

Immunocapture Enzyme-Linked Immunosorbent Assay for Assessment of *In Vitro* Potency of Recombinant Hepatitis B Vaccines[∇]

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Quantification of hepatitis B surface antigen (HBsAg) or relative *in vitro* potency in the final vaccines is a prerequisite for hepatitis B vaccine batch release. The commercial kit for automated analysis (AxSYM) is expensive, and an alternative is required for the estimation of HBsAg in hepatitis B vaccines. Mouse monoclonal antibodies (MAbs) specific for HBsAg were developed and characterized. One of the monoclonal antibodies (HBs06) was used in development of an immunocapture ELISA (IC-ELISA) as an unlabeled capture antibody and biotin-labeled detection antibody. The IC-ELISA was standardized and validated using experimental hepatitis B vaccine batches with various HBsAg concentrations per dose and commercial vaccines. The vaccine was treated with an alkaline solubilizer to desorb the HBsAg from Algel-adsorbed vaccines before testing, and the sensitivity of the test was 5 ng/ml. A good correlation could be observed between the HBsAg estimates derived by both formats, except for the higher HBsAg concentration range, where the IC-ELISA format could estimate closer to the actual values than AxSYM. There was a significant correlation between the estimated relative potencies of the two methods. There was lack of correlation between the *in vivo* potency and the relative *in vitro* potency. However, the estimates of IC-ELISA were comparable to the *in vivo* values when compared with the estimates of AxSYM. The IC-ELISA can therefore be considered to be a reliable test for deriving *in vitro* relative potency and antigen concentration in vaccine batches for batch control and release.

Hepatitis B is a major global health problem caused by hepatitis B virus (HBV), and the disease is characterized by the most serious type of viral hepatitis resulting in cirrhosis and hepatocellular carcinoma. Worldwide, an estimated 2 billion people have been infected with HBV, and more than 350 million have chronic liver infections (26). HBV has a double-stranded DNA (dsDNA) 3.2 kb in size with four reading frames encoding several overlapping viral proteins, including pre-S1, S2, S, core, HBe, X, and polymerase proteins. Hepatitis B envelope protein or surface antigen (HBsAg) is composed of three related envelope proteins covalently linked together. HBV infection can be prevented and controlled by prophylactic vaccination. The earlier generation of vaccine for immunization programs was prepared from human plasma-derived antigen. With the advent of recombinant DNA technology and state of the art expression platforms, HBsAg subunit vaccines were made available for both adult and pediatric use worldwide (20).

The *in vivo* potency of hepatitis B vaccine as a part of quality control is evaluated in laboratory animals, which is correlated with the results of the vaccine clinical trials (25). In addition to this, relative potency is also assessed for each vaccine batch *in vitro* by immunoassays which have been validated using parallel-line assays. The disadvantages of the *in vivo* potency testing

are the inherent variation in results and cost of the animal experiments (11). In addition, the antigenic complexity of HBsAg complicates the evaluation, as estimations of at least 50% seroconversion against HBsAg vary depending upon the subtype of the antigen used in the test (24).

HBsAg contains the common immunodominant “a” determinant shared by all serotypes and genotypes of HBV and two sets of mutually exclusive subtype determinants designated d/y and w/r, resulting in four major subtypes of HBsAg: adw, adr, ayw, and ayr (5, 18). Alteration of residues in the “a” determinant can result in reduced antigenicity and reduced levels of protein expression (13). Measurement of anti-“a” antibodies rather than the antibodies to total HBsAg is believed to be a true indicator of the immunity against HBsAg in the vaccinated subjects (14). Enzyme-linked immunosorbent assay (ELISA)-based methods have been developed for the quantification of group-specific “a” antigen in monovalent hepatitis B vaccine (27) and combination vaccines (10). Commercial ELISA kits, especially the Auszyme kit developed by Abbott Laboratories, have long been used widely for quantifying HBsAg content. The manufacturer has discontinued the kit and replaced it with an expensive method for automated analysis *in vitro*. Subsequent to this, an alternative *in vitro* potency method based on an inhibition ELISA for evaluation of vaccines containing HBsAg has been reported (4). Development of similar in-house ELISA-based procedures for assessing the vaccine’s potency *in vitro* would render the quality assessment of vaccines including the batch release

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economical and avoid the need for relying on expensive kits and equipment.

Monoclonal antibodies (MAbs) play a pivotal role in developing rapid and sensitive ELISA-based methods for antigen and antibody detection, quantification, and characterization of antigen in vaccine research and development (3). *In vitro* production of MAbs has become simpler and inexpensive without having to use laboratory animals for large-scale production. The polyclonal antibodies of immune human and animal origins can be replaced with the HBsAg-specific MAbs for development of highly sensitive and specific ELISAs.

In the present study, we report the development of HBsAg determinant "a"-specific MAbs and the use of one such MAb for the development of an immunocapture ELISA (IC-ELISA) for assessment of the *in vitro* potency of hepatitis B vaccine formulations.

MATERIALS AND METHODS

Hepatitis B purified antigen (HBsAg) and reference standard. Recombinant HBsAg (subtype adw₂) expressed in *Pichia pastoris* obtained from the production department of the Human Biologicals Institute (HBI), Hyderabad, India, was used as a capture antigen for screening the hybridomas. Elovac-B vaccine containing 20 µg of HBsAg obtained from HBI, Hyderabad, was used for hyperimmunization of BALB/c mice to develop hybridomas secreting MAbs and also as the reference standard.

Standards and vaccine samples. (i) Internal reference standard (IRS) vaccine (containing 20 µg/ml of HBsAg) was calibrated using International Reference Standard (NIBSC, United Kingdom) and subsequently used as the reference standard in all of the IC-ELISAs.

(ii) Experimental batches of hepatitis B vaccine with various levels of HBsAg such as 5 to 15 µg/ml, 15 to 20 µg/ml, and 20 to 30 µg/ml were prepared for the analyses by both in-house and commercial ELISA (Abbott Laboratories) methods. In addition, 19 different commercial monovalent hepatitis B vaccines from five different manufacturers (A, B, C, D, and E) were also included in the study.

(iii) Experimental Algel-adsorbed hepatitis B vaccine blends were formulated based on the total protein content of the sterile-filtered bulk antigen, ranging from 100 µg/ml to 10 µg/ml and used for standardization of the assay.

Preparation of monoclonal antibodies. The MAbs were prepared essentially following the method described by Kohler and Milstein (16). Briefly, female BALB/c mice (6 to 8 weeks old) were hyperimmunized with commercial Elovac-B vaccine manufactured by the Human Biologicals Institute, Hyderabad. The splenocytes were harvested and fused with Sp2/0 murine myeloma cells. The polyclonal hybrids were selected based on the reactivity of the antibody secreted by them in an indirect ELISA by coating the ELISA plate with 400 ng of purified recombinant HBsAg per well. The monoclones were selected after two rounds of single cell cloning by limiting dilution. The MAbs were subjected to extensive characterization to determine their isotype, specificity, cross-reactivity, and affinity constant.

(i) **Isotyping.** The heavy- and light-chain isotypes of the MAbs were identified using the Isostrip mouse monoclonal antibody isotyping kit (Roche Applied Sciences).

(ii) **Specificity of the MAb for HBsAg.** HBsAg-coated beads, a component of the AUSAB kit (Abbott Laboratories), were used to determine the specificity of the MAb for HBsAg. In another set of experiments, HBsAg-peroxidase conjugate was allowed to react with each of the MAbs trapped using goat anti-mouse IgG (whole molecule) (Sigma) in an antibody-mediated ELISA. The absorbance values were measured at a 450-nm wavelength using an ELISA reader (Molecular Devices).

(iii) **Specificity of the MAb for the conformational epitope of HBsAg.** The specificity of the MAbs for the conformational epitope of HBsAg was demonstrated by indirect ELISA using purified HBsAg in the native form and denatured form obtained after treatment with SDS and 2-mercaptoethanol. The absorbance values were measured at a 450-nm wavelength using an ELISA reader.

(iv) **Cross-reactivity of the MAb with human plasma-derived HBsAg, yeast host cell proteins, and hepatitis A virus antigen.** The MAbs were checked for their ability to cross-react with the human plasma-derived HBsAg subtypes *viz.* HBsAg/ad and HBsAg/ay (Chemicon) by indirect ELISA. The absorbance values

were measured at a 450-nm wavelength using an ELISA reader. The lack of cross-reactivity of all of the MAbs with the yeast (*Pichia pastoris*) host cell proteins (HCPs) was demonstrated using an immunoenzymatic assay kit (Cygnus Technologies) meant for the measurement of *Pichia pastoris* host cell proteins. The absorbance values were measured at a 450-nm wavelength. The lack of cross-reactivity of the MAbs with the purified hepatitis A viral antigen was demonstrated using a pseudocompetitive immunoassay kit (Mediagnost, Germany) meant for the quantification of hepatitis A viral antigen.

(v) **Test for competitive inhibition.** The specificity of the MAbs for the conformational epitope was also confirmed by an inhibition ELISA using a peroxidase-conjugated MAb provided in the Auszyme kit (Abbott Laboratories). The Auszyme kit consists of a MAb that binds to native HBsAg. The kit is meant for qualitative determination of HBsAg in a sandwich ELISA format using a MAB-peroxidase conjugate. The inhibition ELISA was performed according to the manufacturer's instructions with slight modification. Briefly, standard HBsAg was trapped with a capture MAb on the solid phase. The MAbs were added in increasing concentrations mixed with constant volumes of the MAB-peroxidase conjugate from the kit. The binding of the MAB-peroxidase conjugate was detected by substrate addition, and the percent reduction in absorbance values was calculated.

(vi) **Determination of the affinity constant of the MAb chosen for ELISA.** The kinetic interaction of one of the MAbs, HBs06, identified for the IC-ELISA format was measured using BIAcore surface plasmon resonance (SPR) biosensor (GE, Sweden). The dissociation and association rate constants were obtained by statistical analysis of the data using the BIAcore Evaluation software provided by the manufacturer.

Bulk propagation and purification. One of the MAbs from the panel, HBs06, was propagated in an *in vitro* culture system, FibraStage, containing FibraCel discs (New Brunswick Scientific), using hybridoma serum-free medium (Invitrogen) in order to obtain a highly purified concentrated preparation. The hybridoma supernatants were sampled at different time points to check for the antigen-specific MAb secretion. The MAb in the pooled supernatants was purified using affinity chromatography on MABSelect Sure resin (GE Healthcare, Sweden). The antigen-specific activity of the purified MAbs was confirmed by indirect ELISA, and the purity was checked by nonreducing SDS-PAGE. A part of the purified MAb was labeled with biotin using Ez-LinkSulfo-NHS-SS-biotin (Thermo Scientific) as per the manufacturer's instructions and used for development of the IC-ELISA.

Titration of MAb with HBsAg for determination of optimal dilution. The purified MAb HBs06 chosen for ELISA was titrated with the purified HBsAg as a detection antibody by a sandwich ELISA. Polyclonal guinea pig anti-HBsAg antibody kindly provided by Rick Schuman (Fina Biosciences, LLC) was used to capture the various concentrations of purified unadsorbed antigen, and the trapped antigen was detected by unlabeled MAb HBs06 and biotin-labeled MAb HBs06. The optimal working dilutions of the unlabeled MAb HBs06 for capture and the biotin-labeled MAb HBs06 used for detection of HBsAg were determined by checkerboard titrations.

The assay was performed by coating the plates with polyclonal guinea pig anti-HBsAg antibody diluted in carbonate buffer (pH 9.6). After blocking with bovine gelatin (1% [wt/vol]), purified unadsorbed antigen was added in concentrations ranging from 1,000 ng/ml to 1.5 ng/ml (in phosphate-buffered saline-Tween 20 [PBST]) to duplicate columns. The HBsAg in the wells was detected with unlabeled and biotinylated MAb HBs06. Anti-mouse IgG peroxidase-conjugated antibody (Sigma) and streptavidin-horseradish peroxidase (HRP) (Pierce) were used. Hydrogen peroxide (Merck, India)-activated chromogen (3,3',5,5'-tetramethylbenzidine; Sigma) was added to all wells. The reaction was stopped using 1.25 M sulfuric acid (Merck, India). The optical density (OD) was measured at a 450-nm wavelength using an ELISA reader (Molecular Devices).

Development of IC-ELISA to quantify HBsAg. The appropriately diluted unlabeled and biotin-labeled MAb HBs06 were used for antigen trapping and antigen detection, respectively, in an IC-ELISA format. Different concentrations of purified and adsorbed HBsAg (10 µg/ml to 1.5 ng/ml) were used for development and standardization of the IC-ELISA. The Algel-adsorbed HBsAg was desorbed following a method described by Yamamoto et al. (27) with slight modification. Briefly, 1 volume of the adsorbed vaccine was mixed with 2 volumes of a solubilizer (a mixed solution of 0.4 M sodium phosphate dibasic and 0.45 M sodium citrate, pH 8.5) and incubated at 25°C for 60 min.

IC-ELISA for vaccine batches. The reactivity of the 2-fold-diluted purified and adsorbed recombinant HBsAg (IRS) was calibrated against the international reference standard using the IC-ELISA with the biotin-labeled and unlabeled MAb HBs06 as described earlier. The optical density (OD) was measured at a 450-nm wavelength using an ELISA reader (Molecular De-

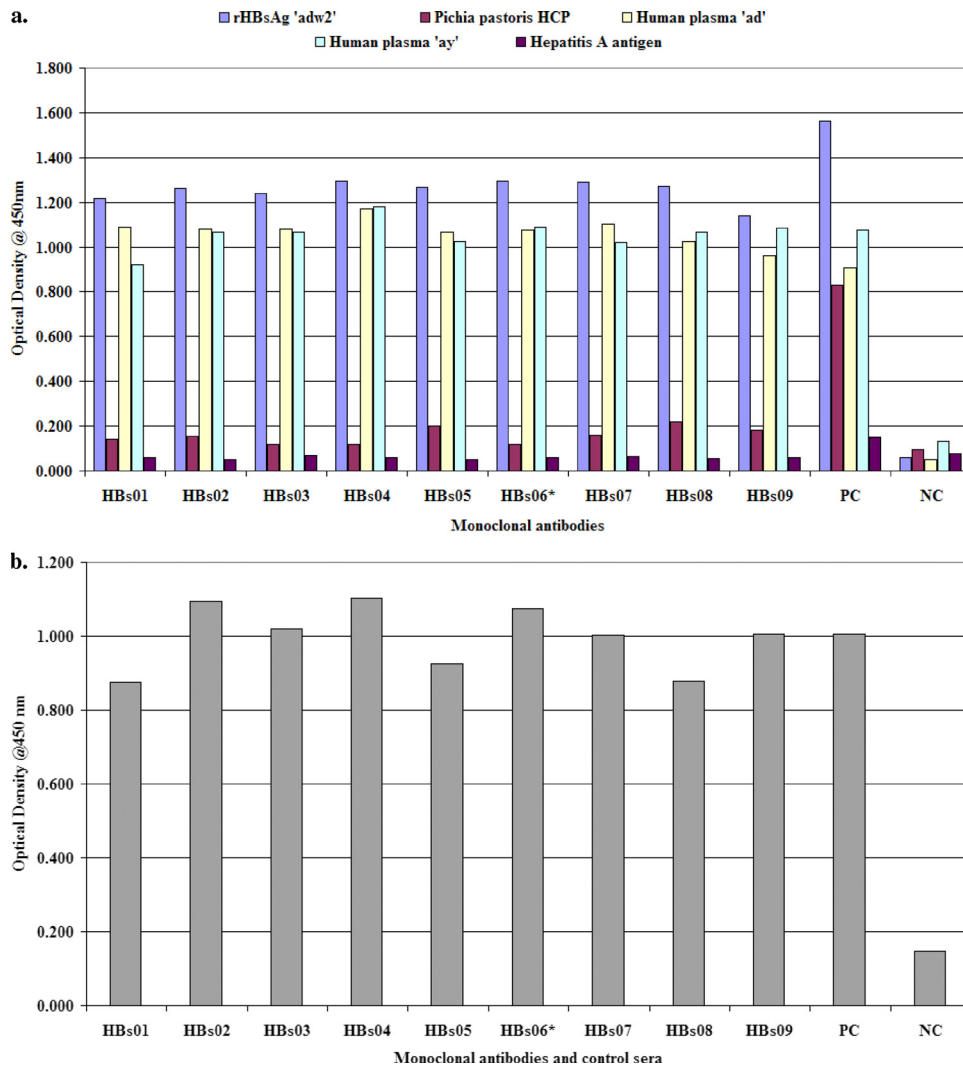


FIG. 1. (a) Indirect ELISA with different subtypes of HBsAg, host cell proteins, and hepatitis A viral antigen to determine the specificity of the anti-HBsAg MAbs. PC, hyperimmune mouse sera as a positive control; NC, growth medium as a negative control. *, MAb HBs06 was chosen for development of IC-ELISA. (b) Indirect ELISA showing specific reactivity of anti-HBsAg MAbs with HBsAg-coated beads from the AUSAB kit.

vices). The HBsAg contents in the final Algel-adsorbed vaccines with 95% confidence interval (CI) and relative potency estimates were derived based on the parallel-line assay (9).

Quantification of HBsAg by AxSYM kit: AxSYM HBsAg (V2). The test is based on microparticle enzyme immunoassay technology and is intended for serological detection of HBsAg (1). Briefly, 150 μ l of the sample, anti-HBs MAb-coated microparticles and biotinylated anti-HBs polyclonal antibody were mixed together and incubated in a reaction vessel. This reaction mixture was later dispensed onto a matrix cell. The anti-biotin-alkaline phosphatase conjugate was then dispensed onto the matrix cell, followed by buffer washes. The alkaline phosphatase activity was determined by the addition of a substrate, 4-methylumbelliferyl phosphate, which was converted to methylumbelliferone, and the fluorescent signal was measured by the AxSYM instrument. The results were calculated using an automated analyzer against the stored AxSYM HBsAg index calibration curve. A test value above a signal/negative value ratio of 2 was considered positive.

Comparison of IC-ELISA with commercial kit and *in vivo* potency. The antigen concentration and relative potency estimate by the IC-ELISA were compared with the estimates of the commercial kit and the *in vivo* potency estimates using standard statistical tools (22).

RESULTS

Characterization of MAb HBs06. The MAb HBs06 was isotyped as IgG1 κ . The specificity and cross-reactivity of the MAb for HBsAg were demonstrated using the in-house recombinant antigens, commercial kit reagents, and human plasma-derived antigens (Fig. 1a). The MAb cross-reacted with different subtypes of hepatitis B virus (adw, ay, and ad). It showed no cross-reactivity with HCPs of *Pichia pastoris* and hepatitis A virus, which is highly endemic in India. It recognized a conformational epitope on HBsAg, as evidenced by its reactivity with native and not the denatured HBsAg in indirect ELISA. The MAb effectively competed with the peroxidase-conjugated MAb of the Auzyme kit, thereby showing the relevance of the epitope, which needs to be targeted for specific detection of antigen (Fig. 1b). The affinity constant of the MAb as determined by BIAcore analysis was 9.58×10^{-9} M (Fig. 2).

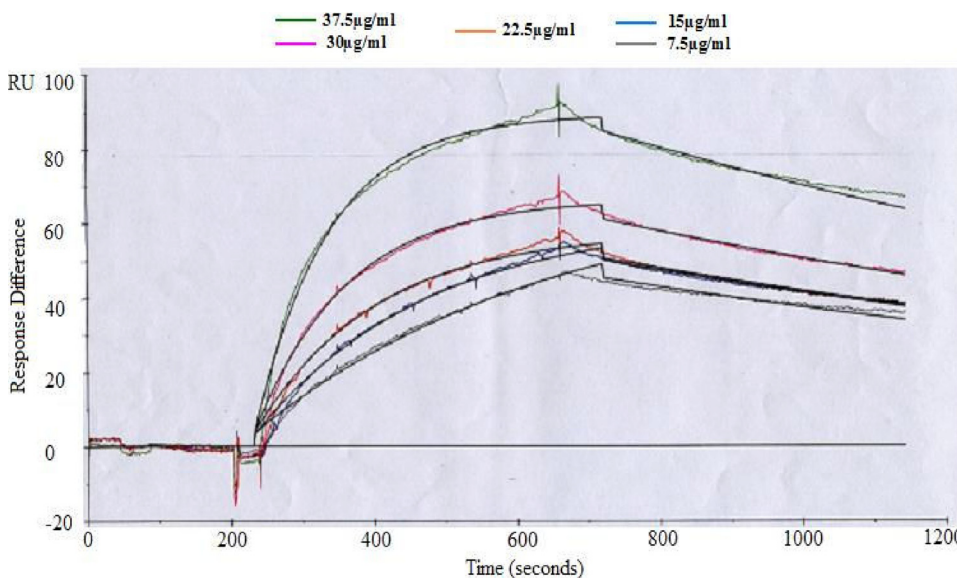


FIG. 2. Sensorgram of the BIAcore assay showing kinetic binding curves of MAb HBs06 with different HBsAg concentrations, as indicated by a different color for each concentration. RU, response units.

Sandwich ELISA in optimization of MAb HBs06 as the detection antibody. The results of indirect ELISA revealed the ability of MAb HBs06 to bind with the solid-phase HBsAg. The sandwich ELISA results using the polyclonal guinea pig anti-HBsAg antibody for antigen capture could help in ascertaining the suitability of the biotin-labeled MAb HBs06 for detection. The detection range of the assay from 1,000 ng/ml to 3.2 ng/ml resulted in an acceptable correlation coefficient (r^2) of ≥ 0.99 for the standard curve. Analytical recovery (AR) was within 20% of the nominal concentrations, and the coefficient of variation (CV) of replicates was $\leq 20\%$. An AR of 75% to 125% and CV of 25% were found to be acceptable at the lowest detection limit of 3.2 ng/ml. Samples whose concentrations fall outside the range of the curve were reassayed at an appropriate dilution. The assay precision and accuracy are summarized as in Table 1.

IC-ELISA for the estimation of HBsAg in vaccine batches. The IC-ELISA developed using MAb HBs06 was used to quantify the HBsAg content in the hepatitis B vaccines. An adsorbed HBsAg IRS (20 µg/ml) was used for the HBsAg quantification in the test samples. Samples of adsorbed hepatitis B vaccines were analyzed by IC-ELISA after desorption

using an alkaline solubilizer followed by eight serial 2-fold dilutions. HBsAg in the adsorbed vaccines could not be detected without desorption.

A typical dose response curve was observed when the HBsAg IRS was added in the concentration range of 670 ng/ml to 5.2 ng/ml (Fig. 3). The fiducial limits of the ELISA for the quantification of HBsAg were 30 µg/ml and 5 ng/ml. The optimal working dilution of the labeled detection antibody was chosen based on a checkerboard titration as 1:32,000, which could yield the best linear fit, with R^2 value of 0.93.

IC-ELISA results of experimental in-house and commercial hepatitis B vaccines. Various experimental batches of hepatitis B vaccines prepared in-house and five different commercial vaccines were analyzed for HBsAg content, both by IC-ELISA and by the AxSYM kit, and the results are presented in Table 2 and Table 3, respectively. A frequency plot of the measured difference between the two estimates showed the distribution of overestimation or underestimation among the different batches tested (Fig. 4). The dose response was clearly evident in the first and second sets of experimental batches (5 to 15 and 15 to 20 µg/ml, respectively). The observed difference between the two estimates was 0.3 to 8 µg/dose. In the third set of experimental batches (20 to 30 µg/dose), the AxSYM method showed underestimation (0.7 to 10 µg/dose) compared to the IC-ELISA, whose estimate was closer to the actual antigen concentration in the vaccine batches of set 3. Among the commercial vaccines, vaccine A had lower HBsAg content compared to the vaccines of manufacturers B, C, and D that were close to the payloads as claimed in the label, while vaccine E had higher antigen payload.

IC-ELISA results from experimental blends of final bulk HBsAg. The HBsAg analysis in the experimental blends formulated based on the total protein estimates could precisely estimate the actual specific protein content of the blends (Table 4). Serial 2-fold dilutions of one of the blends with the maximum total protein content of 100 µg/ml, could back-cal-

TABLE 1. Precision and accuracy of the IC-ELISA performed to qualify MAb HBs06 as the detection antibody^a

Standard	Antibody concn detected (ng/ml)		% AR	% CV	
	Nominal	Mean		Intra-assay	Interassay
	Very low	3.2			
Low	10.0	10.14	9.564	11.93	101.42
Mid	50.0	49.93	6.169	8.148	99.868
Mid	200.0	197.44	6.508	13.89	98.722
High	1000.0	981.907	10.68	11.753	98.191

^a Purified IgG derived from immune guinea pig serum was used as the capture antibody, and purified recombinant HBsAg was used as the antigen.

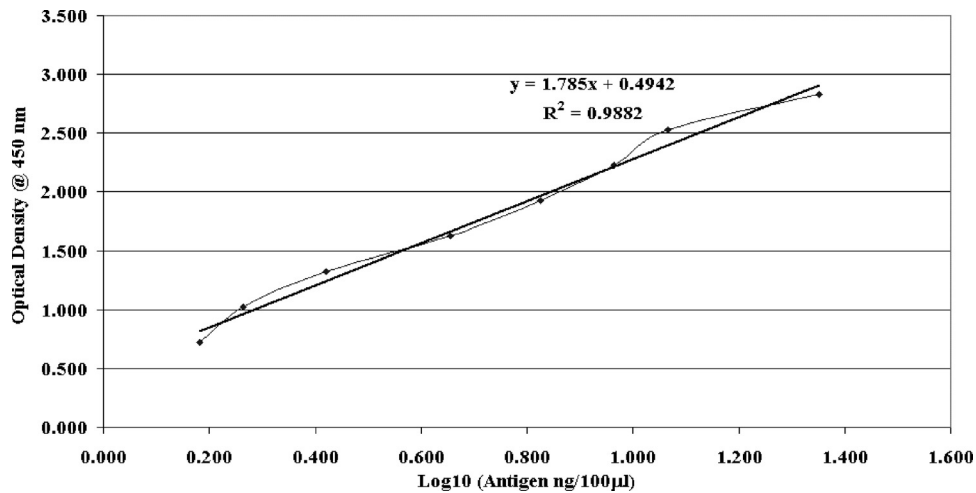


FIG. 3. Standard curve derived using MAb HBs06 against different concentrations of the IRS. The concentrations of HBsAg are shown with logarithmic transformation in the x axis.

culate the specific protein content to the same estimate as that of the neat sample (Table 5).

Correlation of *in vitro* and *in vivo* potency estimates of hepatitis B vaccines. The relative *in vitro* potency values of the

in-house vaccines as derived by *in-house* ELISA and the AxSYM kit were compared to the *in vivo* potency estimates. Regression analysis of IC-ELISA estimates on AxSYM estimates showed good correlation between the two data sets

TABLE 2. Comparison of *in vitro* HBsAg estimates by *in-house* ELISA and AxSYM with *in vivo* potency values

Batch ^a	In-house HBsAg estimate in $\mu\text{g/ml}$ (95% CI)	AxSYM HBsAg estimate in $\mu\text{g/ml}$	<i>In vivo</i> potency	<i>In vitro</i> relative potency by:	
				In-house ELISA	AxSYM
IIL-01	5.56 (6.32–4.85)	8.77	1.06	0.28	0.44
IIL-02	7.43 (8.59–6.34)	8.99	1.10	0.37	0.45
IIL-03	7.78 (9.42–6.29)	15.43	1.31	0.39	0.77
IIL-04	9.05 (11.20–7.13)	22.85	1.56	0.45	1.14
IIL-05	10.16 (11.13–9.24)	12.97	1.14	0.51	0.65
IIL-06	10.19 (11.13–9.39)	18.19	1.18	0.51	0.91
IIL-07	10.74 (11.74–9.80)	17.84	1.35	0.54	0.89
IIL-08	12.74 (14.06–11.50)	16.67	1.20	0.64	0.83
IIL-09	13.19 (14.93–11.60)	15.61	2.17	0.66	0.78
IIL-10	13.20 (14.40–12.07)	15.39	1.95	0.66	0.77
IIL-11	13.26 (14–68–11.95)	19.31	1.36	0.66	0.97
IIL-12	13.28 (14.99–11.70)	18.41	1.23	0.66	0.92
IIL-13	13.29 (14.92–11.77)	18.14	1.18	0.66	0.91
IIL-14	14.57 (16.56–12.75)	12.07	1.36	0.73	0.60
IIL-15	15.00 (16.39–14.00)	17.05	1.19	0.75	0.85
IIL-16	15.33 (16.76–14.00)	16.46	1.06	0.77	0.82
IIL-17	15.35 (16.67–14.12)	15.72	1.13	0.77	0.79
IIL-18	15.73 (17.17–14.39)	17.13	1.39	0.79	0.86
IIL-19	16.12 (19.30–13.39)	23.41	1.08	0.81	1.17
IIL-20	16.21 (17.57–14.95)	15.95	1.07	0.81	0.80
IIL-21	16.95 (18.60–15.44)	24.37	1.11	0.85	1.22
IIL-22	17.02 (19.36–14.93)	20.95	1.11	0.85	1.05
IIL-23	17.31 (18.97–15.78)	24.44	1.14	0.87	1.22
IIL-24	19.09 (21.09–17.28)	18.71	1.07	0.95	0.94
IIL-25	20.73 (22.67–18.96)	19.01	2.71	1.04	0.95
IIL-26	21.81 (26.33–18.11)	22.52	1.44	1.09	1.13
IIL-27	22.19 (26.89–13.67)	19.31	1.15	1.11	0.97
IIL-28	22.59 (24.78–20.61)	20.62	1.10	1.13	1.03
IIL-29	23.68 (25.95–21.63)	16.63	2.87	1.18	0.83
IIL-30	25.76 (29.18–22.81)	19.13	2.67	1.29	0.96
IIL-31	27.09 (30.63–24.04)	20.11	1.08	1.35	1.01
IIL-32	27.41 (30.97–24.35)	17.79	1.66	1.37	0.89
IIL-33	29.67 (33.41–26.46)	16.30	1.95	1.48	0.82

^a *In-house* experimental batches. Batches IIL-01 to IIL-15 contained 5 to 15 $\mu\text{g/ml}$ HBsAg, batches IIL-16 to IIL-25 contained 15 to 20 $\mu\text{g/ml}$, and batches IIL-26 to IIL-33 contained 20 to 30 $\mu\text{g/ml}$.

TABLE 3. Quantification of HBsAg content in commercial hepatitis B vaccines

Commercial vaccine batch	In-house HBsAg estimate in µg/ml (95% CI)	AxSYM HBsAg estimate in µg/ml	<i>In vitro</i> relative potency by:	
			In-house ELISA	AxSYM
A1	8.64 (9.61–7.72)	8.82	0.43	0.44
A2	19.85 (23.03–17.11)	10.12	0.99	0.51
B1	23.35 (26.65–20.50)	29.64	1.17	1.48
B2	23.75 (26.88–27.04)	17.24	1.19	0.86
B3	15.85 (17.11–14.66)	25.32	0.79	1.27
C1	21.76 (25.25–18.79)	22.34	1.09	1.12
C2	20.83 (25.96–16.73)	22.36	1.04	1.12
C3	19.70 (23.94–16.21)	19.00	0.99	0.95
C4	13.31 (15.92–11.01)	20.06	0.67	1.00
C5	10.76 (13.24–8.57)	15.06	0.54	0.75
D1	20.52 (23.70–17.78)	13.86	1.03	0.69
D2	33.35 (39.26–28.61)	16.90	1.67	0.85
E1	21.62 (25.04–18.69)	32.29	1.08	1.61
E2	29.34 (33.45–25.87)	32.29	1.47	1.61
E3	38.24 (44.26–33.34)	33.99	1.91	1.70
E4	29.64 (34.24–25.83)	29.67	1.48	1.48
E5	28.84 (32.83–25.45)	30.36	1.44	1.52
E6	13.53 (15.38–11.83)	23.84	0.68	1.19
E7	13.04 (15.24–11.06)	19.73	0.65	0.99

(adjusted $R^2 = 0.883$), and the fitted line for regression through the origin was $0.987x$ (99% CI, 0.866 to 1.107).

Analysis of variance (ANOVA) performed on the *in vivo* potency and the relative potency values derived from both the tests showed that there was no significant difference between the *in vitro* estimates of HBsAg concentration in the vaccine or the relative potency ($P > 0.05$), whereas there was a highly significant difference between the *in vivo* and *in vitro* estimates ($F = 28.66$; $P < 0.001$). The regression through the origin (RTO) model analysis showed that the adjusted R^2 value for relative potency values derived by the *in vitro* methods and *in vivo* potency values was 0.883. The equations for predicting the *in vivo* potency were $0.946x$ (99% CI, 0.031 to 1.861) and $0.68x$ (99% CI, -0.199 to 1.558) for in-house and AxSYM-derived relative potency, respectively, where x is the estimate of relative potency.

DISCUSSION

Recombinant hepatitis B vaccine has been very popular and effective in preventing several deaths due to hepatitis B worldwide (19). The immunogenicity of HBsAg has become the basis for the first human recombinant vaccine licensed for widespread use (7). Quality assessment of hepatitis B vaccine is central for batch release and also to ensure efficacy and batch consistency. MAbs have the unique property of specific interaction of the paratopes with the epitopes on an antigenic target, which is quite similar on every molecule. This is exploited during the development of immunoassays to provide tests of defined specificity and sensitivity (6).

The present study reports the development of a MAb-based IC-ELISA for quantification of HBsAg content in final vaccine formulations. Several laboratories have produced anti-HBs

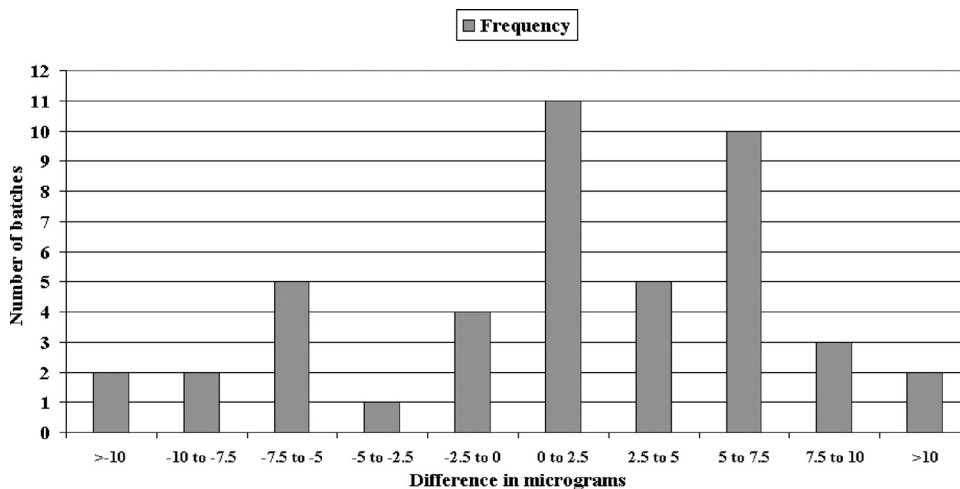


FIG. 4. Frequency plot showing difference between the in-house and AxSYM estimates of HBsAg for the vaccine batches.

TABLE 4. Quantification of HBsAg content in experimental blends prepared based on total protein

Total protein concn in blend (µg/ml)	Actual HBsAg content in blend (µg/ml)	HBsAg estimate (µg/ml) by:		Difference in estimates (µg/ml)
		AxSYM	IC-ELISA	
100	35	35.34	19.51	15.83
80	28	28.19	18.58	9.61
50	17.5	18.45	17.22	1.23
20	7	6.71	8.1	-1.39
10	3.5	3.43	4.29	-0.86

MABs useful for the structural analysis of HBsAg group- or subtype-specific determinants and development of immunodiagnostic kits (8). In the present study, the reactivity of MAB HBs06 with both recombinant and human plasma-derived HBsAg and its cross-reactivity with at least two subtypes of HBsAg clearly indicated its specificity for the group-specific antigenic determinant "a" (27). Although serotype specificity was not a variable in this analysis, the avidity of MAB HBs06 was particularly striking. However, heterogeneity in the pattern of reactivity with different subtype preparations has been reported (15). The MAB recognized an epitope on the HBsAg which is relevant for targeting the vaccine antigen of subtype adw₂, as evidenced by the effective competition with the antibody supplied with the Auszyme kit. This property of the MAB has helped in the development of an ELISA to detect and quantify the antigen content in vaccines.

All serotypes of HBsAg contain the common epitope "a," which consists of 124 to 137 amino acids (aa) and 139 to 147 aa residues of the S protein. This makes the S protein an ideal diagnostic antigen for the detection of HBsAg antibodies against all serotypes (2). The lack of cross-reactivity with hepatitis A viral antigen further confirmed the specificity of the MAB for the defined antigen, as reported elsewhere (23). Reduction of HBsAg caused a significant loss in the binding of MAB to the antigen in ELISA, and it confirmed that the MABs developed in this study recognized only the conformational epitope on the HBsAg. This finding was further proven in the Western blot profiles where the antibody failed to bind to the antigen in the blots.

The importance of reducing the use of laboratory animals and developing useful methods for testing vaccine is widely felt. The *in vitro* methods for determination of antigenic mass have been used to assess the immunogenicity of vaccine and to predict its potency. The results of the sandwich ELISA employing polyclonal antibodies and MABs could reveal the suit-

ability of MAB HBs06 for the standardization of an IC-ELISA format.

Different types of assays for the detection of HBsAg have been developed employing polyclonal antibodies or a combination of polyclonal antibodies and MABs. The MABs have proven time and again their usefulness for development of a sensitive and specific assay format for quality assessment of vaccine antigens. The problems associated with the use of a polyclonal antibody in an assay with regard to its poorly defined nature and batch-to-batch variation have been mitigated by the use of MABs as described in this paper.

The MAB chosen for the development of IC-ELISA belonged to isotype IgG1. This is in agreement with the previous findings (21) which showed the predominance of anti-HBsAg antibody of the IgG1 isotype at the cellular level in human and mouse models. The affinity constant of the MAB was found to be higher (9.58×10^{-9} M), making it an ideal candidate for applications that require immunochemical functions (17).

Reproducible differences were observed in the binding of the biotin-labeled MAB HBs06 to HBsAg, and the lower concentration of the MAB was sufficient to obtain equivalent absorbance in the assay. A single MAB, HBs06, could be used to capture as well as detect the HBsAg due to the presence of multiple copies of monomeric forms with the repeating epitope. The initial capture of HBsAg by the MAB could orient the antigen such that the detection antibody could bind to it without steric hindrance. Various combinations of unlabeled and biotinylated MABs were tried in order to position the MABs to achieve better sensitivity and specificity. A correlation existed between the avidity of the MAB HBs06 in ELISA and the affinity constant as determined by BIAcore, thereby confirming the efficiency of the MAB as a crucial diagnostic reagent.

An IC-ELISA method was developed and validated for the quantification of HBsAg content in the monovalent Adjuvanted hepatitis B vaccines. The IC-ELISA when compared with AxSYM kit showed a very good correlation, especially when the vaccine antigen was desorbed with an alkaline buffer before analysis by IC-ELISA. Adsorbed hepatitis B vaccines without any treatment could not be analyzed by IC-ELISA, which can be attributed either to inhibiting substances in the final vaccine or to steric hindrance by Adjuv. Hence, the adsorbed hepatitis B vaccines were treated with an alkaline buffer before estimating the HBsAg content. The sensitivity significantly improved when the treated vaccine was allowed to react with the coated MAB for 2 h at 37°C, as opposed to 1 h at 37°C (data not shown). The various amounts of vaccine

TABLE 5. Quantification of HBsAg content in experimental blend prepared using 100 µg of total protein

Dilution in blend	HBsAg content in blend (µg/ml)	AxSYM HBsAg estimate (µg/ml)		IC-ELISA HBsAg estimate (µg/ml)		Difference in estimates (µg/ml)
		Calculated	Actual	Calculated	Actual	
		None (neat)	35	34.15	34.15	
1:2	17.5	15.29	31.22	15.24	30.48	0.74
1:4	8.75	7.52	29.44	6.32	25.28	4.16
1:6	5.83	4.51	28.62	4.88	29.28	-0.66
1:8	4.38	4.24	34.72	3.53	28.24	6.48

antigens that could be recovered seemed to depend on the quality of the HBsAg and the methods of HBsAg adsorption to the Algel during vaccine manufacture.

The AxSYM method, basically a microparticle-based enzyme immunoassay, is currently used for the serological detection of HBsAg. The method has been updated recently with a fully automated version, which demands the need for expensive equipment along with the reagent pack for the analysis. Each dilution of the vaccine needs a separate reaction vessel, which limits the number of samples under testing. The testing of duplicate samples for each dilution of the vaccine further increases the cost of the analysis. Although the method is intended for use in detection of HBsAg in sera, it is widely used by most of the manufacturers for detection of the antigen in the vaccine formulations. The in-house-developed IC-ELISA format is simple, more robust, and can accommodate a larger number of samples. Repetition of tests to reconfirm the results becomes expensive in the commercial kit compared to the IC-ELISA.

Comparison of the data generated by both the in-house and commercial formats clearly showed that the results derived by the in-house IC-ELISA were in agreement with the theoretical values of the experimental formulations, unlike the AxSYM estimates. The discrimination between the vaccine batches containing low, optimal, and high relative potencies was quite possible. It has also been shown that both methods were able to evaluate vaccines of different sources and payloads. The batch-to-batch variation in the commercial vaccines could be due to the variation in their expiration dates and the stability of the protein in the respective formulations over a period of time. The claims made by the vaccine manufacturers cannot be rechecked by the regulators in the case of the commercial kit and equipment due to the lack of facilities, whereas the IC-ELISA can be readily adapted for reconfirming the manufacturer's claims.

The interference caused by the different excipients used in the formulation and the methods used for the antigen adsorption to Algel could be attributed to the various detection levels of the antigen. Also, it is important to consider the fact that the two methods are based on different principles, and, hence, some differences in the responses could be expected (4).

The actual HBsAg content could be estimated by two methods from blends prepared based on total protein content, which indicated the specificity of both methods. Also, the dilutions of one of the experimental blends could be used to back-calculate the specific protein content to the same estimate as that of the neat sample, thereby showing the reliability of the assay.

Comparison of the *in vitro* relative potencies of both the methods with the *in vivo* potency values showed a lack of correlation. However, the estimates of IC-ELISA were comparable with the *in vivo* values when compared to the estimates derived by the commercial kit. Hendriksen et al. (12) have explained that for some vaccine products, *in vitro* assays will not be able to completely replace animal models (*in vivo* assays) for the demonstration of efficacy for the foreseeable future. Therefore, it will be difficult to adopt *in vitro* methods or serologically based approaches where protection is not dependent on antibody response. Nevertheless, it is likely that *in vitro* assays can be validated for certain specific purposes: for

instance, to monitor certain aspects of consistency in production. In the classical concept of quality control (the uniqueness of the vaccine batch produced), *in vitro* antigenicity models are generally considered not to be valid replacements for *in vivo* assays because they quantify the amount of antigen and do not reflect the immunogenicity of an antigen, and they do not reflect the rather complex cascade of responses after *in vivo* immunization. So the IC-ELISA can therefore be considered as a reliable test for deriving *in vitro* relative potency and antigen concentration in vaccine batches for batch control and release. Also, vaccine manufacturers who do not have access to the AxSYM method can use the IC-ELISA-based HBsAg estimation as an alternative *in vitro* potency test for hepatitis B vaccine batch release.

The IC-ELISA using MAb HBs06 can be a potential alternative method for arriving at the HBsAg concentration in vaccines where the commercial automated analysis is neither affordable nor readily available.

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