African Journal of Microbiology Research Vol. 5(1), pp. 28-33, 4 January, 2011
Available online http://www.academicjournals.org/ajmr
DOI: 10.5897/AJMR10.641
ISSN 1996-0808 ©2011 Academic Journals

Full Length Research Paper

A magnetic microparticle-based immunoassay for hepatitis C virus NS3 antigen

Li Xie[1]*, Yue-ping Guang[2], Fang Liu[1], Hong-bo Shi[1], Li Yan[1] and Jin-li Lou[1]

[1]Beijing You'an Hospital, Capital Medical University, Beijing 100069, China.
[2]University of Science and Technology Beijing, Beijing 100083, China.

Accepted 9 December, 2010

In order to detect Hepatitis C virus NS3 antigen (HCAg-NS3) in human serum, we developed a magnetic microparticle (MMP) based immunoassay by using biotin-streptavidin amplification, MMP in combination with immunoassay. Results showed that the HCV NS3 monoclonal antibody had a high immobility rate (87.1%) on the surface of MMPs; the assay procedure required smaller volumes of reagents (only 20 µl of immune magnetic bead); the detection could be completed within 30 min; this method could detect 10 pg/ml HCAg-NS3 (sensitivity: 5.2 pg/ml) and had favorable stability; the mean coefficient of variances (CVs) of intra-assay and inter-assay were 5.35 and 7.15%, respectively. The results of this method were also related to those in HCV RNA detection with a linear correlation coefficient of 0.570 ($P < 0.01$). These findings demonstrate this assay which has high specificity, sensitivity, precision and stability may be promising in clinical application.

Key words: Hepatitis C virus NS3 antigen, magnetic microparticle, biotin-streptavidin sytem (ABS), magnetic immunoassay.

INTRODUCTION

Hepatitis C is a health-threatening blood-borne infectious disease caused by hepatitis C virus (HCV) infection (Choo et al., 1989). About more than 50% of HCV patients will develop chronic hepatitis and cirrhosis is observed in about 10-20% of HCV patients who also have high risk for liver cancer (Tobler et al., 2005). In the immunoprophylaxis and clinical treatment of hepatitis C, no reliable vaccine has been prepared and the outcome is not favorable. Currently, diagnosis of hepatitis C is mainly based on nucleic acid testing and antigen testing. It may take up to about 70 days for antibodies to appear in the blood following infection, which is known as the 'window period' (Di, 2000). Therefore, there remains misdiagnosis to a certain extent. Although nucleic acid testing for the detection of HCV RNA can be performed at the early stage, and has advantages in sensitivity and specificity, this method has a high requirement in technique and a low detection rate, and it is time-consuming (Fabrizi et al., 2005; Laperche et al., 2005).

The presence of HCV NS3 antigen (HCAg NS3) is a marker of viral replication and can be observed at 1 week following infection (Muerhoff et al., 2002). However, the concentration of HCAg NS3 in the serum (10^2-10^3 CID/ml) (Huang and Ren, 2000; Moradpour et al., 1996; Hilfenhaus et al., 1992) is usually lower than the detection limit of commonly used ELISA (0.1 ng/ml^{-1} µg/ml) (Jia et al., 2009), which significantly limits the detection of HCAg NS3.

Currently, magnetic microparticles have been hot focus of current research, technique development and industrial development of biology, medicine and pharmacology (Tudorache and Bala, 2007). Recently, an American company (Abbott) developed a chemiluminescent, magnetic particle-based immunoassay for the detection of hepatitis C virus core antigen in human serum or plasma (Leary et al., 2006). However, the complex procedures and high cost limit its application in China. To date, no study has reported the

*Corresponding author. E-mail: profli1996@gmail.com. Tel: +86-10-83997427. Fax: +86-10-63053484.

detection of HCAg by microparticles.

In our previous study, we prepared 5 types of HCV NS3 monoclonal antibodies targeting different epitopes on HCAg and performed the detection of HCAg NS3 with these antibodies by ELISA (Xie et al., 2005). In this study, based on a previously described technique (Xie et al., 2009), biotin-streptavidin amplification (ABS) was used to specifically couple the HCAg NS3 monoclonal antibody to the microparticles aiming to prepare immune magnetic bead (MIB) which can replace the conventional microplate. Then, double antibody sandwich ELISA was employed to detect HCAg NS3. Our study may provide basis for the early diagnosis of HCV infection by magnetic immunoassay.

MATERIALS AND METHODS

Reagents and instruments

Purified HCAg NS3 (Institute of Virology of Chinese Academy of Preventive Medicine), horseradish peroxidase (HRP), biotin conjugated HCAg NS3 monclonal antibody (our department), bovine serum albumin (BSA) (Roche), TMB (Sigma), TMB substrate buffer (our department), HCV RNA reagents (Roche), streptavidin biotin magnetic bead (Dynal Biotech) and double distilled water were used in this study. Other reagents were purchased from Chinese company (analytically pure).

Microplate reader (Model 680) (BIO-RAD), UV-VIS Spectrophotometer (UV-2550) (SHIMADZU), magnetic separators (123.31D DynaMag and 123.20D) and Thermostatic oscillation incubator (ZHWY-2102C) (Shanghai Zhicheng) were used for detection.

MIB washing buffer (pH 7.4) included 0.01 mol/L PBS and 0.05% Tween20, and MIB conservation buffer (pH 7.4) included 0.01 mol/L PBS, 0.5% BSA and 0.1% protein stabilizer.

Sample collection

Serum and EDTA anticoagulated plasma were collected from 42 HCV inpatients and outpatients from January 2009 to January 2010 in the Beijing You'an Hospital. Informed consent was obtained before study. In addition, serum and plasma were also collected from 50 healthy subjects. All samples were stored at -80°C. Informed consent was obtained from each subject before sampling and the study was approved by the Ethnics Committee of Beijing You'an Hospital, Capital Medical University.

RNA extraction and polymerase chain reaction (PCR)

HCV RNA was extracted from 200 µl EDTA anticoagulated plasma by using a RNA extraction kit (Roche, USA) according to manufactures' instructions. cDNA was prepared by Reverse transcription PCR using M-MLV reverse transcriptase (Fermentas, USA). The amplified cDNA was further subjected to two rounds of PCR amplifications using nested primers. The conditions for the first round PCR were as follows; An initial denaturation step at 95°C for 2 min followed by 30 cycles of 94°C for 45 s, 54°C for 45 s, and 72°C for 1 min performed in a thermal cycler (Eppendorf, Germany). The conditions for the 2nd round PCR were the same except that a different set of inner primers was used and the annealing temperature was raised to 62°C in order to amplify the 1st round product.

Preparation of MIB

Streptavidin biotin magnetic beads (1 mg) were washed with MIB washing buffer once and then mixed with 100 µl of biotin conjugated HCAg NS3 monoclonal antibody followed by incubation at room temperature for 30 min. Magnetic separation was performed and supernatant was obtained followed by determination of the protein concentration. Magnetic beads were washed with MIB washing buffer three times and stored in 1 ml of MIB conservation buffer at 4°C for use.

Magnetic immunoassay of HCAg NS3

The purified HCAg NS3 served as positive control and serum from healthy subjects as negative control. Then, 20 µl of HCAg NS3, 20 µl of MIB, 50 µl of HRP conjugated HCAg NS3 monoclonal antibody and 50 µl of reaction buffer were added to a centrifuge tube follow by vortex and subsequent incubation at 37°C for 30 min. After magnetic separation, washing with 0.02 mol/L PBST was performed three times and then TMB substrate was added followed by incubation at 37°C for 10 min. Reaction was terminated by adding 2 mol/L H_2SO_4. Magnetic separation was performed and absorbance (OD) of supernatant was determined at 450 nm.

Definition of the positive value

According to the ratio of $OD_{positive\ sample}$ to $OD_{negative\ control}$ (P/N), P/N≥2.1 was defined as positive (Herrmann et al., 1979).

Statistical analysis

Statistical analysis was performed with SPSS version 13.0 statistic software package. Correlation coefficient test was used to test the significance. A value of $P < 0.05$ was considered statistically significant. All experiments were repeated three times and the values were averaged.

RESULTS

Adherence of antibody to magnetic beads

UV-VIS Spectrophotometer was used to detect the OD of antibody solution before adherence (pre), supernatant after adherence (post) and washing buffer during adherence (wash) at 280 nm. The OD_{pre}, OD_{post} and OD_{wash} were 0.325, 0.040 and 0.002, respectively. The adherence efficiency was calculated as follow:

$$\text{adherence efficiency} = \frac{ODpre - ODpost - \sum ODwash}{ODpre} \times 100\%$$

The adherence efficiency was 87.1%, which suggests biotin conjugated HCAg NS3 monoclonal antibody can efficiently capture streptavidin conjugated magnetic bead forming MIB.

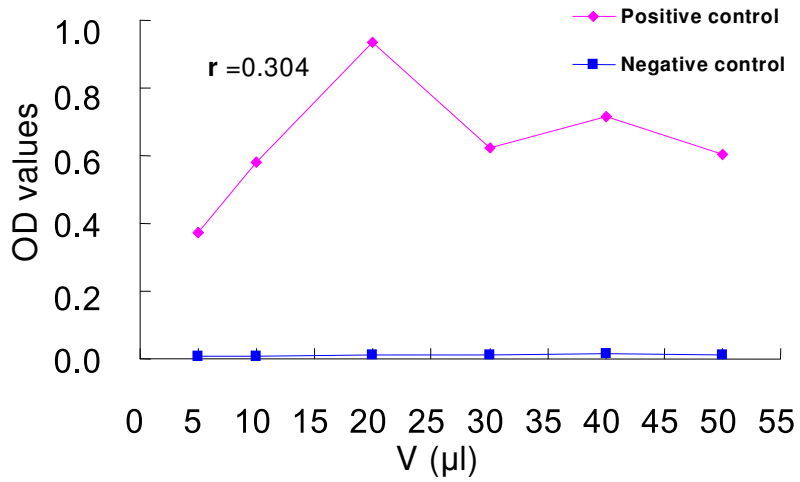

Figure 1. Effects of dose of MIB on the assay performance.

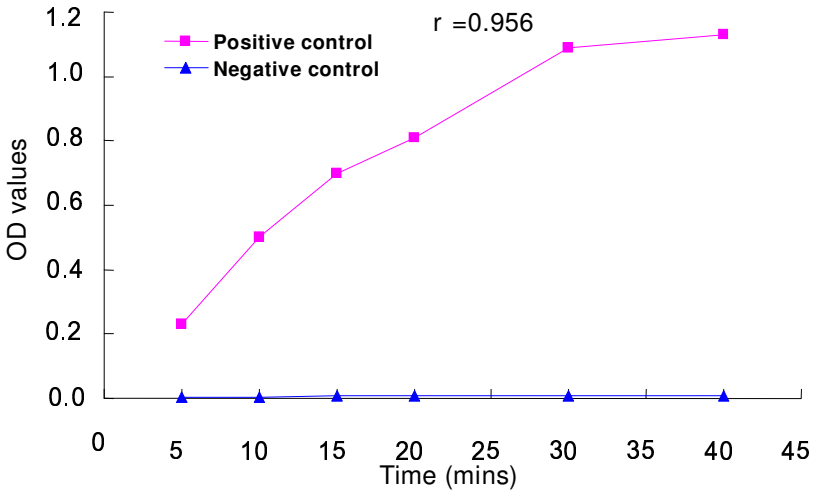

Figure 2. Optimization of the incubation time.

Dose of MIB used for detection

After adding 5, 10, 20, 30, 40 and 50 µl of MIB, the ODs of positive sample and negative controls were determined (Figure 1). Statistical analysis showed no significant difference ($r = 0.304$, $P > 0.05$). With the increase in the MIB amount, the OD of positive samples elevated to a certain extent and subsequently decreased. However, the OD of negative controls was not obviously changed. In the following experiments, 20 µl of MIB were used for detection.

Selection of incubation time

After different incubation times (5, 10, 15, 20, 30 and 40 min), the ODs of positive samples and negative controls were determined (Figure 2). Statistical analysis showed significant difference ($r = 0.956$, $P < 0.01$). With the prolongation of incubation time, the OD of positive samples increased, but it did not significantly elevate after 30 min of incubation. The negative controls had consistent low ODs after different incubation times. Therefore, incubation was performed for 30 min in the following experiments.

Test of detection stability

The MIB, HRP conjugated antibody, positive samples, negative controls, reaction buffer and substrate buffer were stored at 4℃ for 1, 3, 6, 9 and 12 months, and

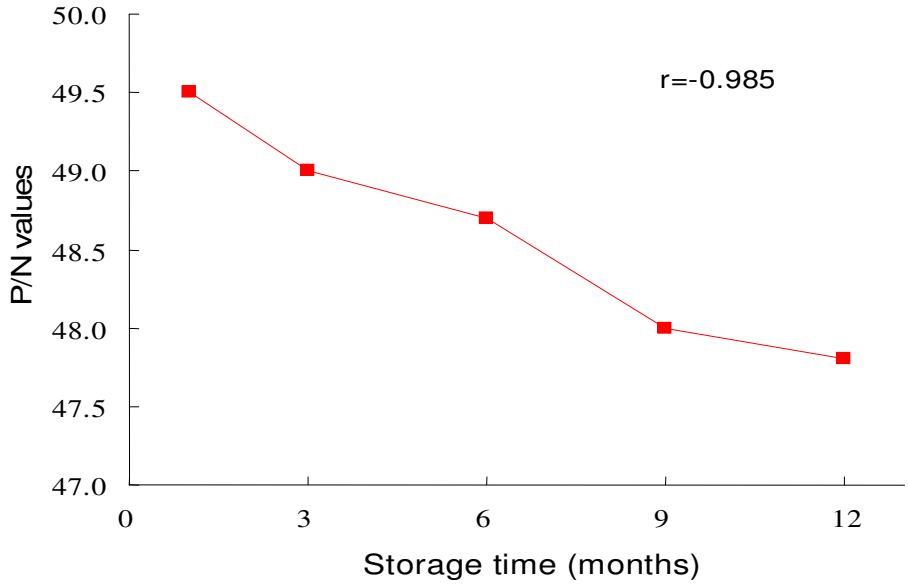

Figure 3. Stability of the assay system at 4℃.

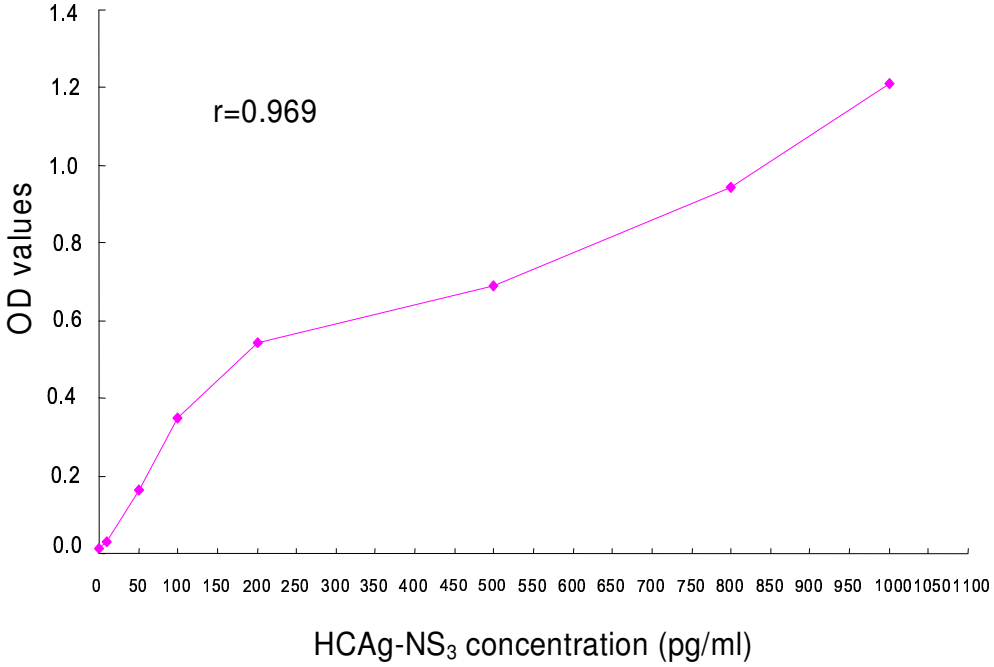

Figure 4. The standard curve for the detection of HCAg-NS_3.

then the ODs of positive samples and negative controls were determined followed by calculation of adherence efficiency (P/N) (Figure 3). Statistical analysis showed significant difference ($r = -0.985$, $P < 0.01$). The P/N was not markedly decreased and it was still higher than 2.1, 12 months later. These findings suggest the assay system has favourable stability.

Standard curve

HCAg NS3 solution of different concentrations (10, 50, 100, 200, 500, 800 and 1000 pg/ml) was prepared with PBS and ODs were determined followed by delineation of the standard curve (Figure 4). Linear regression analysis showed the linear equation was

Table 1. Precision of the assay for HCAg-NS3.

HCAg-NS3 (pg/ml)	Intra-assay (n = 10)			Inter-assay (n = 3)		
	Mean OD	RSD (%)	CV (%)	Mean OD	RSD (%)	CV (%)
100	0.346	1.99	5.73	0.327	2.68	8.19
200	0.575	2.68	4.67	0.539	4.48	8.30
500	0.711	4.01	5.64	0.710	3.51	4.95

$y = 0.001089x + 0.130261$ and the linear correlation coefficient was r=0.969 ($P < 0.01$).

Sensitivity

Standard concentration was detected tem times and the mean OD and its standard deviation were calculated. The difference between mean OD and double standard deviation was substituted into standard equation and the concentration obtained was defined as sensitivity (Ren et al., 2008). Experiment was repeated three times and the mean detection sensitivity was 5.2 pg/ml.

Precision

The deviations in intra-assay and inter-assay were analyzed with MIB and HCAg NS3 solution of different concentrations (100, 200 and 500 pg/ml) (Table 1). Our results showed the deviation was less than 15% suggesting favourable repeatability.

Correlation between magnetic immunoassay and HCV RNA detection

In addition, we detected the HCV RNA in EDTA anticoagulated plasma from 42 patients with reagents from Roche. Results showed 27 patients had HCV RNA positive. Then, the HCAg NS3 in the serum from the same 42 patients was also detected and results revealed 17 patients had positive HCAg NS3 among whom 17 had positive HCV RNA. Further correlation analysis showed the correlation was as high as 81% between results from magnetic immunoassay and HCV RNA detection.

Linear correlation analysis was performed to analyze the correlation between results of HCAg NS3 and HCV RNA. The linear equation was $y = 8.12 \times 10^{-8} x + 0.2216107$ and the linear correlation coefficient was r = 0.570 ($P < 0.01$). This result suggests there is positive correlation between HCAg NS3 and HCV RNA.

DISCUSSION

Immunoassay using immunomagnetic microparticles as the solid phase is a popular and advanced technique developed in recently years. In conventional ELISA, the antibody physically adheres to the polystyrene plate which limits the amount of antibody binding to the plate and the detection sensitivity. In addition, conventional ELISA is time consuming (longer than 24 h).

In the present study, biotin has high affinity to streptavidin which subsequently exerts biomagnification effects. Additionally, the size of magnetic microparticles is one of the critical determinants of the detection sensitivity (Zhuo et al., 2009), and therefore this method can be used in the detection of protein of low concentration (Zhang et al., 2006). In our study, the diameter of microparticles was only 1 μm and so these particles have a large specific surface area. Therefore, more antibodies can adhere to these microparticles, and the antigen binding site of antibodies is fully exposed which reduces steric effects (Ambrosi et al., 2007; Jie et al., 2008). In addition, magnetic microparticles have superparamagnetism and have no remanent magnetism in the absence of additional magnetic field, which abolishes the magnetic reunion and leads to even distribution of particles. Therefore, these advantages effectively and efficiently promote the binding between antigen and antibody (Morisada et al., 2002). The MIB prepared in this way can be bound with more antibodies and has favourable stability. In addition, the dose of MIB in the detection is also small than that used in the ELISA. Furthermore, the incubation can complete within only 30 min. Based on the controllable movement of magnetic particles under magnetic conditions, the products can be easily separated which makes the operation more simple and rapid.

After delineation of the standard curve, the results showed the antigen concentration was linearly correlated with the OD values when the antigen concentration ranged from 0 to 1000 pg/ml ($r = 0.969$, $P < 0.01$). At the lowest detection limit (10 pg/ml), the P/N was still greater than 2.1 ($r = 0.969$, $P < 0.01$), which implies this method can used to detect HCAg NS3 of as low as 10 pg/ml ($OD_{positive} = 0.029$; $OD_{negative} = 0.011$). Therefore, the sensitivity of this method was higher than that of conventional ELISA (1~2 ng/ml) (Xie et al., 2009).

After optimization of the experiment conditions, the results showed the OD of negative controls was consistently lower than 0.1, which suggests our method has extremely low non-specificity.

In the clinical application, the correlation coefficient between this new method and HCV RNA detection was $r = 0.570$ ($P < 0.01$), which suggests there is relationship between two methods. This method could detect HCAg NS3, but more studies are needed to confirm the conclusion.

Taken together, the novel method developed in this study had simplicity, rapidity, high specificity, high sensitivity, and stability in the detection of HCV virus. Our results indicate this method may be promising in clinical application.

ACKNOWLEDGEMENTS

This study was supported by National High Technology Research and Development Program of China (863) (No.2007AA03Z315) and Capital Medical Developing Scientific and Research Fund (No.2007-3080).

REFERENCES

Ambrosi A, Castañeda MT, Killard AJ, Smyth MR, Alegret S, Merkoçi A (2007). Double-codified gold nanolabels for enhanced immunoanalysis. Anal Chem., 79(14): 5232-5240.

Choo QL, Kuo G, Weiner AJ, Overby LR, Bradley DW, Houghton M (1989). Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science, 244: 359-362.

Di Bisceglie AM (2000). Natural history of hepatitis C: Its impact on clinical management. Hepatology, 31(4): 1014-1018.

Fabrizi F, Lunghi G, Aucella F, Mangano S, Barbisoni F, Bisegna S, Vigilante D, Limido A, Martin P (2005). Novel assay using total hepatitis C virus (HCV) core antigen quantification for diagnosis of HCV infection in dialysis patients. J. Clin. Microbiol., 43(1): 414-420.

Herrmann JE, Hendry RM, Collins MF (1979). Factors involved in enzyme-linked immunoassay of viruses and evaluation of the method for identification of enteroviruses. J. Clin. Microbiol., 10(2): 210-217.

Hilfenhaus J, Krupka U, Nowak T, Cummins LB, Fuchs K, Roggendorf M (1992). Follow-up of hepatitis C virus infection in chimpanzees: Determination of viraemia and specific humoral immune response. J. Gen. Virol., 73(4): 1015-1019.

Huang JS, Ren DM (2000). BINGXING GANYAN. Beijing: People's Military Medical Press, p. 363.

Jia CP, Zhong XQ, Hua B, Liu MY, Jing FX, Lou XH, Yao SH, Xiang JQ, Jin QH, Zhao JL (2009). Nano-ELISA for highly sensitive protein detection. Biosens Bioelectron., 24(9): 2836-2841.

Jie G, Huang H, Sun X, Zhu JJ (2008). Electrochemiluminescence of CdSe quantum dots for immunosensing of human prealbumin. Biosens Bioelectron., 23(12): 1896-1899.

Laperche S, Elghouzzi MH, Morel P, Asso-Bonnet M, Le Marrec N, Girault A, Servant-Delmas A, Bouchardeau F, Deschaseaux M, Piquet Y (2005). Is an assay for simultaneous detection of hepatitis C virus core antigen and antibody a valuable alternative to nucleic acid testing? Transfusion, 45(12): 1965-1972.

Leary TP, Gutierrez RA, Muerhoff AS, Birkenmeyer LG, Desai SM, Dawson GJ (2006). A chemiluminescent, magnetic particle-based immunoassay for the detection of hepatitis C virus core antigen in human serum or plasma. J. Med. Virol., 78(11): 1436-1440.

Moradpour D, Wakita T, Tokushige K, Carlson RI, Krawczynski K, Wands JR (1996). Characterization of three novel monoclonal antibodies against hepatitis C virus core protein. J. Med. Virol., 48(3): 234-241.

Morisada S, Miyata N, Iwahori K (2002). Immunomagnetic separation of scum-forming bacteria using polyclonal antibody that recognizes mycolic acids. J. Microbiol. Methods, 51(2): 141-148.

Muerhoff AS, Jiang L, Shah DO, Gutierrez RA, Patel J, Garolis C, Kyrk CR, Leckie G, Frank A, Stewart JL, Dawson GJ (2002). Detection of HCV core antigen in human serum and plasma with an automated chemiluminescent immunoassay. Transfusion, 42(3): 349-356.

Ren SQ, Wang X, Tang BJ (2008). Micro-plate Chemiluminescence Enzyme Immunoassay for Clinical Determination of Progesterone in Human Serum. Anal. Chem., 36(6): 729-734.

Tobler LH, Stramer SL, Lee SR, Baggett D, Wright D, Hirschkorn D, Walsh I, Busch MP (2005). Performance of ORTHO HCV core antigen and trak-C assays for detection of viraemia in pre-seroconversion plasma and whole blood donors. Vox Sang., 89(4): 201-207.

Tudorache M, Bala C (2007). Biosensors based on screen-printing technology, and their applications in environmental and food analysis. Anal. Bioanal. Chem., 388(3): 565-578.

Xie L, Chen WR, Zhang MC (2005). Establishment and Identification of Monoclonal Antibody of Hepatitis C Virus. Tianjing Med. J., 33(8): 492-494.

Xie L, Huang DZ, Chen HL (2009). The clinical application and analysis of hepatitis C virus NS3 antigen detection by ELISA in human serum. Chin. J. Microbiol. Immunol., 29(1): 88-92.

Zhang J, Song S, Zhang L, Wang L, Wu H, Pan D, Fan C (2006). Sequence-specific detection of femtomolar DNA via a chronocoulometric DNA sensor (CDS): effects of nanoparticle-mediated amplification and nanoscale control of DNA assembly at electrodes. J. Am. Chem. Soc., 128(26): 8575-8580.

Zhuo Y, Yuan PX, Yuan R, Chai YQ, Hong CL (2009). Bienzyme functionalized three-layer composite magnetic nanoparticles for electrochemical immunosensors. Biomater., 30(12): 2284-2290.