measured in duplicate to obviate the fluorescence aberrations that occasionally occurred in the TRIFMA.

I-TRIFMA of anti-HCV antibodies. In the I-TRIFMA, C188-coated strips were the same as those used in the DAS-TRIFMA for direct performance comparison. All samples were diluted 1:100 with dilution buffer (50 mM Tris/HCl buffer, containing 0.9% NaCl, 0.05% NaN_3 , 0.05% Tween 20, 0.1% chloracetamide, 0.05% *Escherichia coli* extract, 0.04% EDTA and 20% calf serum). One hundred microlitres of the freshly diluted samples was added in duplicate in microwells and stirred for 30 min at RT. The plate was washed four times. One hundred microlitres of Eu^{3+} -labelled goat anti-hIgG at $0.5 \mu\text{g ml}^{-1}$ in assay buffer (50 mM Tris/HCl buffer, containing 0.9% NaCl, 0.05% NaN_3 , 0.05% Tween 20, 20% calf serum, 0.5% casein and 0.1% fish gelatin) was added and stirred for 30 min. The wells were washed six times with washing buffer, and the fluorescence was detected in the same way as that in the DAS-TRIFMA.

Detection of the anti-HCV IgM. To study the possible contribution of the anti-HCV IgM to the response of the DAS-TRIFMA and to estimate the prevalence of anti-HCV IgM in the DAS-TRIFMA positive samples, 109 positive and 267 negative samples determined by the DAS-TRIFMA were analysed by the anti-HCV IgM TRIFMA as follows. Samples were diluted 1:100 with TSA buffer (50 mmol Tris/HCl l^{-1} , 0.9% NaCl, pH 7.75) containing 4% goat anti-hIgG serum, 0.5% BSA, 0.05% Tween 20 and 0.05% NaN_3 . After incubation for 30 min with slow stirring, the diluted samples were centrifuged at 10 000 g for 10 min. Twenty microlitres of the supernatant sample was transferred to the C188-coated microwells, which were pre-filled with 100 μl TSA buffer containing 0.5% BSA, 0.1% casein, 0.05% Tween 20, 0.1% chloracetamide, 0.05% *E. coli* extract and 0.05% NaN_3 . The mixture was incubated for 1 h under continuous stirring. The wells were washed four times. One hundred microlitres of assay buffer containing Eu^{3+} -labelled monoclonal anti- μ chain of human IgM at 500 ng ml^{-1} was added and incubated for 30 min. The wells were washed and the fluorescence was measured in the same way as described above.

The lowest detection limits. To assess the lowest detection limit of the DAS-TRIFMA, one sample prepared by pooling 17 positive sera was serially diluted and measured by the DAS-TRIFMA, Ortho ELISA and I-TRIFMA simultaneously. The highest dilution rates at which the sample could still be detected as positive were used to evaluate the lowest detection limits of the anti-HCV antibody immunoassays.

Precision of the DAS-TRIFMA. The reproducibility of the DAS-TRIFMA was evaluated by assaying three positive sera with different positive levels within one assay or in different assays. Coefficients of variation were calculated based on the S/Co values and the standard deviations (SD).

Hook effect. The susceptibility of the DAS-TRIFMA to the hook effect (high-dose prozone effect) was evaluated by analysing 56 highly positive anti-HCV sera in their original form and at 1:10 and 1:100 dilutions.

Clinical sample analysis. One thousand and twenty-five clinical samples were analysed by the DAS-TRIFMA, Ortho HCV ELISA 3.0, WAT HCV ELISA and I-TRIFMA. Samples with consistent positive/negative results obtained by the four methods were considered as true anti-HCV antibody positive/negative, and no further study was done. When the assay results were discordant by at least one of above different methods, Inno-LIA and/or HCV RNA analysis was performed. Interpretation of the anti-HCV antibody results of the

discordant samples was according to the MMWR recommendations (Alter *et al.*, 2003): (1) a sample was considered negative or positive when the strip immunoblot assay (Inno-LIA) gave negative or positive results; (2) a sample was considered positive when the sample was Inno-LIA-IND (IND, indeterminate) but PCR-positive; (3) a sample was considered anti-HCV antibody indeterminate when the sample was Inno-LIA-IND but PCR-negative. The indeterminate results were not included in the statistical evaluation. All discordant samples and samples with S/Co values <0.30 by the DAS-TRIFMA but in the range 0.8–1.0 by at least one of the three indirect methods were analysed by PCR-ELISA. The cut-off values of the Ortho ELISA 3.0 and WAT ELISA were calculated according to the instructions included in the kits. To obtain a maximum specificity and sensitivity, the cut-off values of the DAS-TRIFMA, I-TRIFMA and anti-HCV IgM TRIFMA were determined as the mean plus five standard deviations on the basis of analysis of the 870 negative samples.

RESULTS AND DISCUSSION

The lowest detection limits of the different methods

The lowest detection limit of the anti-HCV antibody immunoassay was studied by assaying one positive sample at different dilutions by the DAS-TRIFMA, I-TRIFMA and Ortho HCV ELISA 3.0. The tested positive sample was a pooled serum containing different specificities of anti-HCV antibodies. The maximum dilution of this sample detected as positive by the DAS-TRIFMA was 1:12 500, and was 1:500 and 1:2 500 by the Ortho ELISA and I-TRIFMA, respectively. These data suggested that the DAS-TRIFMA is able to detect HCV antibodies at about a 10-times lower concentration than that detectable by the two indirect methods.

Due to the identical coating antigen used in the DAS-TRIFMA and I-TRIFMA, the improved analytical sensitivity of the DAS-TRIFMA over the I-TRIFMA can be ascribed to the following factors: (1) the HCV antigen labelled with biotin under the described conditions allowed well protection of its binding activity; (2) generally more than 4.2 biotins were coupled to C185, leading to significant signal amplification (Wu *et al.*, 2002); (3) the small bulk of the biotin molecule makes the biotinylated C185 available for binding the target antibodies without serious steric hindrance; (4) the high specificity of the DAS-TRIFMA allowed the use of undiluted sample, while in indirect methods the samples had to be diluted to decrease the interfering molecules that may cause false-positive results, so a low titre of target antibodies in the sample are more likely to be detected by the DAS-TRIFMA than by indirect methods. The enhanced sensitivity of the DAS-TRIFMA may be helpful for detecting the weak antibody response from newly infected patients or patients with suppressed or compromised immunity.

Precision of the DAS-TRIFMA

The reproducibility of the DAS-TRIFMA was studied by assaying three positive anti-HCV sera with S/Co values of

1.3, 5.1 and 16.8, respectively. The within-assay and between-assay coefficients of variation based on S/Co values ($n=12$) were in the range 3.97–6.33 % and 5.31–11.72 %, respectively.

Hook effect

In the one-step procedure of the DAS-TRIFMA, the biotinylated and the solid-phase HCV antigen were incubated simultaneously with the HCV antibodies in the samples. A high concentration of HCV antibodies in samples may simultaneously saturate the epitopes of both the biotinylated and solid-phase HCV antigen, leading to a falsely decreased response. To evaluate whether the DAS-TRIFMA suffered this problem in clinical applications, 56 highly anti-HCV-positive sera were tested in their original form and at dilutions of 1:10 and 1:100. In this way, the hook effect could be spotted if the fluorescence of the diluted sample was stronger than that of the original one. As shown in Table 1, the hook effect was detected in 3 of the 56 samples with the highest fluorescence at a dilution of 1:4, 1:8 and 1:2, respectively. No false-negative was caused by the hook effect in the 56 samples.

Immunoassay of 1025 clinical specimens

One thousand and seven of the 1025 (98.24 %) samples measured by the four immunoassays (DAS-TRIFMA, Ortho HCV ELISA 3.0, WAT HCV ELISA and the I-TRIFMA) gave consistent results, including 899 negative samples and 108 positive samples. The concordance between the DAS-TRIFMA and the three indirect methods was 99.41 % (1019/1025), 98.93 % (1014/1025) and 98.93 % (1014/1025) for Ortho ELISA 3.0, WAT ELISA and I-TRIFMA, respectively. Of the 1007 concordant samples, one sample was negative by all of the four immunoassays but showed an Inno-LIA-IND and PCR-positive result (1.31×10^5 copies ml^{-1}). The S/Co values were 0.87, 0.6, 0.68 and 0.38 by the DAS-TRIFMA, Ortho HCV ELISA 3.0, I-TRIFMA and the WAT HCV ELISA, respectively.

Eighteen of the 1025 samples showed discordant results and were further studied by Inno-LIA and PCR-ELISA. As shown in Fig. 2, 15 of the 18 samples were DAS-TRIFMA-negative and 3 were DAS-TRIFMA-positive. With regard to

the 15 DAS-TRIFMA-negative samples, at least one of the indirect methods gave positive results. Twelve of the 15 DAS-TRIFMA-negative samples were validated as negative by Inno-LIA results. Four of the 18 samples with Inno-LIA-IND and PCR-negative results (samples 1, 2, 8 and 10 in Fig. 2) were excluded from the statistical analysis due to their unclear anti-HCV antibody status. Samples 12 and 13 were correctly detected as positive by the DAS-TRIFMA but missed by at least one of the indirect methods.

By excluding the four samples (samples 1, 2, 8 and 10 in Fig. 2) that showed unclear anti-HCV antibody status, 911 of the 1021 samples were correctly identified as negative by the DAS-TRIFMA, yielding 100 % (911/911) specificity. Meanwhile, the specificity of the Ortho HCV ELISA 3.0, WAT HCV ELISA and I-TRIFMA was 99.34 % (905/911), 99.23 % (904/911) and 99.01 % (902/911), respectively. The sensitivity of the DAS-TRIFMA, Ortho HCV ELISA 3.0, WAT HCV ELISA and I-TRIFMA was 99.09 % (109/110), 98.18 % (108/110), 97.27 % (107/110) and 98.18 % (108/110), respectively. These results indicated that the reliability of the DAS-TRIFMA for anti-HCV antibody detection was significantly improved with respect to both specificity and sensitivity when compared to that of the three indirect methods.

As shown in Fig. 2, 16 of the 21 false-positive events by the three indirect methods had S/Co values less than 3.0, while 5 had S/Co values greater than 3.0, with the highest at 17.92. This result suggested that judging the anti-HCV antibody status based on the S/Co value of an indirect anti-HCV antibody assay may be inadequate, although higher S/Co values in indirect anti-HCV antibody immunoassays is more predictive of true anti-HCV positives (Alter *et al.*, 2003; Dufour *et al.*, 2003a; Ren & Zhuang, 2005).

The S/Co values of the 1025 samples by the DAS-TRIFMA were compared to those of the three indirect methods. As shown in Fig. 3(a, b, c), the positive and negative results were clearly separated by the DAS-TRIFMA, whereas these show obvious overlap by the indirect methods. Of the 911 negative samples, 9, 33, 49 and 61 samples show S/Co values in the range of 0.4–1.0 and 0, 6, 7 and 9 samples were wrongly identified as positive by the DAS-TRIFMA, Ortho ELISA, WAT ELISA and I-TRIFMA, respectively. The overlap of the S/Co values between the negative and

Table 1. Hook effect of the DAS-TRIFMA observed in three samples from 56 highly positive sera

The S/Co values of the 56 sera were in the range 22.3–161.7 by the DAS-TRIFMA.

Sample	Fluorescence for samples at different dilutions (c.p.s.)*					
	Original	1/2	1/4	1/8	1/16	1/32
Sample A	963 847	1 669 083	2 679 378	1 802 764	1 109 238	700 368
Sample B	709 325	1 181 907	1 558 923	2 010 980	1 630 681	976 839
Sample C	578 923	983 092	676 302	439 276	299 342	192 822

*The cut-off of the DAS-TRIFMA was 15 820 c.p.s. in above table.

Fig. 2. S/Co values of the 18 discordant samples obtained by DAS-TRIFMA and three indirect methods. HCV RNA analysis: the concentration of HCV RNA was 7.18×10^6 copies ml^{-1} for sample 13 by PCR-ELISA. No results were obtained for samples 6, 7 and 11 due to the strong PCR inhibition caused by heparin in the plasma. The remaining 14 samples were PCR-ELISA-negative. Inno-LIA analysis: samples 1, 2, 8 and 10 were Inno-LIA-IND; samples 12 and 13 were Inno-LIA-positive; the remaining 12 samples were Inno-LIA-negative.

positive results by indirect methods makes it difficult to estimate the true HCV status, especially when the S/Co values were around 1.0. Moreover, this phenomenon in indirect methods also makes it difficult to set up a reasonable cut-off value, since high cut-off may miss samples with low titres of target antibodies, while low cut-off may give rise to an unacceptably high ratio of false-positives. Due to the enhanced specificity of the DAS-TRIFMA, the weakly positive results by the DAS-TRIFMA (e.g. S/Co values from 1.0 to 2.0), which are prone to be confused by false-positive results by indirect methods, were highly predictive for identifying the existence of HCV antibodies. Taking into account the above findings, it can be expected that further improvement of the analytical sensitivity of the DAS-TRIFMA may be helpful for enhancing its ability to detect trace amounts of HCV antibodies; this work is presently in progress in our laboratory.

Excluding the 18 discordant samples, 14 of the 1025 samples totally nonreactive by the DAS-TRIFMA but with S/Co values in the range 0.8–1.0 by at least one of the indirect methods were analysed by PCR-ELISA; none were PCR-ELISA-positive. Therefore, the possibility that these samples were from newly infected persons with a low concentration of HCV antibodies was excluded. The causes of the elevated signals in the indirect methods were not studied further.

The amino acid sequence and the purity of the HCV antigen used for assay development are important factors influencing both the specificity and sensitivity of anti-HCV antibody immunoassays. Sharing the same solid-phase antigen, the improved specificity of the DAS-TRIFMA over the I-TRIFMA was mainly associated with its DAS format. The DAS format endows the assay two levels of binding selection; namely, the target antibodies must be recognized

by both the coated antigen and labelled antigen and it is then possible to produce a response. In such a case, if some molecules were nonspecifically attached by the coated HCV antigen, it is possible to choose another HCV antigen lacking this attachment as tracer antigen to prevent the 'sandwich' formation. As a result, the possibility that interference molecules bridge the coated and the labelled antigen can be decreased to a low level. The labelled second anti-hIgG antibodies used to trace the captured anti-HCV IgG antibodies in the indirect methods recognize not only HCV-specific IgG but all hIgG molecules. Because of the high IgG concentration in human blood (generally more than 5 mg ml^{-1}), there is a strong tendency for some of these IgG molecules to be bound to the well surface by direct adsorption or by indirect capture via the surface molecules, and then arouse a signal, giving false-positive results. This problem might be more serious when the samples are from patients with systemic lupus erythematosus, portal cirrhosis, rheumatoid arthritis and some infectious diseases due to the very complicated, higher concentration of immunoglobulin components in their blood. To alleviate such a problem, indirect anti-HCV immunoassays usually require a 1:10–1:100-fold sample dilution prior to test. This strategy is effective; however, it does not always work well, and will inevitably be detrimental to the detection sensitivity when the sample contains a very low concentration of target antibodies.

The above investigations proved that the DAS format is an effective strategy for improving the specificity of anti-HCV antibody immunoassays; however, other choices exist for the same purpose. For example, the specificity of the Ortho Anti-HCV Chemiluminescence immunoassay (CLIA) is enhanced compared to the Ortho ELISA, as reported by Dufour *et al.* (2003b). Because both the CLIA and ELISA are indirect format-based, the CLIA specificity improvement can probably be ascribed to factors other than the assay

Fig. 3. Comparison of the S/Co values of the 1025 clinical samples by the DAS-TRIFMA and three indirect methods. The S/Co span between the two dashed lines parallel to the x - or y -axis was 0.7–1.5. The relatively lower S/Co values for the positive samples obtained by the Ortho anti-HCV ELISA 3.0 (b) were partly because of its higher cut-off value, which was generally more than 0.60 (OD₄₉₂) in our experiments.

format, e.g. the differences in the coating materials, the optimization of the assay components and the setting of cut-off values.

Anti-HCV IgM detection by the TRIFMA

Of 109 DAS-TRIFMA-positive samples, 17 (15.60 %) were anti-HCV IgM reactive (mean S/Co=2.782) and 9 (8.26 %) had S/Co values of 0.6–1.0 by the indirect anti-HCV IgM TRIFMA. Of the 17 anti-HCV IgM-positive/DAS-TRIFMA-positive samples, the ratio of the mean S/Co by the DAS-TRIFMA to that of the I-TRIFMA was 2.06, and was only 1.25 for the remaining 92 anti-HCV IgM-negative/DAS-TRIFMA-positive samples. This result suggested that anti-HCV IgM in the 17 samples might have contributed to the response of the DAS-TRIFMA. In addition, the weakly positive sample 13 by the DAS-TRIFMA (S/Co=1.839; Fig. 2) was also anti-HCV IgM reactive with S/Co at 1.76. This result suggested that HCV antibodies in this sample were mainly of the IgM class; this hypothesis could explain why it was detected by the DAS-TRIFMA but missed by all three indirect methods with labelled anti-hIgG as tracer. The possibility that the response of the IgM TRIFMA on the 17 samples was caused by IgG antibodies rather than IgM was excluded since no significant response was observed with the use of labelled goat anti-hIgG in place of the anti- μ second antibodies in the anti-HCV IgM TRIFMA. Based on these observations, it could be rationally deduced that anti-HCV IgM, and perhaps other classes of HCV-specific antibodies which were not investigated in this study, had strengthened the detectability of the target antibodies in the DAS-TRIFMA. The enhanced detectability of the HCV antibodies in the DAS-TRIFMA by antibodies other than those of the IgG class is helpful for more sensitive diagnosis of HCV infection. Of the 267 DAS-TRIFMA-negative samples, 3 gave positive results in the anti-HCV IgM TRIFMA. This discrepancy was perhaps also associated with the indirect format of the anti-HCV IgM TRIFMA, in which any IgM molecules in the sample, if non-specifically attached on the solid-phase surface, may cause an elevated signal by the same mechanism as that described above.

In conclusion, a DAS-TRIFMA was developed and evaluated in this study to determine its ability to detect the total antibodies to HCV. The use of biotin as an indirect label allowed efficient antigen labelling and good preservation of the antigen's immunoreactivity. The DAS-TRIFMA was sensitive, precise and could be completed within 60 min. Although the clinical samples studied in this paper were relatively limited, the results of this study had sufficient statistical power to demonstrate the superiority of the DAS-TRIFMA over the indirect methods with respect to specificity and sensitivity. Such improvements may be useful for screening for HCV infection and other clinical applications. The DAS-TRIFMA omitted the sample pre-dilution due to its excellent specificity, leading to simplification of the assay procedure and a more

sensitive detection of the low concentration of target antibodies. It is anticipated that a DAS immunoassay for anti-HCV antibody detection, as exemplified by the present DAS-TRIFMA, will play an important role in future diagnosis of HCV infection in clinical laboratories and blood banks, as well as for different research purposes.

ACKNOWLEDGEMENTS

We would like to thank Professor Sun LP, Shanghai Public Sanitation Center, Nanjing 85 Hospital and the Second Affiliated Hospital of Nanjing Medical University for providing their valuable specimens. This work was supported by the foundation of High Technology Research Program of Jiangsu Province, China (no. BG2007606) and the foundation for Talents in the Six Main Professions of Jiangsu Province, China (no. 2006130).

REFERENCES

- Abdel-Hamid, M., El-Daly, M., El-Kafrawy, S., Mikhail, N., Strickland, G. T. & Fix, A. D. (2002). Comparison of second- and third-generation enzyme immunoassays for detecting antibodies to hepatitis C virus. *J Clin Microbiol* **40**, 1656–1659.
- Alter, M. J., Kuhnert, W. L. & Finelli, L. (2003). Guidelines for laboratory testing and result reporting of antibody to hepatitis C virus. *MMWR* **52** (RR-3), 1–16.
- Ansari, M. H. K. & Omrani, M. D. (2006). Evaluation of diagnostic value of Elisa method (EIA) & PCR in diagnosis of hepatitis C virus in hemodialysis patients. *Hepatitis Monthly* **6**, 19–23.
- Bürgisser, P., Simon, F., Wernli, M., Wüst, T., Beya, M. F. & Frei, P. C. (1996). Multi-center evaluation of new double-antigen sandwich enzyme immunoassay for measurement of anti-human immunodeficiency virus type 1 and type 2 antibodies. *J Clin Microbiol* **34**, 634–637.
- Carreño, V. (2006). Occult hepatitis C virus infection: a new form of hepatitis C. *World J Gastroenterol* **12**, 6922–6925.
- Carreño, V., Pardo, M., López-Alcorocho, J. M., Rodríguez-Iñigo, E., Bartolomé, J. & Castillo, I. (2006). Detection of hepatitis C virus (HCV) RNA in the liver of healthy, anti-HCV antibody-positive, serum HCV RNA-negative patients with normal alanine aminotransferase levels. *J Infect Dis* **194**, 53–60.
- CDC (2000). Hepatitis C virus infection among firefighters, emergency medical technicians, and paramedics – selected locations, United States, 1991–2000. *MMWR* **49**, 660–665.
- Chevaliez, S. & Pawlotsky, J. M. (2006). Hepatitis C virus serologic and virologic tests and clinical diagnosis of HCV related liver disease. *Int J Med Sci* **3**, 35–40.
- Colin, C., Lanoir, D., Touzet, S., Meyaud-Kraemer, L., Bailly, F. & Trepo, C. (2001). Sensitivity and specificity of third-generation hepatitis C virus antibody detection assays: an analysis of the literature. *J Viral Hepat* **8**, 87–95.
- Dufour, D. R., Talastas, M., Fernandez, M. D. A., Harris, B., Strader, D. B. & Seeff, L. B. (2003a). Low-positive anti-hepatitis C virus enzyme immunoassay results: an important predictor of low likelihood of hepatitis C infection. *Clin Chem* **49**, 479–486.
- Dufour, D. R., Talastas, M., Fernandez, M. D. A. & Harris, B. (2003b). Chemiluminescence assay improves specificity of hepatitis C antibody detection. *Clin Chem* **49**, 940–944.
- Hyams, K. C., Riddle, J., Rubertone, M., Trump, D. H. & Mazzuchi, A. J. (2001). Prevalence and incidence of hepatitis C virus infection in the US military: a seroepidemiologic survey of 21,000 troops. *Am J Epidemiol* **153**, 764–770.
- Lok, A. S. & Gunaratnam, N. T. (1997). Diagnosis of hepatitis C. *Hepatology* **26** (Suppl. 1), 48S–56S.
- Radkowski, M., Horban, A., Gallegos-Orozco, J. F., Pawelczyk, A., Jablonska, J., Wilkinson, J., Adair, D. & Laskus, T. (2005). Evidence for viral persistence in patients who test positive for anti-hepatitis C virus antibodies and have normal alanine aminotransferase levels. *J Infect Dis* **191**, 1730–1733.
- Ren, H. R. & Zhuang, H. (2005). The progress in studying the correlation of S/Co values of anti-hepatitis C virus enzyme immunoassay with the positive results of supplemental assays. *Chin J Lab Med* **8**, 1096–1099 in Chinese.
- Richter, S. S. (2002). Laboratory assays for diagnosis and management of hepatitis C virus infection. *J Clin Microbiol* **40**, 4407–4412.
- Schmidt, B. L., Edjlalipour, M. & Luger, A. (2000). Comparative evaluation of nine different enzyme-linked immunosorbent assays for determination of antibodies against *Treponema pallidum* in patients with primary syphilis. *J Clin Microbiol* **38**, 1279–1282.
- Wu, F. B., Xu, Y. Y., Xu, T., Wang, Y. Z. & Han, S. Q. (1999). Time-resolved fluorescence immunoassay of thyroxine in serum: immobilized antigen approach. *Anal Biochem* **276**, 171–176.
- Wu, F. B., Han, S. Q. & He, Y. F. (2002). Time-resolved immuno-fluorometry of serum hTSH with enhanced sensitivity. *J Immunoassay Immunochem* **23**, 191–210.
- Zylberberg, H. & Pol, S. (1996). Reciprocal interaction between human immunodeficiency virus and hepatitis C infection. *Clin Infect Dis* **23**, 1117–1125.