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Serological detection of hepatitis B viral infection by a panel of solid-phase enzyme-linked immunosorbent assays (ELISA)

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Abstract

Immunoassays for the detection of hepatitis B virus (HBV) in biological samples were developed. Using recombinant HBV antigens (Ags) and HBV-specific antibodies (Abs), we designed and evaluated a panel of enzyme-linked immunosorbent assays (ELISAs) detecting the main hepatitis B-related viral markers, namely HBV surface Ag (HBsAg), HBV e Ag (HBeAg), Abs to HBsAg (anti-HBs), Abs to HBV core Ag (anti-HBc) and Abs to HBeAg (anti-HBe), in blood serum. The ELISAs were validated using a panel of prescreened, by commercial tests, serum samples. In principle, HBV Ags or anti-HBV monoclonal antibodies (mAbs) were immobilised on microplate wells. Horseradish peroxidase (HRP) or biotin were used to prepare labeled Abs. Specifically for the determination of HBsAg and HBeAg, two-site sandwich immunoenzymometric assays were developed. The useful range was estimated at 20–500 ng/ml and human serum samples assayed were diluted 10- and 4-fold for HBsAg and HBeAg, respectively, with phosphate buffered saline (PBS) containing Tween 20 and gelatin. For the detection of Abs to HBs an indirect ELISA was formulated. Sera were similarly 4-fold diluted in the same buffer. Finally, competitive ELISAs were used for detecting anti-HBc and anti-HBe and sera tested were diluted 20- and 5-fold, respectively. All selected dilutions resulted in the accurate and reliable determination of HBV Ags and anti-HBV Abs. Taken altogether, these ELISAs are highly specific and equally sensitive to the circulating tests. However, their design could be very useful for research and/or preclinical studies of selected HBV-infected individuals.
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Keywords: Hepatitis B; ELISA; Solid-phase assays; HBV antigens; HBV antibodies

Abbreviations: Ab, antibody; Ag, antigen; cbc, carbonate–bicarbonate buffer; ELISA, enzyme-linked immunosorbent assay; HBV, hepatitis B virus; HRP, horseradish peroxidase; mAb, monoclonal antibody; OD, optical density; OPD, *ortho*-phenyldiamine; PBS, phosphate buffered saline; PBS–T, PBS–0.1% Tween 20; PBS–T–G, PBS–0.1% Tween 20–0.5% gelatin; TMB, trimethylbenzoate

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1. Introduction

Hepatitis B is a widespread disease and nearly 5% of the world's population is currently infected with hepatitis B virus (HBV). Moreover, almost 400 million are chronic carriers of HBV [1]. HBV is a noncytopathic, enveloped virus, which contains a fully elucidated circular double-stranded DNA genome. HBV-DNA sequencing revealed the presence of four open reading frames, encoding for known and putative proteins, namely S (encoding for HBV-associated main surface protein), C (encoding for the nuclear/core protein), P (encoding for DNA polymerase) and X (gene encoding for a polypeptide which functions as a transcriptional transactivator on various regulatory elements) [2]. As HBV causes acute and chronic necroinflammatory disease and HBV carriers face an increased risk of developing cirrhosis and hepatocellular carcinoma [3], information on either the presence or absence of HBV in the serum of an infected person is necessary. HBV-specific assays are routinely used for the assessment of disease activity in persistent infection, for monitoring therapeutic regimes with antiviral agents and most importantly, for evaluating the infection in a donor's blood to prevent recipients' contamination. As for today, the most accurate diagnostic tool available is the detection of viral DNA, usually performed by sensitive PCR [4]. However, the course of HBV infection is determined by the interplay between viral replication via HBV protein production and the host's immune response, and thus clinical practice diagnosis of HBV infection is established by the serological detection of HBV protein products (antigens; Ags) as well as host-produced antibodies (Abs) (Fig. 1). HBV Ag and anti-HBV Ab detection is often simultaneously carried out in the same serum or plasma specimens, using enzyme-linked immunosorbent assays (ELISAs), highly specific for HBV surface antigen (HBsAg), Ab to HBsAg (anti-HBs), Ab to HBV core Ag (anti-HBc), and HBV e Ag (HBeAg) or Ab to it (anti-HBe). Although recent data suggest that HBsAg correlates only slightly with viral replication [4], HBsAg is the primary diagnostic marker used for screening blood products in hospitals and health-care facilities [5]. HBeAg is considered as a better marker of viral blood infection, whereas the development of host Abs to HBs (anti-HBs) and HBe (anti-HBe) indicate the assessment of immunity and the reduction of viral replication in the infected individual. The persistent presence of host Abs to HBc (anti-HBc) is associated with chronic HBV infection and can select for HBV-infected samples in the absence of HBsAg and/or anti-HBs [5].

In this study, we report the in-house development of a panel of ELISAs for the detection of HBV infection markers using a series of commercial recombinant HBV Ags and anti-HBV Abs. Various assay types were used for setting the HBsAg, HBeAg, anti-HBs, anti-HBc and anti-HBe ELISAs, some of which differ in principle from the commercial assays. We specifically selected the development of ELISAs since this type of solid-phase assay systems, beside their considerable spectrum of applications [6], are currently

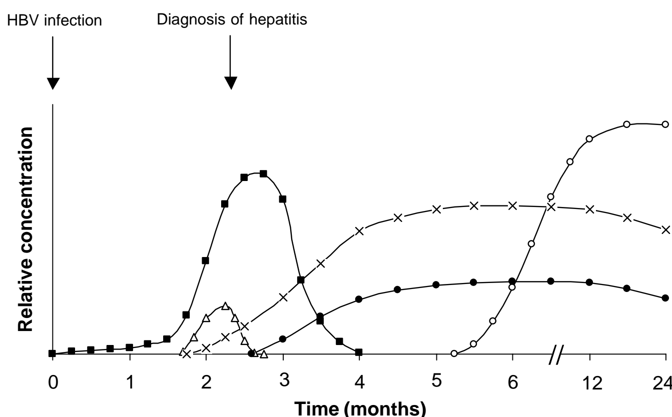

Fig. 1. Schematic representation of the time sequence of HBV serological markers observed in patients with acute hepatitis B. (■) HBsAg; (△) HBeAg; (×) anti-HBc; (●) anti-HBe; (○) anti-HBs.

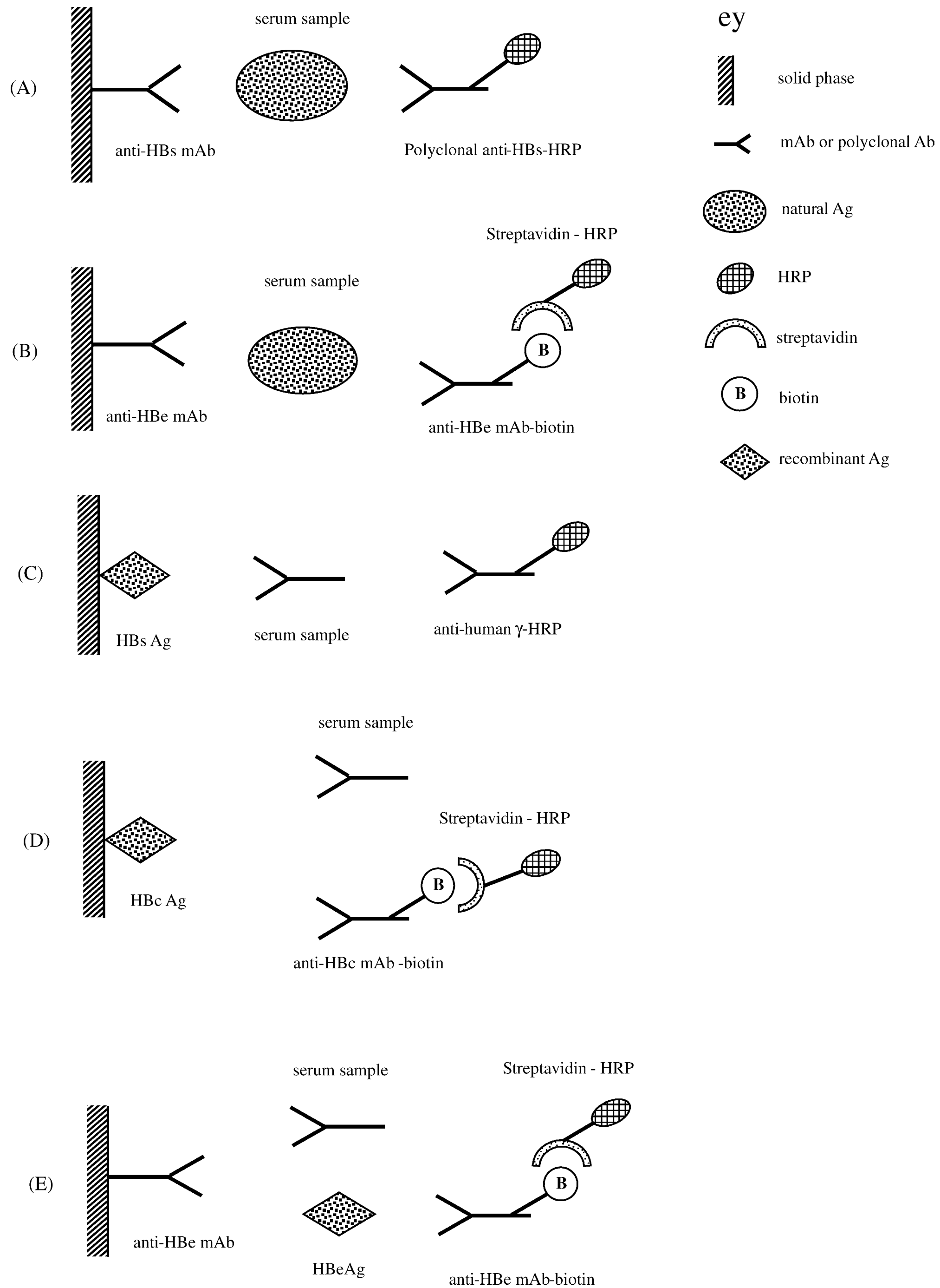

Fig. 2. Schematic overview of ELISA principle used for the design of HBV-specific assays. (A) Sandwich ELISA for the detection of HBsAg; (B) sandwich ELISA for the detection of HBeAg; (C) indirect ELISA for anti-HBs determination; (D) competitive ELISA for anti-HBc detection; (E) competitive ELISA for anti-HBe detection.

ranked among the most sensitive laboratory methods that allow the rapid, easy, reliable and accurate detection of viral Ags and Abs.

Although the basic concept of the ELISA methodology remains the same since its initial development in 1970s, many assay variations have evolved [7,8]. For the detection of HBeAg an Ag capture assay was selected. A capturing monoclonal Ab (mAb) specific for HBeAg is adsorbed onto the solid matrix. The Ag–Ab complex formed, is determined by the subsequent binding of a detection anti-HBe Ab conjugated to an indicator molecule (e.g. biotin), to allow for quantitative analysis of binding (for schematic representation see Fig. 2B). Appropriate controls ensure that the detection Ab does not bind non-specifically to the Ag or to impurities within the sample and that the detection Ab does not cross-react in the absence of Ag with the solid phase. When cross-reaction cannot be avoided, as in the HBsAg ELISA, capture and detection Ab from different host animals are selected, to prevent same species Ab binding (Fig. 2A).

The HBV Ab-detecting ELISAs are designed based on similar principle. For the anti-HBs ELISA, the solid matrix is pre-coated with recombinant HBsAg. The solid-phase-bound Ab is detected using a secondary Ab directed against the constant region (Fc) of the specific Ab isotype (indirect ELISA; Fig. 2C). A competitive ELISA was designed for anti-HBc detection. Serum and an anti-HBc-specific Ab–biotin conjugate were co-incubated with the capture Ag. The amount of color developed, is inversely proportional to the amount of serum anti-HBc (Fig. 2D). Finally, a variation of this format was selected for the anti-HBe detection assay. Specific anti-HBe Ab was bound on the solid phase and a standardized, constant amount of HBeAg was used as competitor (Fig. 2E). In the absence of serum anti-HBe, the conjugate anti-HBe–biotin binds on the HBeAg, already attached to the solid phase and the amount of HBe–anti-HBe complexes formed is inversely proportional to the degree of inhibition determined.

By screening a panel of HBV-infected sera samples, we show that the aforementioned ELISAs do not differ, in terms of sensitivity, specificity, accuracy and reproducibility, from the circulating tests. Moreover, the HBV-specific ELISAs developed herein require less amount of serum (4- to 20-fold, compared to the commercial tests) and by using the appropriate Ag or Ab standard solutions, can quantify the respective HBV marker. They can be therefore potentially used for research screening of HBV-infected blood samples or for determining HBV-related Ags or Abs in selected infected individuals where quantification could be useful for follow-up monitoring.

2. Experimental

2.1. Antigens and antibodies

HBV Ag and Ab to HBV Ag were purchased from Fitzgerald Industries International, Inc. (MA). Recombinant HBsAg (30-AH37), HBcAg (30-AH39) and HBeAg (30-AH18) were produced in yeast or *E. coli*. Anti-HBs (clone M701079) and anti-HBe (clone M410512) are mouse subclass IgG1 mAbs. Anti-HBc (clone M41058) and anti-HBe (clone M410513) are mouse subclass IgG2a mAbs. Anti-HBs–HRP is an affinity-purified goat polyclonal Ab conjugated to HRP. Goat anti-human IgG (γ-specific)–HRP was purchased from Sigma.

2.2. Reagents

All chemicals, including horseradish peroxidase (HRP), biotin, streptavidin–HRP, trimethylbenzidine (TMB), *ortho*-phenyldiamine (OPD), Tween 20 and gelatin were from Sigma.

2.3. Source of samples

A panel of 50 human serum samples from patients with clinical indications of HBV infection, HBV carriers and HBV negative individuals was used in this study. The samples derived from the Department of Epidemiology and Medical Statistics, Athens School of Hygiene, Athens, Greece and were previously tested for HBV markers by commercial HBV ELISA kits. The characteristics of these samples are summarized in Table 1. Briefly, the samples were categorized into four groups based on the serological profiles of the individuals as follows:

- group A, six individuals with high viral replication profile whose sera contained HBsAg, anti-HBc and HBeAg;

Table 1
Serological profile of serum panel according to HBV positive (+) and negative (−) markers as tested by commercial HBV ELISAs (see Section 2.3)

	n	HBsAg	Anti-HBs	Anti-HBc	HBeAg	Anti-HBe
Group A	6	+	−	+	+	−
Group B	12	+	−	+	−	+
Group C	12	−	+	+	−	+
Group D	20	−	−	−	−	−
Total	50	18[a]	12[a]	30[a]	6[a]	24[a]

n: number of samples.
[a] Number of positive samples for each specific HBV marker.

- group B, 12 individuals with low viral replication profile whose sera were positive (+) for HBsAg, anti-HBc and anti-HBe but negative (−) for HBeAg;
- group C, 12 individuals with an atypical and protection markers profile, with sera that tested (+) for anti-HBs, anti-HBc, anti-HBe and had any of these markers as (+) alone or in combination; and
- group D, 20 age and sex-matched control samples with a negative profile, with sera tested (−) for all HBV markers.

2.4. Preparation of anti-HBe–biotin and anti-HBc–biotin conjugates

Two milligrams of each Ab (anti-HBe, M410512 and anti-HBc), were dialyzed overnight at 4 °C over $NaHCO_3$ solution (0.1 M). One milligram of biotin-*N*-hydroxysuccinimide ester was diluted just prior to use in 30 μl DMSO, and 10 μl of the solution was added to the Ab. The mixture was incubated for 1 h at room temperature and dialyzed overnight at 4 °C over phosphate buffered saline (PBS; 10 mM, pH 7.4, containing 150 mM NaCl). Glycerol (1:1 v/v) was added and aliquoted conjugates were stored at −70 °C.

Optimal dilutions of conjugates were predetermined from titration experiments, using standard Ag concentrations. The selected dilutions, hereafter used in the ELISAs, correspond to 50% binding.

2.5. Direct ELISA for the detection of HBs antigen

Monoclonal anti-HBs was dissolved in carbonate–bicarbonate (cbc) buffer (0.1 M, pH 9.5) at a final concentration of 5 μg/ml. The wells of 96-well flat-bottomed microtiter plates (Maxisorb, Nunc, Rocksilde, Denmark) were coated with anti-HBs (100 μl per well) and incubated for 1 h at 37 °C and overnight at 4 °C. The optimal Ab concentration was determined by checkerboard titration. After coating, wells were washed three times with PBS and free binding sites were blocked with PBS containing 0.5% gelatin (PBS–G; 150 μl per well), for 45 min at 37 °C. Wells were washed three times with PBS containing 0.05% Tween 20 (PBS–T) and pat dry. For Ag testing, HBsAg was serially diluted (2 μg/ml to 2.7 ng/ml) in PBS–T–gelatin (PBS–T–G), and 100 μl of each dilution was added to the wells of the plate. Selected human sera samples were also serially diluted (1:5–1:320) in the same solution and 100 μl of each dilution was added to the wells. Plates were incubated at 37 °C for 90 min, washed four times with PBS–T and 100 μl of HRP-conjugated goat anti-HBs diluted 1:1000 in PBS–T–G was added to each well. After incubation at 37 °C for 90 min, plates were washed four times with PBS–T and once with PBS. The assay was completed by the addition of the enzymic substrate-chromogen H_2O_2-TMB (100 μl per well). The reaction was permitted to proceed for 10 min at room temperature in the dark, and the reaction stopped by the addition of 2N H_2SO_4 (50 μl per well). Light absorption was determined at 450 nm using a Dynatech MR 5000 photometer (Dynatech, Chantilly, VA).

The panel of sera, previously screened for HBsAg by Hepanostika HBsAg Uni-Form II (Organon Teknica Diagnostics), were diluted 1:10 with PBS–T–G and assayed with the developed ELISA. Samples with an optical density at 450 nm ($OD_{450\,nm}$) > 2.5 × standard deviations (SD) above the mean absorbance of (−) control sera were considered as (+) for HBsAg.

2.6. Direct ELISA for the detection of HBe antigen

Monoclonal anti-HBe (M410513) was dissolved in cbc buffer at a final concentration of 1 μg/ml as determined by titration experiments. Plates were coated with the diluted anti-HBe (100 μl per well), incubated, washed and saturated as previously described for the HBsAg ELISA. For Ag testing, HBeAg was serially diluted (1 μg/ml to 0.45 ng/ml) in PBS–T–G, and 100 μl of each dilution was added to the wells. Selected human sera samples were serially diluted (1:2–1:64) in PBS–T–G and 100 μl of each dilution was added to the wells. Plates were incubated at 37 °C for 1 h and washed four times with PBS–T. Anti-HBe (M410512)–biotin conjugate diluted at a final concentration of 2 μg/ml in PBS–T–G was added (100 μl per well). After incubation for 1 h at 37 °C, the plates were washed four times with PBS–T. Streptavidin–HRP was diluted 1:10,000 in PBS–T–G, 100 μl of the solution/well was added and the plates were incubated for 45 min at 37 °C. Following four washes with PBS–T and two washes with PBS, the assay was completed by the addition of H_2O_2-TMB (100 μl per well) following the same protocol as for HBsAg determination. As for intra-assay negative controls, anti-HBe–biotin conjugate and streptavidin–HRP or only streptavidin–HRP were added to coated plates, without the concomitant addition of Ag.

The panel of sera, prescreened for HBe antigen by the commercial test HBe (rDNA) EIA (Abbott Laboratories) were diluted 1:4 with PBS–T–G and assayed. Samples with an $OD_{450\,nm} > 2.5 \times SD$ above the mean absorbance of (−) control sera were considered as (+) for HBeAg.

2.7. Indirect ELISA for the detection of anti-HBs antibodies

The wells of a 96-well plate were coated with HBsAg diluted in cbc buffer at a final concentration of 2 μg/ml (100 μl per well) and proceeded as mentioned previously (see Section 2.5). After three washes with PBS–T, 100 μl per well of serially diluted selected serum samples (1:2–1:32) was added and incubated for 90 min at 37 °C. The plates were washed five times with PBS–T. Following conjugate titration, anti-human IgG–HRP was diluted 1:2000 with PBS–T–G, added to the plate (100 μl per well) and incubated for 1 h at 37 °C. Plates were further washed four times with PBS–T and twice with PBS. The assay was completed by adding H_2O_2-TMB and as described above. Marginal absorbance was recorded in control wells coated with Ag, containing only conjugate.

The panel of sera tested with the developed anti-HBs assay was initially screened with AUSAB EIA (Abbott Laboratories). Samples were diluted 1:4 with PBS–T–G and assayed. Anti-HBs (+) sera were considered, when they presented $OD_{450\,nm}$ values > $2.5 \times SD$ above (−) control samples.

2.8. Competitive ELISA for the detection of anti-HBc antibodies

HBcAg was diluted at 2 μg/ml in cbc buffer and added to the microplate (100 μl per well). Washing and blocking steps were performed as already described. Sera samples (diluted 1:20–1:320 in PBS–T–G) and the conjugate anti-HBc–biotin (diluted 1:45,000 in PBS–T–G) were simultaneously added to the wells (50 μl of each/well) and incubated for 90 min at 37 °C. Wells were washed five times with PBS–T, dried, streptavidin–HRP was added and the assay was completed as described in the HBeAg ELISA. $OD_{450\,nm}$ was determined. To the (−) control Ag-coated wells, anti-HBc–biotin conjugate and streptavidin–HRP or only streptavidin–HRP were added, and the absorbance recorded in the former corresponded to 100% of binding of the biotinylated mAb to the HBcAg.

The panel of serum samples was screened by MONOLISA anti-HBc (Sanofi-Pasteur). The same samples were diluted 1:20 with PBS–T–G and assayed with the developed test. Anti-HBc (+) sera were selected when inhibition (%) determined was >40%.

2.9. Competitive ELISA for the detection of anti-HBe antibodies

The wells of 96-well microtiter plates were coated with anti-HBe (M410513), washed and blocked following the same procedure as described for the HBe ELISA (Section 2.6). Recombinant HBeAg at a final concentration of 100 ng/ml (referred to as neutralizing concentration) (50 μl per well) and serum samples, serially diluted in PBS–T–G (1:5–1:320) (50 μl per

well), were concomitantly added to each well. Plates were incubated at 37 °C for 1 h and washed four times with PBS–T. The assay further proceeded as previously described for the HBeAg ELISA and the absorbance was recorded at 450 nm. Coated wells incubated with HBeAg at a final concentration of 100 ng/ml were used as for intra-assay controls and the $OD_{450\,nm}$ obtained from these wells represents 100% binding between the neutralizing reagent and the anti-HBe antibodies. Results are expressed as percentage of inhibition of 100% binding in the presence of anti-HBe antibodies in the assayed test sera.

The panel of sera was screened for anti-HBe by HBe (rDNA) EIA (Abbott Laboratories). The same sera were diluted 1:5 with PBS–T–G and assayed with the developed ELISA. Samples presenting over 40% inhibition were considered as (+) for anti-HBe.

3. Results and discussion

3.1. Design and evaluation of ELISA for HBsAg

Sandwich ELISA is very popular for the routine screening of biological samples, due to its reliability and its ease in operation without using radioactivity. The combination of two Abs, recognizing different epitopes of the same Ag, increases both the sensitivity and the specificity of the assay. Herein, a polyclonal enzyme-labeled anti-HBs Ab was selected as detection Ab and a mAb anti-HBs was used for immobilization because of its stronger reactivity for HBsAg. The combination of different Ab-producing host animals (goat and mouse) resulted in the generation of a highly specific and sensitive ELISA, which yielded no cross-reactivity between HBs and other HBV-related (e.g. HBeAg, anti-HBc, anti-HBe), or HBV-irrelevant substances present within the serum samples assayed (data not shown).

The concentration of mAb anti-HBs to be used in the assay was determined by initial titration experiments, where plates were coated with 5–0.1 ng/ml. As expected, the highest OD resulted from the highest concentrations of anti-HBs (5 ng/ml) (data not shown). Although lower antibody concentrations (up to 1 ng/ml) yielded a clear discrimination between (+) and (−) samples, the highest concentration (5 ng/ml) was selected to coat the plates in the remainder of the study in order to increase the sensitivity of our method. Using recombinant HBs as Ag, the useful range of our assay was estimated at 20–500 ng/ml (Fig. 3A).

We then examined the optimum serum dilution for use in the HBsAg assay. Selected HBsAg (+) and HBsAg (−) samples were serially diluted 1:5–1:320 in the assay's solution (PBS–T–G). As shown in Fig. 3B, 1:5 and 1:10 dilution of the samples resulted in high ODs, and clear-cut discrimination between (+) and (−) samples could be reliably detected. Consequently, a serum dilution of 1:10 was selected to screen the sera panel with the formulated HBsAg assay.

Of the four sample groups assayed at random it was found that:

(i) eighteen HBsAg (+) samples assayed, were (+) for HBsAg;
(ii) twelve HBsAg (−) samples (group C; Table 1) were (−) for HBsAg; and
(iii) all 20 HBsAg (−) control samples were (−) for HBs.

The cut-off value of the ELISA was determined by calculating the OD of the control group (group D). Their mean OD plus 2.5 × SD was selected as cut-off point, and therefore assayed samples yielding an OD above this value were considered as (+). Even when the cut-off was set by adding 3 × SD to the mean OD of control samples, the same percentage of (+) samples was detected, indicating the high specificity of our assay.

Compared to Hepanostika HBsAg Uni-Form II ELISA (Organon Teknika Diagnostics), we observed that our test presents indeed equal sensitivity and specificity. The comparison of our assay with Hepanostika was deliberately made, due to the same ELISA principle used. In both cases, Abs from different host animals (murine and goat in our test, murine and ovine in Hepanostika) are used as capturing and detecting reagents, respectively. However, polyclonal anti-HBs was selected to be used as detecting Ab, which adds a crucial parameter. Recognition of various HBs antigenic determinants (deriving from the four major HBsAg subtypes, designated adw, adr, ayw and ayr [1,9]) by the polyclonally-derived Abs enhances the binding ability of the conjugated reagent and eliminates non-specific cross-reaction between irrelevant epitopes present in the serum and the de-

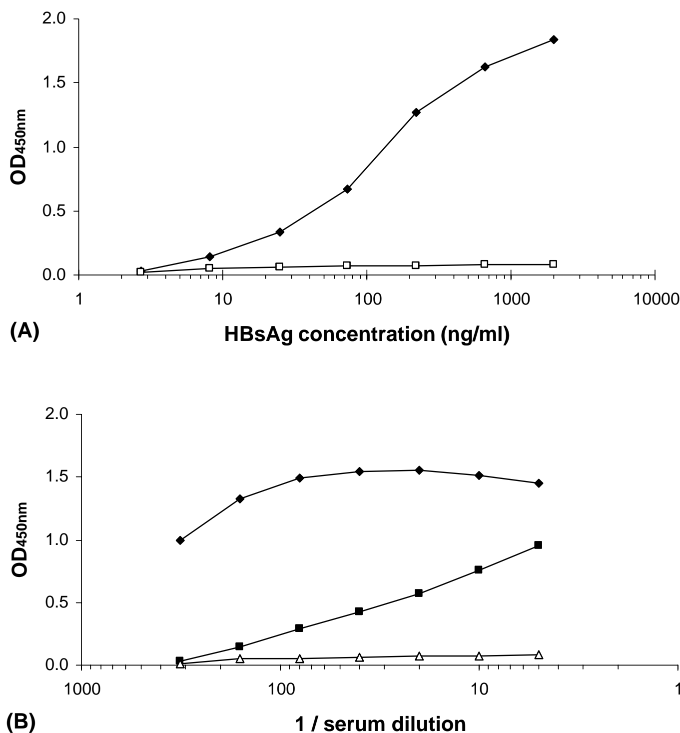

Fig. 3. ELISA for the detection of HBsAg. Increasing concentrations of recombinant HBsAg (■) or HBeAg (□) (A) and different dilution of two HBsAg (+), (◆) and (■), and one HBsAg (−) (△) serum samples were tested for binding with anti-HBs mAb, immobilized on the solid support (B). Bound Ags were detected with anti-HBs HRP-conjugate, followed by the substrate H_2O_2-TMB. ODs were read at 450 nm. For further details see Section 2. Mean values from triplicate analysis are given.

tecting antibodies. A similar principle is used in Auszyme HBsAg EIA (Abbott Laboratories), where a mixture of murine anti-HBs mAbs–HRP conjugates, is used as detecting reagent, resulting in the increase of the assay's sensitivity.

The differences in the OD values determined between the commercial tests and our ELISA could be due to the significantly prolonged incubation periods and/or more washing steps, which are eliminated in routine practice. Furthermore, the plates were coated with the relatively high, however optimal concentration of Ab (5 ng/ml), as was shown by titration experiments. All these parameters resulted in the formulation of a sensitive HBsAg assay, which is equally sensitive and specific to the circulating tests.

Taking into consideration the prolonged duration of our test in its present form, we do not suggest that it could substitute any commercial assay, used for high throughput sera screening. On the contrary, our assay presents an alternative methodology, which can be used for evaluating the clinical outcome of selected HBV-infected patients where besides easiness, high sensitivity is required. It also offers an additional benefit of quantitative measurement of HBsAg concentration in the assayed samples.

3.2. Design and evaluation of ELISA for HBeAg

The ELISA for the detection of HBeAg is also a sandwich assay. Two murine mAbs for HBeAg were used. Coating was performed with the mAb which presented the highest affinity for the Ag. Immobilized Ab gave satisfactory response at concentrations ranging from 2 to 0.5 μg/ml (data not shown). For practical reasons 1 μg/ml was selected for coating, since this concentration is sufficient for the accurate HBeAg determination without the concomitant consumption of high amount of anti-HBe. The detecting Ab was cou-

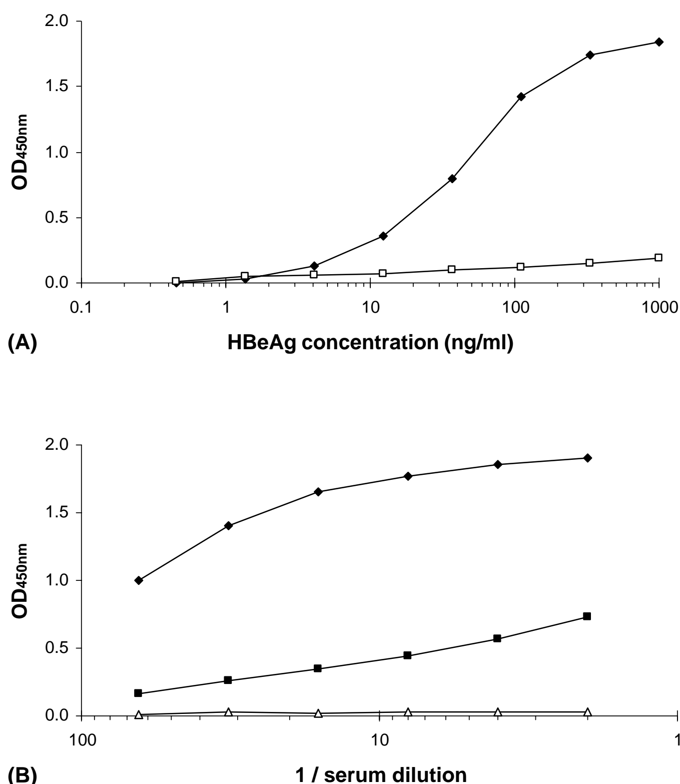

Fig. 4. ELISA for the detection of HBeAg. Increasing concentrations of recombinant HBeAg (■) or HBsAg (□) (A) and different dilution of two HBeAg (+), (◆) and (■), and one HBeAg (−) (△) serum samples were tested for binding with anti-HBe mAb (B). Other details as in Fig. 3.

pled to biotin, because the biotinylation procedure is much simpler and the yield of biotinylated Ab tends to be higher compared to HRP-labeled Ab [10]. This indirect labeling of the Ab and its detection through biotin–streptavidin interaction, although it requires additional incubation and washing steps, significantly improved the ELISA's sensitivity. Therefore, the useful range of the HBeAg assay did not differ much from the HBsAg assay and ranged between 10 and 500 ng HBeAg/ml (Fig. 4A).

This was better proven by screening HBeAg selected (+) and (−) samples. Serial dilutions of 1:2–1:64 showed that 1:4 serum dilution presented clear-cut discrimination between (+) and (−) samples (Fig. 4B). The difference observed in the dilution used herein and in the HBsAg ELISA (1:4 versus 1:10, respectively) reflects partly the differences in the coating concentration of the respective monoclonal Ab (1 μg/ml versus 5 μg/ml). The serum panel was therefore diluted 1:4, tested and the results showed that:

(i) six HBeAg (+) sera were (+) for HBeAg also with our assay;
(ii) twenty-four HBeAg (−) sera (groups B and C; Table 1) were (−) for HBeAg; and
(iii) the 20 HBV(−) control samples were, as expected, HBeAg (−).

As in the previous ELISA, HBeAg (+) samples presented OD value of the cut-off, calculated as mean OD value of negative controls $+2.5 \times$ SD, albeit the addition of $3 \times$ SD did not subvert the outcome in any of the assayed samples.

Immunoreactivity comparison of our assay with the HBe(rDNA) EIA (Abbott), which uses the same ELISA principle, demonstrated that both assays can meet clinical needs for sensitivity and specificity. For reasons related to the prolonged duration and the more

than 20 steps of reagent addition necessary, we do not suggest that this conventional ELISA can be used for the massive screening of serum samples. However, the option of formulating an HBeAg-specific test for research purposes with the potential of accurately quantifying Ag concentration, makes our test useful.

3.3. Design and evaluation of anti-HBs ELISA

For the detection of host-produced Abs to HBsAg, an indirect ELISA was designed. Recombinant HBsAg was used as capturing agent and plates were coated with optimal amount of Ag (2 μg/ml). This concentration was selected on the basis of checkerboard titration against a known anti-HBs (+) and an anti-HBs (−) sample and yielded a satisfactory binding ability. We selected to coat the plates with purified full length HBsAg, since host B cells recognize both, conformational and linear antigenic epitopes on HBsAg [11]. The development of an ELISA using short synthetic peptides spanning linear epitopes of HBV surface protein as the Ag target could significantly restrict Ag recognition by different HBV genotypes, reduce the accuracy of the test and consequently the induction of an anti-HBV immune response could be overlooked [9,12]. Specific binding of anti-HBs present in the serum to immobilized HBsAg was further detected using a goat anti-human γ-specific Ab, which selectively binds to human IgGs. We specifically selected this reagent, since anti-HBs antibodies of IgG isotype, render the host, immune to further HBV infection and are of crucial clinical importance.

By testing selected anti-HBs (+) and (−) samples serially diluted from 1:2 to 1:32, we observed that an optimal serum dilution, which would result in reliable anti-HBs determination, would be 1:4 (Fig. 5). Next, with the developed assay, the available serum panel was screened and the results are as follows:

(i) twelve anti-HBs (+) samples were also found (+) with our assay;
(ii) eighteen anti-HBs (−) samples (groups A and B; Table 1) were anti-HBs (−); and
(iii) all 20 (−) control samples were anti-HBs (−).

The cut-off value of the assay, either calculated as mean OD of (−) control samples +2.5 × SD or +3 × SD, did not alter any of the results.

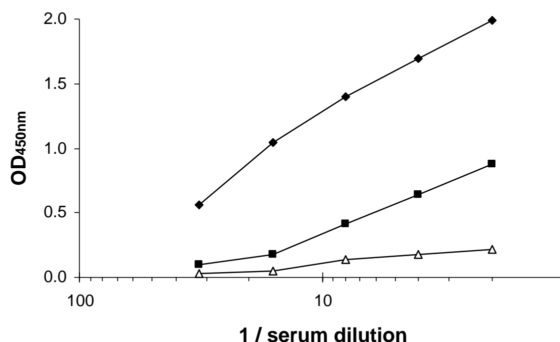

Fig. 5. ELISA for the detection of anti-HBs. Sera from anti-HBs (+), (◆) and (■), and an anti-HBs (−) (△) individuals were serially diluted and tested for binding to immobilized HBsAg. Bound Abs were detected by anti-human γ-specific Abs labeled with HRP. Mean values from quandruplicate analysis are presented.

Commercial anti-HBs assays (e.g. AUSAB EIA, Abbott Laboratories) are based on the principle of competitive ELISA. We present herein an indirect anti-HBs ELISA, which is easy to develop and has high sensitivity, comparable to that of the circulating tests. It would be interesting to assay borderline samples with the designed assay, to precisely elucidate its potential clinical use. Additionally, our anti-HBs ELISA may be proven useful for testing samples of limited volume, since it requires 1/4 of serum used in the commercial tests.

3.4. Design and evaluation of ELISA for the detection of anti-HBc

Antibodies to HBV core Ag are important markers that show de novo acquisition of HBV infection [9]. Anti-HBc determinations are useful for monitoring the progress of HBV infection and in cases of acute hepatitis B may be the only detectable serological marker. The importance of its early detection in blood products classified as HBV (−) due to the absence of HBsAg and/or anti-HBs [13], resulted in the generation of highly sensitive ELISA tests. Herein, we used the same ELISA principle as the commercial tests CORZYME (Abbott Laboratories) and MONOLISA anti-HBc (Sanofi-Pasteur). In the competitive ELISA designed, we used immobilized recombinant HBcAg as capture reagent, and biotinylated mAb anti-HBc as conjugate competing with anti-HBc present in the

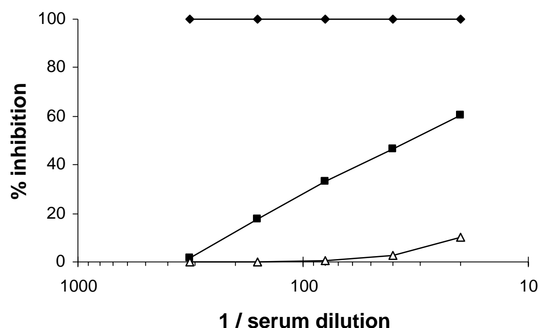

Fig. 6. ELISA for the detection of anti-HBc. Inhibition of binding of anti-HBc–biotin conjugate to immobilized recombinant HBcAg by anti-HBc present in sera of anti-HBc (+) (◆) and (■) individuals is shown. Diluted samples from an anti-HBc (−) (△) serum simultaneously assayed, shows no inhibition. The curves indicate inhibition (%) plotted vs. serum dilution. Mean values from triplicate analysis are presented.

specimen for the limited number of HBcAg binding sites in the well. The concentration of the coating Ag (2 μg/ml) was selected following titration experiments (data not shown). Similarly to the anti-HBs ELISA, this Ag concentration yielded high OD values when (+) sera (diluted 1:20–1:320) were assayed. In this type of competitive assay, results are usually expressed as inhibition (%) of Ab binding to the Ag. Forty percent inhibition was selected as cut-off value in our assay. As can be noticed, sera could be clearly distinguished in anti-HBc (+) and anti-HBc (−) samples when diluted 1:40 with the assay buffer (PBS–T–G) (Fig. 6). We selected as optimal sample dilution 1:20 in order to determine safely HBV infection. The panel of sera collected was used for the evaluation of the formulated assay. The results were as follows:

(i) thirty anti-HBc (+) sera, yielding an inhibition (%) above the cut-off value (groups A, B and C; Table 1) were considered as (+) also with our anti-HBc ELISA; and
(ii) the 20 anti-HBc (−) control sera were anti-HBc (−) with our test as well.

Our assay detects both anti-HBc IgM and IgG Abs, since in the competitive ELISA set up, there is no Ig class discrimination. Different assay formulation would be required for detecting specifically either anti-HBc IgM or anti-HBc IgG classes, preferably by the indirect ELISA principle, as previously mentioned for anti-HBs Abs. However, the presented assay meets the clinical needs for sensitivity and specificity compared to the commercial tests and can be safely used for serum screening. In this design, the streptavidin–biotin system was introduced. Compared to HRP, biotin detection system favors the sensitivity of the ELISA [10]. Furthermore, the use of substrate-chromogen H_2O_2-TMB instead of H_2O_2-OPD results in the measurement of higher absorbance values with our assay compared to CORZYME. Indeed, in optimization experiments set to test the binding affinity of the anti-HBc conjugate to HBcAg, TMB presented higher sensitivity over OPD (50% binding of the conjugate to the Ag was achieved with 100 μl per well TMB diluted at 1:8000 or OPD diluted 1:4000; data not shown).

As already mentioned for the previous ELISAs, the use of 1/20 the amount of serum for valid anti-HBc results is the major advantage of our assay. However, the duration needed for its completion eliminates its potential use for large-scale surveys.

3.5. Design and evaluation of ELISA for the detection of anti-HBe

The anti-HBe detecting ELISA is also a competitive assay. However, in this design, plates are coated with anti-HBe mAb, which competes with anti-HBe, if present in the serum, for binding to a standardized amount of HBeAg, referred to as neutralizing agent. It is evident that the amount of the neutralizing Ag added, should be proportional to the Ab concentration used for coating. The same detection system (biotin–streptavidin) as in the HBeAg ELISA was used and the results were compared with the HBe (rDNA) EIA (Abbott) test, utilizing the same principle.

Selected sera were assayed at different dilutions (1:5–1:320). Forty percent inhibition was selected as cut-off value in this assay. As is shown in Fig. 7, sera diluted 1:5 showed good discrimination and (+) specimens could be reliably distinguished from anti-HBe (−) samples. The same serum panel used throughout the study was also assayed herein. Indeed,

(i) twenty-four anti-HBe (+) samples were also detected as (+) with our assay;
(ii) six anti-HBe (−) samples (group A; Table 1) were (−) for anti-HBe; and

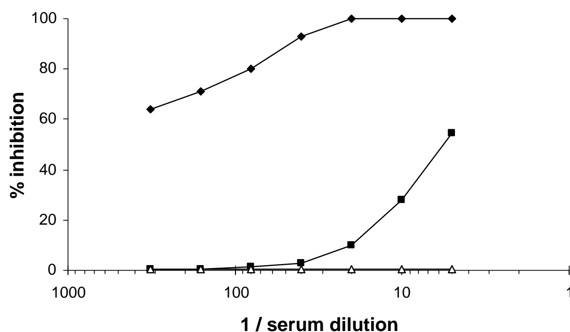

Fig. 7. ELISA for the detection of anti-HBe. Inhibition of binding of neutralizing recombinant HBeAg to immobilized anti-HBe by anti-HBe Abs present in sera of anti-HBe (+) (◆) and (■) individuals is shown. Diluted serum samples from an anti-HBe (−) (△) individual, shows no inhibition. Other details as in Fig. 6.

(iii) the 20 (−) control samples were determined as (−) with our assay.

Finally, as already mentioned in the preceding paragraphs, the novelty added by our assay to the anti-HBe-specific commercial ELISAs, is the possibility of using diluted (1:5 in this case) serum samples, and this dilution does not reduce or eliminate the sensitivity, specificity or accuracy of the test.

4. Conclusions

We designed and developed a panel of immunoassays for the serological determination of the most important HBV markers, using a commercially available set of recombinant HBV Ags and specific anti-HBV Abs. The ELISAs gave similar assay performances as the circulating tests, in terms of specificity and sensitivity. Furthermore, in contrast to commercial tests, human serum samples can be assayed diluted, with no alterations observed in the HBV serodiagnosed profile. The simplified, cost-effective procedure for HBV-specific ELISA set up as described herein, offers the possibility of their in-house development. Therefore, these ELISAs can be used mainly for experimental purposes and/or preclinical studies. They may also be helpful as supplementary assays to resolve indeterminate results, particularly if HBV Ag or anti-HBV titers are examined. It would be of interest to assay borderline samples, where the respective Ag or Ab levels in serum of infected individuals are very close to cut-off points, in order to evaluate the possible use of our assays more widely.

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