Validation of Analytical Method for Hepatitis B Component in Pentavalent Vaccine as Well as Hepatitis B Vaccine (Monovalent) by Sandwich Elisa Method

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Abstract: *For evaluating the antigen content in recombinant Hepatitis B Vaccine (Monovalent and Pentavalent vaccines), National Control Laboratory, Kasauli has taken an initiative to develop a uniform method for evaluation of antigen content of Hepatitis B vaccine. All the manufacturers in India are having different method for evaluating Relative Potency of Hepatitis B Vaccine. In the present study the samples of different manufacturers were tested for potency by Sandwich ELISA method. This method was validated to check the suitability for all the manufacturers. Various validation parameters were evaluated to verify the fulfilled defined acceptance criteria. This validation method of Potency estimation can be transferred to different manufacturers throughout India to maintain the synchronization and harmonization of potency estimation of Hepatitis B vaccine. For the in- vitro potency assay of Hepatitis B Vaccine Adaltis EIAgen HBsAg Kit was used to determine the Relative Potency. Validation Parameters were evaluated following the International Conference on Harmonization (ICH) guidelines. Samples of different Manufacturers were tested for validation parameters. It was confirmed that the ELISA Kit when used for vaccine potency testing meets the criteria of specificity , accuracy, linearity, precision (Intra assay precision- (repeatability), Inter assay precision- (reproducibility & ruggedness).The method was found specific based on the results generated by ELISA Kit, when used for potency testing , meets the criteria for accuracy (80-125%), linearity $R^2 \geq 0.99$, precision (repeatability , with a CV% less than 15% and reproducibility with a CV% less than 15%).*

Keywords: ELISA. Relative Potency, HBsAg, Vaccines

1. Introduction

Viral Hepatitis is a major global public health problem and is an important cause of morbidity and mortality in large parts of tropical and developing countries and also in regions of Europe. It is an inflammatory system diseases of liver caused by any more than half a dozen viruses: Hepatitis- A (infectious Hepatitis), Hepatitis-B (serum Hepatitis), Hepatitis-C (transfusion associated Hepatitis), Hepatitis-D (delta Hepatitis), Hepatitis E (enterically transmitted hepatitis), Hepatitis-G (acute sporadic hepatitis) and new emerging hepatitis viruses; like TT virus and SEN virus [1]-[2].

Hepatitis B Virus (HBV) is a double stranded DNA virus in the *Hepadnaviridae* family. One of the five hepatitis B Viruses, Hepatitis B Virus causes acute and chronic hepatitis in humans, despite the current availability of an effective vaccine, almost 1.2 million people worldwide still die each year from HBV related diseases [3].Since there is no specific treatment , prevention has been the major aim in managing viral Hepatitis B.

The development of Hepatitis B vaccine is considered to be one of the major achievements of modern medicine. The three different classes of vaccines are available based upon how they are derived (from Plasma, yeast , mammalian cells).The most effective vaccine is the yeast cderived vaccine and was licensed for the first time in USA in 1987, the recombinant DNA vaccine elaborated from cultures of yeast cloned with HBsAg s-gene. The genetically engineered vaccine is as immunogenic safe and effective and is more cost effective than the plasma derived vaccine [4]. The fact that this vaccine does not depend on the scare plasma resource is an added advantage [5].

Recombinant Hepatitis B vaccine available in the market is in two forms one is thiomersal free and another is with thiomersal as a preservative. Two yeast derived vaccine Recombivax HB and Engerix-B are widely available [6].In India presently the manufacturers are producing both vaccines thiomersal free and with thiomersal and the yeast used is either *Hansenula Polymorpha* or *Pischia Pastoris*. Bevac-B, Elovac-B & Hivac-B are produced by *Pischia Pistoris* and Genevac –B is produced by *Hansenula Polymorpha*.[13]

Latest vaccines available in the market are in the Combination form with Hepatitis B as component in it e.g. Hexaxim (®) (DTaP-IPV-Hep B-Hib) is a new, thiomersal-free, fully liquid, hexavalent combination pediatric vaccine containing diphtheria and tetanus toxoids, acellular pertussis, inactivated poliovirus, recombinant hepatitis B virus surface antigen produced in the yeast *Hansenula polymorpha*, and Haemophilus influenzae type b polysaccharide (polyribosylribitol phosphate) conjugated to tetanus toxoid. Thus, Hexaxim (®) provides effective seroprotection or seroconversion against six major childhood diseases simultaneously, both as primary and booster vaccination, and offers the benefits and convenience of a fully liquid, ready-to-use vaccine [7]. Most of the Indian manufacturers have produced Pentavalent Vaccines with Hepatitis B as Component in it e.g. Pentavalent vaccine (DTP-HepB-Hib) or only four components (DTP+Hep B).

Biological reference standards are used in Qualifying or validating test procedures to ensure uniformity in the designation of potency or activity of biological preparation including vaccines. These are required to ensure lot to lot consistency of production and to minimize systematic deviation of assay. Internal reference standards are used as working standards and they will therefore, need to be formulated so that they are stable through their use. WHO/National/EDQM standards (if available), or a commercial vaccine with known potency may be used as reference standard and shall further be used to calibrate the secondary standards.

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 [8]. In India, production batches of the Hepatitis B vaccine should be tested by the National Control Laboratory (NCL) before being released to the market, in terms of their potency. This can be done either by means of the mouse immunogenicity (in-vivo) method, which is a time-consuming and labor intensive process, or by an in-vitro method with acceptable analytical performance and with specifications determined based on the results obtained from testing some batches at final lot of the vaccine with proven efficacy.[9] National Control Laboratory for Immunobiologicals in India is to ensure the use of safe, potent and quality Immunobiologicals in the country. To achieve this goal , NCL performs tests on the representative samples of requirements or specifications established and approved by National Regulatory Authority (NRA) during registration and Licensing process. It is most important to have reliable and reproducible results to ensure the quality and safety of the immunobiolgicals.

Validation of an analytical method should demonstrate that the method is reliable and intended to use. In order to validate a new method parameters of validation include accuracy, precision linearity and specificity should be recommended.[10] Depending on the type of analytical methods different parameters of validation should be assessed.[11].For Relative potency determination there is no need to calculate detection limit and quantification limit.

Usually the method validation is carried out by the manufacturer and is transferred to National control Laboratory for testing of the vaccine. If, for any reason the method validated by the manufacturer cannot be transferred to the NCL (non availability of reagents or instruments) a new method should be validated by the NCL before the product is authorized for public [12-13].

There are different methods used for Potency testing of Hepatitis B vaccine.[14] Hepatitis B Relative Potency is presently being tested by different methods by the manufacturers by in vitro method i.e. ELISA. Hence there is an urgent need for a uniform method for estimation of potency of Hepatitis B Component.[15] The commercial kit for automated analysis (AxSYM) is expensive, and an alternative is required for the estimation of HBsAg in hepatitis B vaccines[16]. The Sandwich 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. Here we report the feasibility of using sandwich ELISA method for determination of antigen content.

2. Materials and Methods

2.1 Vaccine Samples and Reagents

The samples received of Hepatitis B vaccine (Monovalent as well as Pentavalent) from different Indian manufacturers at Central Drugs Laboratory Kasauli were taken for this study. To avoid any bias and conflict the Manufacturers names are not disclosed. It should be noted that there is no National Reference Standard available for Hepatitis B vaccine. Hence In house reference standards received from the manufactures were used for the validation of the assay. Relative Potency was assigned in micro grams and the summary lot protocol for batch manufacture as well as validation data was provided by manufacturer. The PBS containing 0.05% Tween 20 were used as diluents to prepare dilutions of samples and reference preparation.

AdaltisEIAgen HBs Ag Kit was used for HBsAg concentration determination.

2.2 Sandwich ELISA for In-vitro potency determination

The In –Vitro method of potency testing of Hepatitis B vaccine is based on direct quantization of HBs content to evaluate the relative potency of vaccine to find the suitable range of dilutions in which the response curves are linear. A wider range of reference and vaccine samples were examined as a result the reference and the test samples are diluted from 1:5000 to1:80000 in PBS containing (0.05% Tween 20). Samples and reference were tested in triplicate. The preparation was evaluated by ELISA (Adaltis EIAgen HBs Ag Kit) to identify the HBs Ag content. Final relative potency was calculated by Parallel Line analysis programme (Bioassay Assist Software).This programme is able to perform both parallel line assay and Probit Analysis. The validation parameters followed in the course study are mentioned hereafter.

2.3 Validation Methodology

Validation of a method is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled [17]. Validation is important as it defines whether the test will produce reliable results in the context of its intended use. For the present study six batches of Hepatitis B vaccine of four manufacturers were taken and studied for different parameters of validation.

2.3.1 Specificity

Specificity is the ability to assess unequivocally the target pathogen or analyte in the presence of components which might be expected to be present [18]. The specificity of an assay is the capability of the assay to differentiate similar organisms or analytes or other interference from matrix elements that could have a positive of negative effect on the assay value. To evaluate the specificity of Hepatitis B

Component both in Monovalent and Combination Vaccines (Liquid Pentavalent Vaccine) by sandwich ELISA using EIAgen HBs Ag Kit. Rabies Vaccine was taken as a Control for which this method is not specific**.** The OD values were observed at 420 -650 Wavelength.

2.3.2 Accuracy

Accuracy is the agreement between value found and an excepted reference value. This requires a "gold" standard or method but in the absence of a gold standard or method, comparison to established reference labs may substitute. [19].Accuracy was reported by three time testing of each batch of Hepatitis B of different Manufacturers. The estimated R.P values were calculated. The percentage of recovery was evaluated. The analyte recovery of the samples should have to be within 80 to 125% of the expected value.[20]

2.3.3 Linearity

Linearity is the ability of the assay to return values that are directly proportional to the concentration of the target pathogen or analyte in the sample. Mathematical data transformations, to promote linearity, may be allowed if there is scientific evidence that the transformation is appropriate for the method. [18-19]To demonstrate the Linearity of the assay, In House Reference Standards of different manufactures were taken. The R^2 values of IHRS of three different days were taken. The Coefficient of Linearity determination (R^2) should be $\geq$ 0.99 and the statistical analysis shows no significant deviation from parallelism or linearity.

2.3.4 Precision

Precision is defined as "The closeness of agreement between independent test results obtained under stipulated conditions"[21]. There are three different types of precisions depending on the stipulated conditions and these are repeatability (r), intermediate precision (Rw) and reproducibility (R). Repeatability is the variability observed when as many factors as possible, e.g., laboratory, technician, days, instrument, reagent lot are held constant and the time between the measurements is kept to a minimum as opposed to reproducibility conditions where all factors are varied and measurements are carried out over several days. For intermediate precision, all factors except laboratory are allowed to vary and for clarity the factors changed should be stated in the validation report. Repeatability is sometimes called within-run or within-day precision while intermediate precision is also known as between-run or between day repeatability.

Precision is difficult to quantify and it is therefore the inversely related imprecision that is commonly reported. As measures of the imprecision it is usual to report both the SD and coefficient of variation (%CV) for the different levels of the measured investigated with the condition as a subscript, e.g., %CV_{Rw}. Analysis of variance (ANOVA) is used in the estimation of the imprecision and to facilitate in the calculations an excel file (Data Sheet S3 in Supplementary Material) has been created using the formulas in ISO 5725-2 [22].

2.3.5 For Intra assay precision- (repeatability)

The precision of the analytical method is the degree of agreement among individual test results when the method is applied repeatedly to multiple samplings of homogenous sample [18-19]. It should be done by at least one analyst. The percentage CV between the final in vitro potency/antigen content values of all six in dependant assays should be equal to or not more than 15 %.

2.3.6 For Inter assay precision- (reproducibility & ruggedness)

The ruggedness of an analytical method is the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of conditions, such as different analyst, different days etc.[23] It should be done at least two analysts (Analyst-1 and Analyst-2) on two different days (Day-1 and Day-2).

The percentage CV between the final in vitro potency/antigen content values of all six independent assays and between the two analysts should be equal to or not more than 15 %.

3. Results

Four Indian Manufacturers (I, II, III, and IV) of Hepatitis B vaccine (Monovalent and Combined Vaccine) were taken for potency estimation of sandwich ELISA method. To avoid any bias and conflict the name of manufacturers are not disclosed for the current study.

3.1 Specificity

The ELISA Kit applied for the in –vitro assay is used for diagnostic purposes. All the four samples results complied with the acceptance criteria and the method validated to be specific. This method was not specific for rabies vaccine which was taken as a control.

3.2 Accuracy

To demonstrate the accuracy of the test method three independent assays were carried by single analyst on three different days. In house reference standards of different manufacturers were taken and assay was performed. The mean of the results of different manufactures are **Manufacturer I** 113%, **Manufacturer II** 106% , **Manufacturer III** 113% and **Manufacturer IV** 111%.These values of recoveries of the samples were between 80 to 125% as is of the expected value. Hence all the results were within acceptance criteria. The results of accuracy are summarized in **Table 1.**

Table 1: Results of Accuracy

Mfgs	No. of Replicates	Expected Value (R.P.)	Observed Value	% Recovery
I	1	0.923	1.219	98
	2	0.923	1.119	121
	3	0.923	1.123	121
II	1	1.53	1.556	101
	2	1.53	1.583	103
	3	1.53	1.592	104
III	1	1.09	1.046	95
	2	1.09	1.352	124
	3	1.09	1.336	122
IV	1	1.119	1.355	121
	2	1.119	1.319	117
	3	1.119	1.078	96

3.3 Linearity

To demonstrate the linearity of the assay, data were collected from the Accuracy study. For the validated assay the Coefficient of Linearity determination (R^2) should be ≥ 0.99 and the statistical analysis should show no significant deviation from parallelism or linearity. **Manufacturer I** the observed R^2 value of IHRS was ≥ 0.99, **Manufacturer II** the Observed R^2 value of IHRS was ≥ 0.99, **Manufacturer III** the observed R^2 value of IHRS was ≥ 0.99& **Manufacturer IV** the observed R^2 value of IHRS was ≥ 0.99.The observed R^2 values of IHRS of different manufacturers at three different days were ≥ 0.99.Figure 1 to 12 represents the graphical representation of R^2Value and linearity of different Manufacturers at three different days. Hence, it shall be anticipated that the parameter of linearity is being followed in the given validation assay.
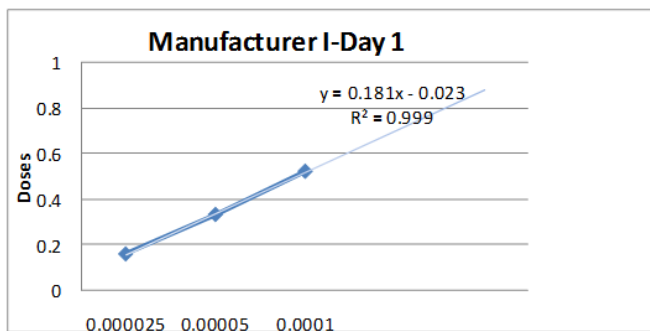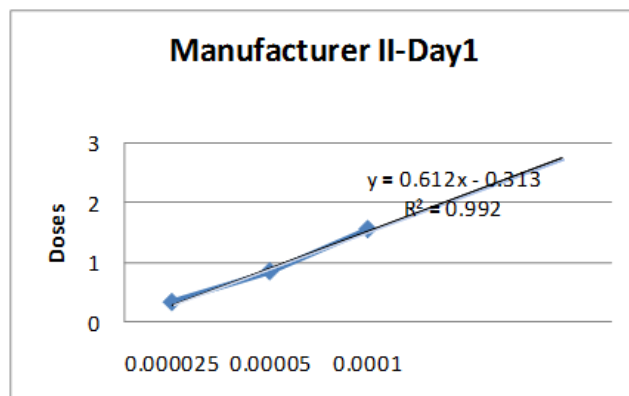

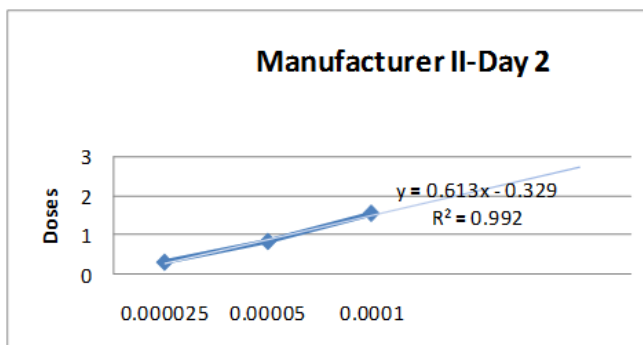**Figure 1:** Graph represents Linearity at Day 1 of Mfg I
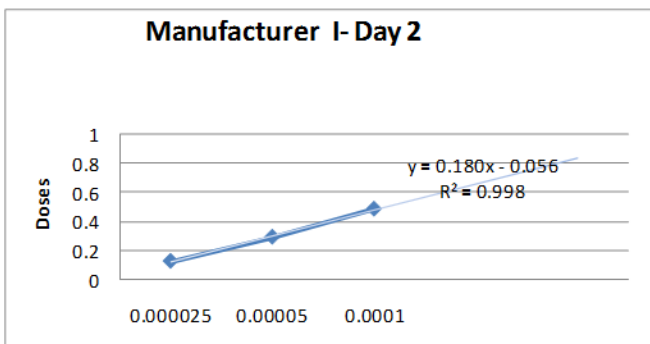
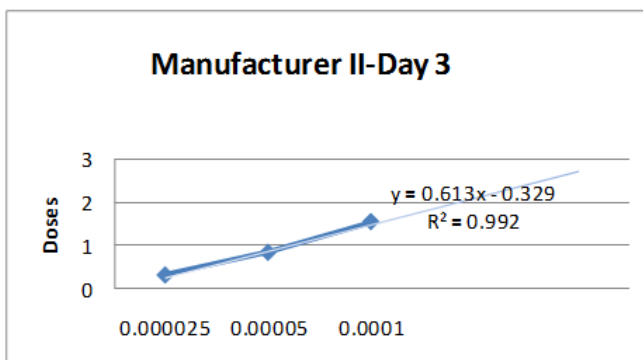**Figure 2:** Graph represents Linearity at Day 2 of Mfg I
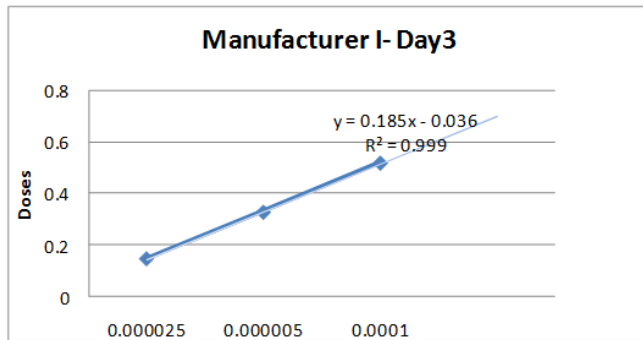
Figure 3: Graph represents Linearity at Day 3 of Mfg I

Figure 4: Graph represents Linearity at Day 1 of Mfg I

Figure 5: Graph represents Linearity at Day 2 of Mfg II

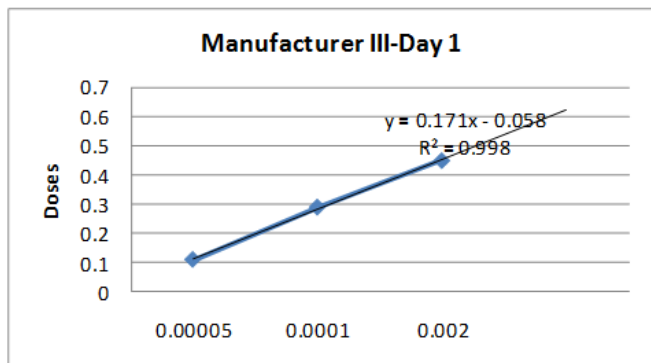**Figure 6:** Graph represents Linearity at Day 3 of Mfg II
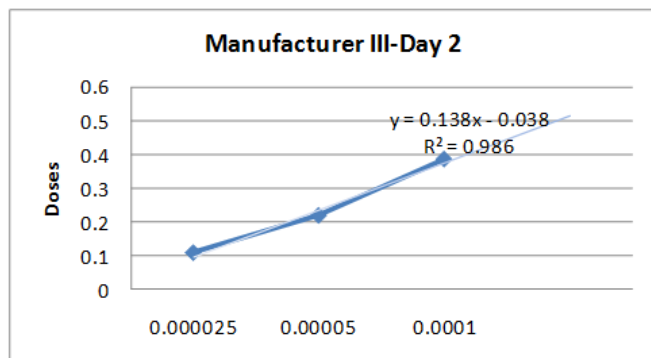
Figure 7: Graph represents Linearity at Day 1 of Mfg III

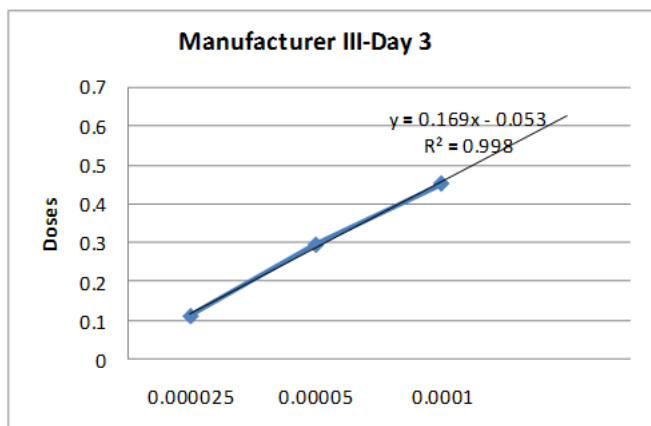**Figure 8:** Graph represents Linearity at Day 2 of Mfg III

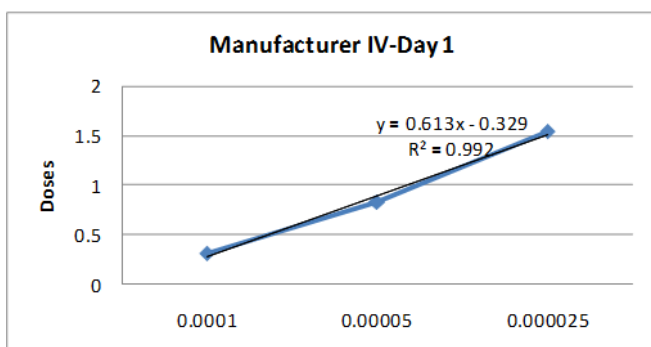**Figure 9:** Graph represents Linearity at Day 3 of Mfg III

Figure 10: Graph represents Linearity at Day 1 of Mfg IV
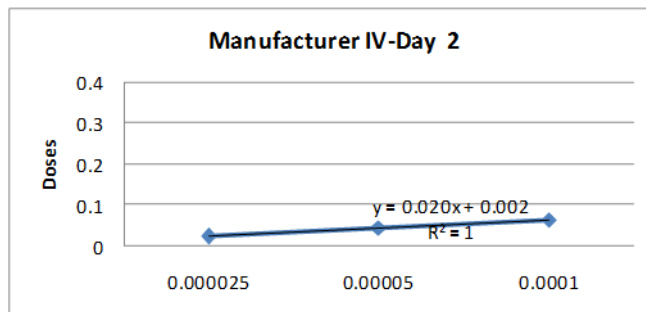

Figure 11: Graph represents Linearity at Day 2 of Mfg IV
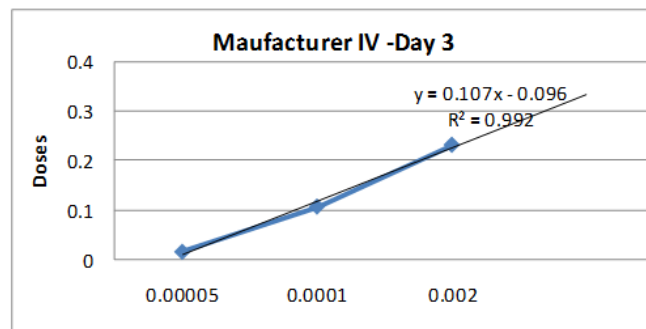

Figure 12: Graph represents Linearity at Day 3 of Mfg IV

3.4 Precision

3.4.1 Intra assay precision- (repeatability)

The results of the Intra assay precision (repeatability) of the six independent assay of four different manufacturers are summarized in **Table 2**. The percentage CV between the final in vitro potency/antigen content values of all six independent assays should be equal to or not more than 15 %.It was observed from the data that the mean Relative Potency of **Manufacturer I**: 1.544 ± 0.088 and the %CV is 5.6 , **Manufacturer II :** 1.582 ± 0.08 and the %CV is 4.9 , **Manufacturer III:** 1.254 ± 0.167 and the %CV is 12.8 & **Manufacturer IV:** 1.424 ± 0.062 and the %CV is 4.3. This is complying with the acceptance criteria of % CV equal to or not more than 15%.

Table 2: Results of repeatability

No. of Assays	MfgI	Mfg II	Mfg III	Mfg IV
1	1.490	1.680	1.069	1.355
2	1.592	1.459	1.068	1.506
3	1.670	1.596	1.355	1.483
.4	1.483	1.534	1.187	1.428
5	1.459	1.587	1.428	1.416
6	1.634	1.636	1.416	1.355
Mean	1.544	1.582	1.254	1.424
S.D.	0.088	0.080	0.167	0.062
%CV	5.6	4.9	12.8	4.3

3.4.2 Inter assay precision- (reproducibility & ruggedness)

To evaluate the intra assay precision, demonstrating laboratory variations, the test were performed by analyst in different days. Average, Standard deviation and coefficient of variation was calculated Moreover the test were repeated by two analysts and the results of each were evaluated by t test.

The acceptance criterion of reproducibility is same as repeatability beside the results of t test for should not be significant. In intermediate precision study, it was observed that for **Manufacturer I :** The mean RP observed among assay carried out by different analysts on the same day ranges from 1.4862 ± 0.33 to 1.652 ± 1.5 and % CV ranges from 0.33 to 1.5.The mean RP observed among the assays carried out by a single analyst on different days are 1.584 ± 0.090 and 1.52± 0.094 and the % of CV ranges from 5.6 to6.2.The mean of RP observed among all assays carried out by analyst on two different days i.e. mean RP of six determinations is 1.55 ± 0.088 and the % CV is 5.7. **Manufacturer II:** The mean RP observed among assay carried out by different analysts on the same day ranges from 1.4862 ± 0.3 to 1.652 ± 1.5 and % CV ranges from 0.004 to 0.094.The mean RP observed among the assays carried out by a single analyst on different days are 1.578 ± 0.111 and 1.585± 0.051 and the % of CV ranges from 3.16 to 7.0.The mean of RP observed among all assays carried out by analyst on two different days on two different days i.e. mean RP of six determinations is 1.58 ± 0.077 and the % CV is 4.8. **Manufacturer III:** In intermediate precision study , it was observed that the mean RP observed among assay carried out by different analysts on the same day ranges from 1.248 ± 0.317 to 1.3857 ± 3.0 and % CV ranges from 2.5to 7.3.The mean RP observed among the assays carried out by a single analyst on different days are 1.164 ± 0.16 and 1.34 ± 0.13 and the % of CV ranges from 14.2 , 10.08.The mean of RP observed among all assays carried out by analyst on two different days on two different days i.e. mean RP of six determinations is 1.25 ± 0.16 and the % CV is 13. **Manufacturer IV:** In intermediate precision study , it was observed that the mean RP observed among assay carried out by different analysts on the same day ranges from 1.48 ± 0.042 to 1.65 ± 0.025 and % CV ranges from 0.33 to 1.54.The mean RP observed among the assays carried out by a single analyst on different days are 1.588 ± 0.088 and 1.506 ± 0.079 and the % of CV ranges from 5.24 to 5.54.The mean of RP observed among all assays carried out by analyst on two different days on two different days i.e. mean RP of six determinations is 1.55 ± 0.088 and the % CV is 5.6.

Table 3: Results of reproducibility of Manufacturers

Total Assay Performed (Six	*Mfg I*	*Mfg II*	*Mfg III*	*Mfg IV*
Mean of R.P of Analyst -1	1.584	1.578	1.164	1.588
Mean of R.P of Analyst -2	1.525	1.585	1.343	1.506
Standard Deviation of Analyst-1	0.090	0.111	0.165	0.088
Standard Deviation of Analyst-2	0.094	0.0510	0.135	0.079
%CVAnalyst-1	5.6	7.0	14.2	5.54
%CVAnalyst-2	6.2	3.16	10.08	5.24
Mean of 6determination	1.554	1.582	1.254	1.554
S.D. of 6determination	0.088	0.077	0.167164	0.0888
%CV of 6determination	5.7	4.8	13	5.6

4. Discussion

The Quality control of Hepatitis B vaccine revolves around the major parameter of potency, previously till 1996only in –vivo testing of Hepatitis B was permitted by WHO. According to WHO and European Pharmacopeia, specifications of the in vitro method must be determined in validation studied against in vivo method [24]. This was mainly based on the idea that the animal test could predict the efficacy in humans. Several groups of mice were injected with the different dilution of vaccine in order to establish an ED_{50}.Inspite of inherited problem of in –vivo assay, this assay remains the main stay for potency estimation of Hepatitis B vaccine particularly for new manufacturer before establishment for consistency for subsequent use of in –vivo assay for in process and final lot it has to be established that there is good correlation between the final lot. In addition to the international agreement for reducing animal use for quality control of vaccines , [25-26] there is a current tendency for using alternative methods to animal for potency determination as it is variable , expensive , time consuming and more intensive[27].

The need for different method arises especially when the manufacturer uses highly expensive instruments for testing purposes. This poses challenge to the National Control Laboratories for determination of potency specification for Hepatitis B vaccines from different sources. Thus NCL should focus on In house developed method with communication with the manufacturers during the few lots obtained during the registration and licensing period.

Indian Hepatitis B vaccine is used for immunisation in our country and by other countries. There is a need to harmonise the quality control procedures, mainly the potency tests. Taking into account that the in vivo potency test is more expensive, variable and longer than the alternative methods and that the commercial kits are expensive.[28] A commercial HBsAg Kit different from that used by the manufacturers of the Hepatitis B recombinant vaccine was used for potency testing of Hepatitis B (Monovalent and Pentavalent) produced by different manufacturers and validated for routine lot release.[29-30] Precision of the test was assessed as repeatability and intermediate precision, Accuracy was checked by percentage recovery approach, specificity was tested by using different product without hepatitis B component in it. All the validation Parameter predetermined for validation parameters were met.

Manufacturing process of rDNA Hepatitis B vaccine for different Manufacturers is different. The yeast used for the production is different for different manufacturers, some of the manufacturers used *Pischia Pistaria* and *Hansenula Polymorpha* [13] further the flow chart for the production is different , some of the Hepatitis B are with Thiomersal and without Thiomersal. During the validation of potency test of vaccine, there is lot of variation in the results as already discussed of different manufacturers but are in range of the accepted limits The aim of our Laboratory was to evaluate the validation parameters such as specificity, linearity, accuracy, and precision which gave outstanding results with four

Manufacturers and to have a uniform method for potency testing of Hepatitis B Vaccine.

5. Conflict of Interest

It is declared that this study raises no conflict of interest to any other person involved to this study and in the department.

Acknowledgment

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