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ScienceDirect
Analytical Biochemistry 362 (2007) 69–75
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

A simple, rapid, and sensitive integrated protein microarray for simultaneous detection of multiple antigens and antibodies of five human hepatitis viruses (HBV, HCV, HDV, HEV, and HGV)

Rongzhen Xu [a,*], Xiaoxian Gan [b], Yongming Fang [a], Shu Zheng [a], Qi Dong [a]

[a] *Second Affiliated Hospital, Cancer Institute, School of Medicine, Zhejiang University, Hangzhou 310009, China*
[b] *Zhejiang Academy of Medical Sciences, Hangzhou 310013, China*

Received 7 September 2006
Available online 29 December 2006

Abstract

Protein microarrays for parallel detection of multiple viral antigens and antibodies have not yet been described in the field of human hepatitis virus infections. Here, we describe a simple, rapid, and sensitive integrated protein microarray with three different reaction models. The integrated protein microarray could simultaneously determine in human sera two viral antigens (HBsAg, HBeAg) and seven viral antibodies (HBsAb, HBcAb, HBeAb, HCVAb, HDVAb, HEVAb, HGVAb) of human hepatitis viruses within 20 min. The results of the protein microarray were assessed directly by the naked eye but can also be analyzed by a quantitative detector. The detection limit of this protein microarray was 0.1 ng/ml for HBsAg. Overall, >85% concordance was observed between the integrated protein microarrays and an enzyme-linked immunosorbent assay for above hepatitis viral antigen and antibody detections in human sera. This integrated protein microarray can be easily optimized for clinical use and epidemiological screening for multiple hepatitis virus infections.

Keywords: Integrated protein microarray; Hepatitis viruses; Antibody; Antigen

Hepatitis virus infections are common public health problems affecting millions of people worldwide. It has been known that there are at least seven different types of hepatitis viruses that can cause human diseases, including hepatitis A (HAV)[1], B (HBV), C (HCV), D (HDV), E (HEV), G (HGV), and transfusion transmitted (TTV) viruses [1–7]. Some hepatitis viral infections cause serious problems. For instance, HBV and HCV infections not only can cause hepatitis but also significantly increase the rate of hepatocellular carcinoma (HCC) [8]. In addition, coinfection of different hepatitis viruses are frequent in several patients since the same routes of transmission are shared by these viruses (drug abusers, blood transfusion, etc.) [9–12]. Viral antigens and antibodies in serum are essential biomarkers for diagnosis and therapy follow-up of these viral diseases [13–19]. For example, in the case of HBV infection, the presence of plasma hepatitis B surface antigen (HBsAg) indicates an active HBV infection [18]. On the other hand, the appearance of anti-HBs antibodies is an indicator of patient recovery [19].

Currently, certain commercial tests, such as EIA-based tests and colloidal- gold-based immunochromatographic strip tests, permit the detection of each of these parameters in separate assays. EIA-based tests have sensitive and

* Corresponding author. Fax: +86 571 87214404.
E-mail address: xurongzhen@hzcnc.com (R. Xu).

[1] *Abbreviations used:* HAV, hepatitis virus A; HBV, hepatitis virus B; HBsAg, hepatitis virus B surface antigen; HBsAb, hepatitis virus B surface antibody; HBcAb, hepatitis virus B core antibody; HBeAg, hepatitis virus B e antigen; HBeAb, hepatitis virus B e antibody; HCV, hepatitis virus C; HCVAb, hepatitis virus C antibody; HDV, hepatitis virus D; HDVAb, hepatitis virus D antibody; HEV, hepatitis virus E; HEVAb, hepatitis virus E antibody; HGV, hepatitis virus G; HGVAb, hepatitis virus G antibody; TTV, transfusion transmitted virus; HCC, hepatocellular carcinoma; EIA, enzyme immunoassay; Ag, antigen; Ab, antibody; McAb, monoclonal antibody; HRP, horseradish peroxidase; CV, coefficient of variation; RCA, rolling-circle amplification; TSA, tyramide signal amplification; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; DAB, diaminobenzidine; PBS, phosphate-buffered saline.

0003-2697/$ - see front matter © 2007 Published by Elsevier Inc.
doi:10.1016/j.ab.2006.12.008

specific features, but these assays are time consuming and involve complicated procedures. The colloidal-gold-based immunochromatographic strip test is rapid and simple and can be used for point-of-care testing, but its low sensitivity limits its wide application for some low-abundance antigens or antibodies in serum samples. In addition, these assays require large quantities of both sample and reagents, thus it is very hard to perform mass screening and profiling of viral coinfection using these assays. Protein microarrays may circumvent these limitations by allowing simultaneous and multiparametric analysis of serum biomarkers. Indeed, a variety of protein microarrays have been used to measure biomarkers in different fluid samples over the past decade [20–30], but their application in clinical diagnostics remains difficult because of their complex processes, expense and the requirement of sophisticated devices. Protein microarrays for parallel detection of multiple viral antigens and antibodies present in sera have not yet been described in the field of human hepatitis virus infections.

Here, we report a novel integrated protein microarray that can simultaneously detect in human sera nine different kinds of viral antigens and antibodies of HBV, HCV, HDV, HEV, and HGV with simple, flexible, cost-effective, and sensitive features. Its performance was validated with a collection of sera previously characterized with commercial ELISA kits for their reactivity for these viral antigens and antibodies.

Materials and methods

Viral antigens, antibodies, and other reagents

HBsAg was obtained from Chemicon (Temecula, CA, USA); goat anti-HBsAb, HBeAg, HBeMcAb1, HBcAg, HRP–HBsMcAb, HRP–HBsAg, HRP–HBeMcAb, and HRP–HBcMcAb were obtained from Shanghai Hua Tai Biotechnology Industrial Co., Ltd (Shanghai, China); recombinant HCV antigen with multiple antigen epitopes (HCV MpAg) was from Wandergen Bio-Medicine Technology Co., Ltd. (Beijing, China); HDV, HEV, and HGV antigens were from Beijing Kewei Diagnostic Reagent Co., Ltd. (Beijing, China); EZ-link sulfo-NHS-LC-Biotin, avidin–HRP conjugate, and ImmunoPure Metal Enhanced DAB Substrate kit were from Pierce Chemical Co. (Rockford, IL, USA).

Membranes for preparation of protein microarrays

The membrane used for preparation of protein microarrays was nitrocellulose membrane PROTRAN BA 83, which was obtained from Schleicher & Schuell (0.2-μm pore size, Lot No. BJ0606-1; Germany).

Serum samples

Serum samples were obtained from individuals in Second Affiliated Hospital, School of Medicine, Zhejiang University, China, who had given informed consent and were stored at −80 °C until use. Serum samples, which were characterized with commercial ELISA kits for their reactivity for these viral antigens and antibodies, contained different hepatitis virus infection and healthy subjects.

Preparation of biotinylated viral antigens

Preparation of biotinylated viral antigens (HCV, HDV, HEV, and HGV) was carried out using EZ-link sulfo-NHS-LC-Biotin according to the manufacturer's instructions for biotinylation of proteins.

Method design for simultaneous detection of multiple viral antigens and antibodies

To avoid false-positive results due to high cross-reactivities of several viral antibodies such as HBeAb and HBcAb, which must be performed using competition immunoassay, we designed three different antigen–antibody reaction systems widely used in current immunoassays for the detection of viral antigens and antibodies of hepatitis viruses in human sera (Table 1). The antigen–antibody reaction models set up for protein microarrays to simultaneously detect multiple viral antigens and antibodies are shown in Fig. 1.

Protein microarray setup

To simultaneously detect these viral antigens and antibodies in one chip, we designed an integrated protein microarray with three separate 3×3 spot arrays in three separate reaction wells in a special apparatus (Figs. 2A and 2B), which is similar to a device for immunofiltration assay but had three separate reaction wells in one chip. Schematic representation of the array is presented in Fig. 2C, and the left three spots of each array contained biotinylated BSA protein for array location and quality control. The working principle of the protein microarray was designed based on the enzyme immunofiltration assay, and the protein microarray assays were carried out in the designed apparatus (Fig. 2). HRP conjugates and DAB development system were used in this array assay. Thus, the array assay results were assessed directly by the naked eye but can also be analyzed by a quantitative detector.

Table 1
Detection methods for viral antigens and antibodies of hepatitis viruses

Detection targets	Methods	Capture probes	Detecting probes
HBsAg	Ab-sandwich assay	Goat-anti-HBsAb	HRP-HBsMcAb
HBsAb	Ag-sandwich assay	HBsAg	HRP-HBsAg
HBeAg	Ab-sandwich assay	HBeMcAb1	HRP-HBeMcAb2
HBeAb	Competition assay	HBeAg	HRP-HBeMcAb
HBcAb	Competition assay	HBcAg	HRP-HBcMcAb
HCVAb	Ag-sandwich assay	HCV MpAg	Biotinylated HCV Ag
HDV Ab	Ag-sandwich assay	HDV Ag	Biotinylated HDV Ag
HEVAb	Ag-sandwich assay	HEV Ag	Biotinylated HEV Ag
HGVAb	Ag-sandwich assay	HGV Ag	Biotinylated HGV Ag