

Development of a Highly Sensitive ELISA for Quantification of Hepatitis B Virus (HBV) Surface Antigen (HBsAg)

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Background: Hepatitis B Virus (HBV) infection is of global importance and many studies are aimed to develop more sensitive diagnostic tools and improve therapeutic options. HBV infection is diagnosed and followed-up basically by serological studies, among which the enzyme-linked immunosorbent assay (ELISA) is widely used.

Aim: To develop a sensitive HBsAg ELISA for both research and diagnostic purposes.

Materials and Methods: A hybridoma (clone 4D1) secreting anti-HBs IgG2a monoclonal antibody (mAb) was used for the establishment of our in-house ELISA. Tests were performed for both cell culture medium and human sera. The hepatoma cell lines of human origin PLC/PRF/5 and Hep G2 were used for validation assays. Tests for human sera were run concurrently with the Access® Immunoassay System.

Results: The in-house ELISA system had an analytical sensitivity less than 0.41 ng/ml for both human sera and cell culture medium. The performance comparison of our in-house ELISA system with the Access® Immunoassay System even for samples with s/co levels of 1.5-13.7 gave a significant correlation.

Conclusions: These reliable results of the newly established HBsAg ELISA system make it a promising candidate for diagnostic as well as research purposes.

Key Words: HBsAg, monoclonal antibody, ELISA

Hepatit B Virus (HBV) Yüzey Antijeninin (HBsAg) Ölçümü Amacı ile Yüksek Düzeyde Hassas Bir ELISA Kitinin Geliştirilmesi

Giriş ve Amaç: Hepatit B Virus (HBV) enfeksiyonları dünya çapında büyük öneme sahiptir. Terapötik seçeneklerin iyileştirilmesi ve daha hassas tanı araçlarının geliştirilmesi için birçok araştırma yürütülmektedir. Biz, bu çalışmamızda gerek araştırma gerekse tanısall amaçlı kullanılacak hassas bir HBV yüzey antijeni (HBsAg) ELISA kiti geliştirmeyi amaçladık.

Yöntem ve Gereç: ELISA kitinin geliştirilmesi için immünglobulin G2a izotipinde monoklonal antikor salgılayan bir hibridoma klonu (Klon 4D1) kullanıldı. Testler hem hücre kültür besiyeri hem de insan serumu için gerçekleştirildi. İnsan kökenli PLC/PRF/5 ve Hep G2 hepatoma hücreleri kullanılarak geçerlilik çalışması yapıldı. İnsan serumu için yapılan testler aynı zamanda "Access® Immunoassay System" ile de çalışıldı.

Bulgular: Laboratuvarımızda oluşturduğumuz ELISA sistemi ile 0,41 ng/ml'den daha düşük bir analitik sensitivite saptandı. Oluşturduğumuz ELISA sisteminin s/co düzeyleri 1,5-13,7 olan serumlar için yapılan performans karşılaştırmasında önemli bir korrelasyon elde edildi.

Sonuç: Oluşturduğumuz ELISA sistemine dair elde edilen güvenilir sonuçlar, bu testin gerek tanısall gerekse araştırma amaçları için uygun olabildiğini göstermektedir.

Anahtar Sözcükler: HBsAg, Monoklonal antikor, ELISA

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Introduction

Hepatitis B Virus (HBV) infection is of global epidemiological concern, with about 350 million people chronically infected. The endemicity, which varies geographically, is considered high when chronic infection prevalence is about 8%-15%. This means a high cost for HBV infection related diseases and the main goal is prevention of transmission and thus chronic liver diseases due to HBV infection. Vaccination remains the principal means of prevention (1,2).

In order to determine the infection status, various serological markers like HBsAg, anti-HBs, and anti-HBc are assessed. In the pre-nucleic acid testing era, quantitation of HBsAg was found to be a significant tool for predicting the outcome of an acute HBV infection (3). Acute HBV infection patients who show a decrease in HBsAg concentration by more than 50% in the first 3 weeks develop a clearance of HBsAg from the circulation within 6 months, whereas patients who do not show such a decrease become carriers (4). Newer generation, more sensitive enzyme-linked immunosorbent assays (ELISAs) have shortened the window period of HBV infection course by several days in comparison to less sensitive ELISAs. The availability of more sensitive HBsAg ELISAs provides an important means for earlier detection of blood donors who display the window period of HBV infection and thus prevents transfusion related transmissions. Nucleic acid testings show a greater sensitivity in comparison to ELISAs in earlier detection of HBV infection (5). However, the ease of availability and increasing sensitivities of ELISAs make these methods more affordable and applicable in comparison to expensive and labor-intensive assays. The present study aimed to develop an in-house ELISA system for the quantification of HBsAg in human serum and in cell culture medium.

Materials and Methods

Cell lines and monoclonal antibody purification

Previously established "a" epitope specific anti-HBsAg monoclonal IgG2a secreting hybridoma clone 4D1 was cultured in flasks (CellStar®, Greiner Bio-one GmbH, Germany) in RPMI-1640 (Biochrom, Berlin, Germany) supplemented with 10% IgG-depleted FBS (FBS- Gold, PAA Laboratories, Linz, Austria) (6). The culture supernatant was precipitated with 50% (v/v) of saturated ammonium sulfate overnight and dialyzed (Spectra/Por®, 12-14 kDa, Spectrum Medical, USA) against 10 mM, pH 7.4 phosphate buffered saline (PBS) overnight and filtered (0.2 µm filter, Corning Incorporated, Corning, Germany) afterwards. The filtrate was loaded onto a Protein G column (Protein G-Agarose, 2 ml, Roche Diagnostics GmbH, Mannheim, Germany) attached to a high-pressure liquid chromatography system (1100 Series, Agilent Technologies, Waldbronn, Germany) and IgG2a was eluted with 0.1 M citric acid, pH 2.4, and

neutralized immediately with Trizma® base (Tris (hydroxymethyl) aminomethane, Sigma). The peak fraction of the eluate was run through a 0.7 x 30 cm Sephadex G-25 Fine column. Protein content of the purified monoclonal antibody was determined using bovine serum albumin (BSA) as the standard. Anti-HBs value of the purified mAb was assessed by Access® Autoanalyzer.

In order to determine the performance of the developed ELISA with cell culture medium, human hepatocellular carcinoma derived cell line supernatants were tested for their HBsAg content. For this purpose, a hepatoma cell line known to secrete HBsAg, PLC/PRF/5 (ATCC® Number: CRL-8024™), and a hepatoma cell line known not to secrete HBsAg, Hep G2 (ATCC® Number: HB-8065™), were used. Cell lines were thawed and cultured in RPMI-1640 supplemented with 10% FBS in 24-well cell culture plates. Supernatants were collected when 70%-80% confluency was observed for each cell line using an inverted microscope.

Human sera and antigen

Human sera used in this study were collected randomly from residual sera sent to our laboratory for diagnostic purposes. Each sample was tested for its HBsAg, anti-HBs, HBeAg, anti-HBe, anti-HBc (total) content by Access® (Beckmann Instruments Inc., Access® Immunoassay System, which will be referred to hereafter as Access®) and classified according to their HBsAg presence. All samples were stored at -80 °C until study. "ad" and "ay" subtypes HBs antigens were commercially obtained (Human plasma derived, purified, Biodesign, Biodesign International, Saco, ME, USA).

Enzyme-Linked Immunosorbent Assay

The performance of the in-house HBsAg ELISA was evaluated for cell culture medium (RPMI-1640, supplemented with 10% FBS) and pooled human sera (HBsAg negative as determined by Access®) separately. High-binding capacity ELISA microtiter plates (Costar, Corning Inc., USA) were coated with anti-HBs IgG2a at 2 µg/ml in 0.05 M carbonate-bicarbonate buffer (CBB), pH 9.6, 100 µl/well, by overnight incubation at +4 °C. Plates were blocked with 1% BSA-PBS, 200 µl/well for 3 h at RT and washed 3 times with PBS containing 0.05%

Tween 20 (PBS-T). Standard antigen was prepared by mixing equal amounts of "ad" and "ay" subtypes and was serially diluted either in culture medium (RPMI-1640, supplemented with 10% FBS) or human sera. Plates were incubated for 1 h at RT and then washed (ELISA Washer, Columbus Plus, Tecan, Austria) 3 times with PBS-T. To each well was added 100 μ l of biotinylated anti-HBs IgG2a (clone 4D1 derived mAb) at a concentration of 50 ng/ml, which was determined in preliminary assays exploiting a checkerboard system. After the plates were incubated for 1 h at RT, they were washed 3 times with PBS-T. Streptavidin-horse radish peroxidase was added (100 μ l/well) and incubated for 30 min at RT and washed 3 times with PBS-T afterwards. 3,3',5,5' Tetramethylbenzidine (TMB) solution was added (100 μ l/well) and incubated for 30 min at RT and the revealed reaction was stopped by adding 100 μ l of 1 M H₂SO₄ per well. Plates were read at 450/620 nm by an ELISA reader (ELISA Reader, Sunrise, Tecan, Austria).

The minimum detection limit of ELISA was calculated by adding 2 x SD to the mean optical density (OD) obtained with HBsAg negative human sera. Thereafter, to evaluate the performance characteristics of the in-house ELISA, Access[®]-verified human sera were used. Human serum samples were randomly selected from HBsAg positive and HBsAg negative human sera assessed by Access[®] for their antigen content. A total of 48 HBsAg negative and 21 HBsAg positive human sera were studied with the in-house ELISA for their HBsAg amount. Results obtained by our in-house ELISA were analyzed for correlation with the antigen levels measured by Access[®]. Additionally, to test the cut-off value of the in-house ELISA and to compare it with Access[®], HBsAg antigens ("ad": "ay" = 1:1) diluted in Access[®] verified and pooled HBsAg negative human sera were run concurrently with both Access[®] and the in-house ELISA.

Statistical analyses

Regression-correlation analyses were performed and inter- and intra-assay coefficients of variation (CV) were calculated. SPSS for Windows, version 10.0, was used for the statistical calculations.

Results

Analytical sensitivity of the in-house HBsAg ELISA format was evaluated for cell culture medium and pooled

human sera (HBsAg negative as determined by Access[®]) separately. Hepatitis B virus surface antigens ("ad": "ay" = 1:1) at various concentrations were prepared in either culture medium or human sera and were run according to the ELISA protocol described above. As depicted in Figure 1a and 1b, the models gave comparable results. The cut-off values of cell culture medium and human serum ELISA formats were determined by adding 2 standard deviations to the mean OD obtained when antigen negative wells were assayed 4 times. This yielded a cut-off value of 0.091 and 0.085 for human sera and cell culture medium ELISA formats, respectively. According to these baseline data, the analytical sensitivity of the HBsAg ELISA format for both human sera (mean OD = 0.1) and cell culture medium (mean OD = 0.115) was less than 0.41 ng/ml.

Quality control of the in-house ELISA format was evaluated by calculating its CVs for intra- and inter-assay differences in ODs at certain concentrations of antigen.

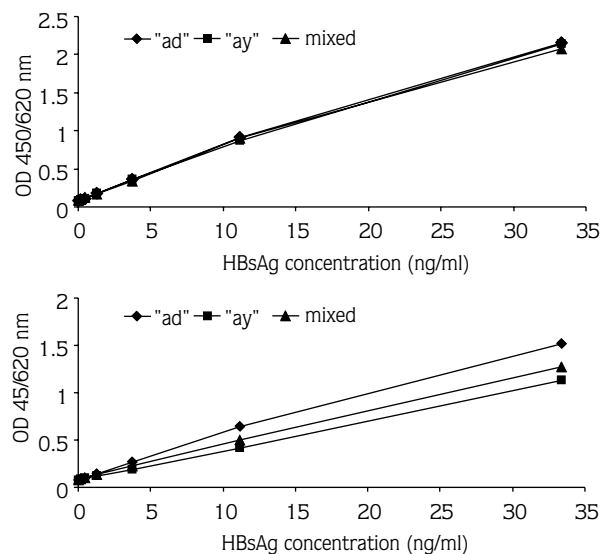


Figure 1. Enzyme-linked immunosorbent assay of HBsAg. Anti-HBs monoclonal antibody-coated wells were incubated with varying amounts of HBsAg subtypes "ad", "ay" or mixed ("ad": "ay" = 1:1). Characteristics of the in-house ELISA format for antigen subtypes and mixed content in cell culture medium and human sera are shown. Figure 1a depicts HBsAg ("ad", "ay", and "ad": "ay" = 1:1) dilution in cell culture medium (for mixed content, $r^2 = 0.994$, $P < 0.01$) and Figure 1b depicts the dilution of the HBsAg in pooled human sera (for mixed content, $r^2 = 0.999$, $P < 0.001$). The overlapping ODs relevant to certain antigen concentrations make symbols overlap especially for antigen concentrations below 5 ng/ml. Results for both depictions are the mean of quadruplicate observations.

This procedure was performed for both cell culture medium and pooled human serum separately. As can be seen in the Table in detail, favorable low variations below 10% were observed for both HBsAg ELISA formats.

The HBsAg ELISA system developed for human sera was also checked for its performance characteristics with Access® predefined human sera. Human sera defined as HBsAg negative (n = 48) and HBsAg positive (n = 21, among them 13 sera with s/co values of 1.5-13.7) by Access® were assayed with the in-house ELISA format. All of the 48 samples determined as negative by Access® were also defined as negative with the in-house ELISA. It was also the case for HBsAg positive human sera and all samples were determined as positive by the in-house ELISA. A high correlation coefficient (Pearson 0.91, P < 0.001) was yielded when the assays were compared for s/co levels of 1.5-13.7, as shown in Figure 2.

When the supernatants of the hepatoma cell lines cultured in RPMI-1640 supplemented with 10% FBS were evaluated by the in-house ELISA, it was observed that the supernatants of PLC/PRF/5 had a prominent high OD. On the other hand, supernatants of Hep G2 had an OD equivalent to the cell culture medium without antigen. Tested supernatants of the cell lines and their relevant ODs are depicted in Figure 3.

In order to test defined concentrations of HBsAg in human sera, HBsAg (“ad”:“ay” = 1:1) was diluted in pooled human sera, which was predefined by Access® as HBsAg negative. Samples were run concurrently with Access® and the in-house ELISA. As shown in Figure 4,

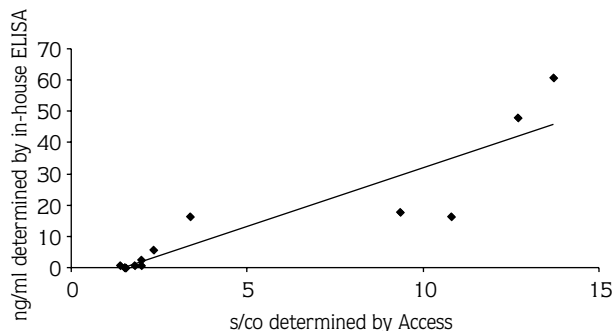


Figure 2. HBsAg serum samples with s/co levels of 1.5-13.7, as determined by Access®, were assayed with the in-house ELISA. As detailed in the results section, the in-house ELISA correctly defined 13 out of 13 serum samples. A high correlation coefficient was yielded between the assays (Pearson correlation 0.91, two-tailed, P < 0.001).

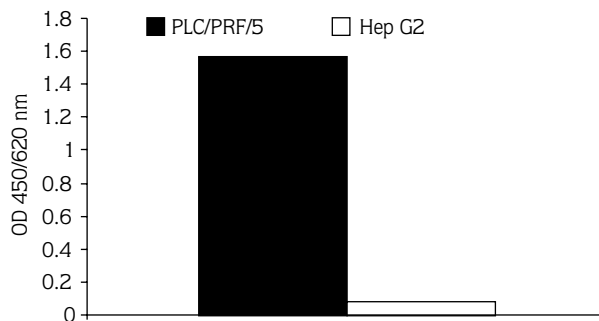


Figure 3. Human hepatoma cell lines PLC/PRF/5 and Hep G2 were cultured in RPMI-1640 supplemented with 10% FBS and supernatants were evaluated for HBsAg content. The mean OD (450/620 nm) of PLC/PRF/5 (dark bar) was found to be 1.58 (equivalent to 24.4 ng/ml mixed antigen content, i.e. “ad”+“ay”). Tested supernatants of Hep G2 cells (empty bar) revealed a mean OD of 0.085, which was below the detection limit of the in-house ELISA and similar to the ODs of cell culture medium without antigen. Results are the mean of quadruplicate observations.

the in-house ELISA could measure the lowest concentration (0.41 ng/ml) that gave a s/co of 1.23 with Access®. The antigen concentration of 0.14 ng/ml was not detectable with Access® or the in-house ELISA. Statistical correlation gave a coefficient of 0.99 (Pearson, P < 0.001).

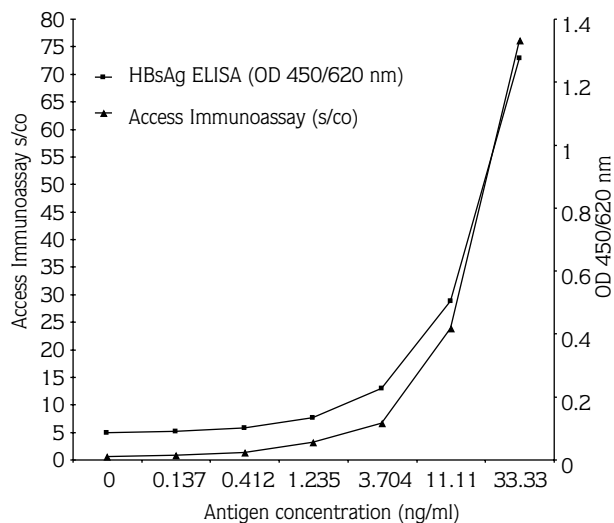


Figure 4. Comparison of HBsAg diluted to various concentrations in human sera with both Access® and in-house HBsAg ELISA. HBsAg antigens (“ad”:“ay” = 1:1) were diluted in Access® verified and pooled HBsAg negative human sera and were run concurrently with both Access® and in-house ELISA. Solid line represents the s/co of one for Access® and dashed line represents the cut-off value of the in-house ELISA (0.091). The correlation coefficient was statistically significant (Pearson correlation 0.99, 2-tailed, P < 0.001).

Discussion

In order to define the infection status in HBV infected individuals or to screen donors for potential HBV infections, several markers are assayed by various methods. Long before the onset of clinical symptoms, HBsAg is one of the serum markers that can be detected in the course of HBV infection. Either for screening or for follow-up purposes, there are many HBsAg detection systems commercially available and ELISAs are widely used for these purposes (7,8). However, there is a continuous challenge regarding the sensitivities of the HBsAg detection systems. By using more sensitive assay systems, low levels of HBsAg can be detected, so that the residual risk of transfusion associated HBV infection can be minimized and the window period can be further reduced. Although there are more sensitive assays available, such as nucleic acid amplification techniques, their limitations make them difficult for wide-range use in blood screening laboratories and thus easily applied methods are being investigated (7). In addition, clinical practices aimed to foresee the HBV infection course, not just the presence but also the quantitation of HBsAg, may also be an important tool in certain research areas (3,9). Several cell lines that secrete HBsAg into the culture medium are being exploited for various hypothesis testing, including anti-viral research, and efficiency of transfections by measuring HBsAg quantitatively (10,11).

The in-house HBsAg ELISA system we report in this study has a sensitive level of measurement of HBsAg with a significant correlation with regard to the validation tests (for human sera, $r^2 = 0.999$, $P < 0.001$; for cell culture medium, $r^2 = 0.994$, $P < 0.01$). Additionally, as detailed in the Table, CVs of both human sera and cell culture

medium formats were good reflections of assay precision and reproducibility (12,13). In order to evaluate the performance characteristics of our in-house ELISA further, we assayed human sera defined by Access[®] as HBsAg negative and HBsAg positive. The results obtained were satisfactory, defining all negative and all positive samples correctly. For sera samples with s/co levels of 1.5-13.7, our ELISA format gave a high correlation with the established Access[®] (Pearson, 0.91, $P < 0.001$). We also assayed 30 PCR verified HBV DNA positive human sera reflecting a certain range of DNA copies/ml (data not shown) and found no correlation with the amount of HBV DNA and HBsAg titers, as also stated in the literature (14). Our in-house HBsAg ELISA system also showed an efficient performance with supernatant of PLC/PRF/5 and Hep G2 cell lines cultured in RPMI-1640 supplemented with 10% FBS. As expected, it measured HBsAg content in the supernatants of PLC/PRF/5 cells but no antigen in the supernatant of Hep G2 cells. When antigens at certain concentrations (diluted in pooled, Access[®] predefined HBsAg negative human sera) were tested for the comparison of cut-off values, it was observed that the in-house ELISA system was as good as Access[®]. Concentrations lower than 0.41 ng/ml fell beyond the cut-off values for both systems, and overall a very high correlation (Pearson correlation 0.99, $P < 0.001$) was observed.

In conclusion, the results of our in-house developed HBsAg ELISA reflect a reliable and cost-effective alternative for certain purposes. It has good sensitivity for HBsAg detection and quantitation. It is promising for investigational analysis of HBsAg even at low concentrations in both human sera and cell culture medium.

Table. Intra- and inter-assay coefficients of variation (CV) of the in-house HBsAg ELISA for cell culture medium and pooled human sera.

	Antigen ^a (ng/ml)	Intra-assay CV% (n = 4)	Inter-assay CV% (n = 4)
Cell culture medium	33	2.1	1.6
	11	1.3	2
	3.7	1.2	2.6
	1.2	0.8	1.2
Human sera	33	0.6	1.1
	11	2.2	1.6
	3.7	4.9	4.5
	1.2	6.8	5.5

^a Hepatitis B virus surface antigens "ad" and "ay" = 1:1

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