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Development and Standardization of ELISA as Pre-screen Test for the Potency Estimation during Commercial Production of Antisnake Venom Serum (ASVS)

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Abstract

In vivo neutralization assay is the only accepted method for the potency testing of antisnake venom serum (ASVS), till today. Due to non-availability of any other methods, this assay is considered as gold standard for potency testing. However, it requires large number of animals. There is an increasing need over the years to develop an *in vitro* alternate method so as to reduce/abolish the use of animals. Further, newer method may prove less complicated, sensitive, less expensive and less laborious for potency testing of antivenom. The present study was planned to develop, standardize an ELISA assay for the potency testing of ASVS and to validate its utility as a pre-screen test during commercial production. The ELISA assay was standardized and validated critically and, was applied to test the potency of purified anti Cobra venom serum and polyvalent ASVS. Different variables were tested to determine optimal antigen–antibody concentrations, antigen-coating conditions and the assay validation as per the WHO recommendations. The ELISA assay was found to be specific and sensitive with marked reproducibility. Further comparison of *in vitro* and *in vivo* result indicated that ELISA method can quantitate the anticobra Ig’s in the serum, accurately as high degree of correlation > 0.9 was obtained between both the assays. The result suggests that ELISA can be used as a pre-screen assay to the conventional *in vivo* assay during initial stages of antivenom production.

Keywords: Antisnake venom serum, ELISA, Cobra venom, Neutralization assay

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1. Introduction

Since the inception of human civilization, snakes have always been objects of fear and superstition with veneration and respect. Poisonous snakes are common health hazard all over the world including Africa, Asia, Latin America and New Guinea [1]. Worldwide, about 65 species pose serious health hazards to man [2]. In India, a total of 216 snake species have been reported, out of which, Cobra (*Naja naja*), Krait (*Bungarus caeruleus*), Russell's viper (*Vipra russelli*) and Saw Scaled viper (*Echis carinatus*) are responsible for most of the snakebite deaths [2, 3]. Poisonous snakebite requires prompt and specific measures in terms of potent specific anti snake venom serum (ASVS), which is the only scientific approach to effectively treat these cases. The polyvalent ASVS prepared at Central Research Institute, Kasauli, HP, India is an enzyme refined globulin $F(ab')_2$, prepared from the plasma of equines hyper immunized with venoms of above four medically important snakes.

The principle of antivenom therapy relies on neutralization of snake venom with specific antivenom serum. According to Indian pharmacopoeial requirements, 1 ml of ASVS should be able to neutralize not less than 100 intravenous mouse LD_{50} or 0.6mg each of Cobra and Russell viper venom and 0.45mg each of Krait and Echis venoms. Till date, the potency testing of ASVS is based on protection and neutralization tests in animals. Although neutralization assay with 50% end point (median effective dose, ED_{50}) has been accepted worldwide as standard bench mark [1, 4] but due to cost constraints and animals usage, the need for reliable *in-vitro* assays is always of interest. Moreover, traditionally employed animal assays are laborious, expensive and time consuming **[5].** Therefore, considering the economical and ethical aspects, there is a necessity to develop *in-vitro* alternatives wherever possible to minimize the cost, time and use of laboratory animals [1, 6].

Standardization of *in vitro* assay for ASVS screening is going on, worldwide [7, 8]. *In vitro* assay are easy to perform with consistent and reproducible results, which are difficult to obtain with *in vivo* assay [9]. *In vitro* test can be used for the evaluation of biological activity of different preparations such as venom and its antivenom [10]. Many *in vitro* assays have been reported till date including ELISA and cell line assay [9, 11-17]. To the best of our knowledge, none of these have been adapted in routine practice, so far. Keeping this in view, study was undertaken primarily to standardize ELISA assay for the potency estimation of ASVS using cobra venom as reference; and finally to validate ELISA to exploit its utility as a pre-screen assay to minimize the use of laboratory animals during antivenom efficacy testing at in-process production level. The in-vivo assay may sustain in routine testing at final production level.

2. Materials and methods

2.1 Reagents

All the reagents prepared were of highest purity (Analytical Grade). Solutions were prepared in high purity triple distilled water.

2.2 Cobra venom and sera samples

Certified pure cobra venom and polyvalent equine antisnake venom serum (ASVS) in the form either of (a) hyper immune serum samples and (b) hyper immune plasma obtained after bleeding of equines or (c) purified ASVS were obtained from the Antisera Division, Central Research Institute, Kasauli, H.P. (India). Lyophilized monovalent Cobra antivenom serum (reference standard), was obtained from Central Drugs laboratory, Central Research Institute, Kasauli, H.P. (India).

2.3 Development and Standardization of ELISA

Standard Cobra antivenom serum was used as reference. Checkerboard titrations were made to optimize the concentration of the antigen and antibody.

2.4 Antigen coating

100 µl of cobra venom antigen (0.025mg/ml in 50mM carbonate-bicarbonate buffer-pH 9.6) was added to first well of each column of

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microtiter plate (Greiner, USA). 11th and 12th columns were used as controls. Serial 2-fold dilutions of antigen were carried out while moving in rows from A to H. The plate was incubated at 4°C for overnight.

2.5 Blocking

After incubation, wells were washed 5 times with PBST (PBS- 0.01M- pH 7.2 with Tween-20, Sigma Chemicals, USA) using ELISA washer (TECAN, Model: Columbus Plus-Basic, Austria) and blot dried. 100µl of blocking reagent (1% w/v BSA, Sigma Chemicals, USA) in PBST was added in each well. Plate was incubated further at 37°C for 2 hours.

2.6 Antibody dilution

After incubation, wells were washed 5 times and blot dried as described earlier. Reference serum was diluted 1:32 in PBST and further 2-fold dilutions were prepared in PBS (pH 7.2). Finally the each dilution of serum was added to wells of each column. Antigen control, positive control, negative control, BSA control, conjugate control, buffer control and plate controls were also processed simultaneously. The plate was incubated at 37°C for another 2 hours.

2.7 Addition of conjugate and substrate

Anti-horse IgG –HRP conjugate (Sigma chemicals, USA) was used at 1:10,000 dilution as per the manufacturer's instructions. The plate was incubated at 37°C for 1hour followed by washing. To each well, 100µl of freshly prepared substrate (*viz.,* H_2O_2-o-phenylenediamine dihydrochloride, Sigma Chemicals, USA) was added and the plate was incubated in dark at 22°C for 30 minutes. Reaction was stopped by adding 50µl of 1N H_2SO_4 and absorbance was read at 492nm using ELISA plate reader (TECAN, Model: Sunrise-Basic, Austria).

2.8 Determination of Cut off value

For determination of cut-off value for ELISA assay, the discrimination level for positive and negative results either may be set at 0.15 or 0.20 or taken as the Mean plus two or three standard deviations (SD) [18, 19]. In the present study the cut off value was calculated as mean absorbance of negative control wells plus 2SD. Similar method was used for the standardization of ELISA assay for the estimation of antibody titer of Cobra antivenom serum and different ASVS samples.

2.9 Validation of ELISA assay

For the validation of ELISA assay, different parameters which may affect the performance of ELISA such as specificity, sensitivity, linearity, accuracy, precision (intra and inter assay) and robustness were critically evaluated as per the requirements of WHO [20, 21] and Eurachem guidelines [22].

2.10 Venom toxicity assay

Swiss Albino mice (17-20g, age 3-4weeks), were used for potency estimation of monovalent and polyvalent antisera. Study was duly approved by institutional ethical committee (CPCSEA, registration no. 04-05/2006). Serial 20% decrement dilutions of Cobra venom (10mg/ml) were prepared in physiological saline. 0.5ml of each dilution was injected intravenously to each mouse in group of six. Mice survivals (L) and deaths (D) were observed for 48 hours and LD_{50} of the venom was calculated using Spearman-Karber Method [1].

2.11 Potency testing of Cobra antivenom serum (monovalent) and ASVS (polyvalent)

2.11.1 In vivo neutralization assay (ED_{50} assay)

Potency testing of purified and in-process Cobra antivenom serum was carried with $6LD_{50}$ of Cobra venom mixed with different dilutions of antivenom. Each reaction was incubated at 37°C for 30 minutes followed by injecting 0.5ml of each mixture I.V. in mice. The control group of mice was given a mixture of venom (challenge dose) with saline solution. Mice were observed for survival or death after 48hrs. Results were calculated by Probit analysis [23]. Potency was expressed in terms of milligram of cobra venom neutralized by 1.0ml of the monovalent or polyvalent anti venom serum.

2.11.2 In vitro potency test (ELISA)

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Potency testing of polyvalent ASVS, hyper immune equine plasma and serum was carried out by ELISA. For this, samples were appropriately diluted (1:32 to 1:2038) and were titrated against 0.00625µg/ml of antigen i.e. venom. The titer of each sample was calculated using anti Cobra antivenom serum as standard reference.

2.12 Statistical analysis

All assays were performed in triplicate and results obtained were evaluated statistically using software Graphpad prism ver. 11.05.

3. Results

3.1 Standardization of ELISA

3.1.1 Optimal antigen/antibody concentration

Checker board titrations were performed to determine the optimum concentration of antigen (Cobra venom) and antibody (reference anti cobra venom serum). The optimum concentration of antigen was observed to be 0.00625µg and that of antibody was 1:128. Mean cut-off value of 0.159 was determined after 5 independent assays.

3.1.2 Optimum incubation temperature for antigen coating

Of the four different time and temperature combinations worked out for optimum antigen coating, incubation at 37°C for two hours followed by storage at 4°C overnight was found to be the best ($p<0.0001$, Table 1).

3.1.3 Titration of standard Cobra antivenom serum and ASVS using ELISA

At different dilutions of the reference Cobra antivenom (monovalent) and ASVS (polyvalent) antiserum, assay was found to be linear between 1:32 to 1:32768 dilutions. Absorbance was plotted against the logarithm of inverse of sera dilutions. At both lower (≤1:16) and higher dilutions (≥1:65536), assay response was observed to be non-linear (Fig. 1). The titers of monovalent and polyvalent antiserum were calculated to be 1:1799 and 1:1915.5 respectively using in-house standard curve. The respective potencies of monovalent and polyvalent sera were found to be 0.66mg and 0.70mg of Cobra venom when tested through *in vivo* neutralization assay.

Table 1: Optimization of incubation temperature for antigen coating for the assay.

Temperature and time period	Absorbance[a] (Mean ± SD)[b]	CV (%)
A. 37°C for 3 hours	1.490 ± 0.077	5.1
B. 4°C overnight	0.284 ± 0.017	5.9
C. 37°C for 2 hours followed by 4°C overnight	1.776 ± 0.106[c]	6.0
D. 37°C for 3 hours followed by 4°C overnight	1.595 ± 0.107	6.7

The concentration of antigen, antibody dilution (Anti Cobra Venom Serum - reference standard) and conjugate (HRP) were fixed to 0.00625 µg/ml, 1:128 and 1:10,000 respectively.

a Absorbance measured at 492nm.

b Mean and standard deviation of n=20 absorbance values.

c Statistically significant difference ($p < 0.0001$) (calculated using Graphpad online calculator) from temperature and time period A, B and D.

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Table 2. Validation of Specificity of indirect-ELISA assay.

Serum Samples	Absorbance at 492nm		
	Mean[d]	**SD**	**CV**
Normal horse serum[a]	0.057	0.0019	3.3
Diphtheria antitoxin[a]	0.060	0.0036	7.2
Anti krait venom serum[a]	0.058	0.0032	5.4
Anti Echis venom serum[a]	0.058	0.0028	4.8
Anti Russell venom serum[a]	0.057	0.0030	5.4
Tetanus antitoxin[a]	0.064	0.0035	5.5
Antirabies serum[a]	0.090	0.0038	4.3
Anti cobra venom serum[b]	1.308	0.0217	1.7
Polyvalent ASVS[c]	1.604	0.0207	1.3

Specificity was performed by coating the 96-well microtitre plate with 0.00625μg/ml of antigen concentration and using nine different sera samples. The assay was repeated ten times per sera sample.

ASVS= Anti Snake Venom Serum

a Negative control serum;

b Reference standard serum;

c Test serum ;

d Mean of n= 10 absorbance values for each serum.

Table 3. Validation of accuracy of the indirect-ELISA assay.

Assay	Mean Absorbance	Standard deviation	Upper fiducial limit[a]	Lower fiducial limit[b]
I	1.463	0.029	1.479	1.447
II	1.458	0.035	1.478	1.439
III	1.440	0.041	1.463	1.417

The accuracy of indirect-ELISA assay is explained in terms of fiducial limits at 95% level. The assay was performed three times using the reference serum at a dilution of 1:64. The mean absorbance of 12 wells containing the above mentioned serum dilution was calculated. Each absorbance value was in between the upper and lower fiducial limits.

a Upper fiducial limit = Mean + 1.96 (Standard error)

b Lower fiducial limit = Mean - 1.96 (Standard error)

3.2 Validation of ELISA

3.2.1 Sensitivity and Specificity

Sensitivity of assay was determined by limit of detection (LOD) and limit of quantitation (LOQ). The LOD and LOQ were found upto serum dilution of 1:64748 and 1:56694, respectively. Specificity of ELISA assay was ascertained by using different antitoxins and their products [specific Cobra antivenom serum (mono

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and polyvalent) and non-specific (Diphtheria antitoxin, tetanus antitoxin, anti-rabies serum, anti-Krait venom serum, anti-Echis carinatus venom serum and anti-Russell's viper venom serum]. Assay was repeated 10-times with negative and positive control and was found to be highly specific (Table 2).

3.2.2 Linearity

The assay was found to be nearly linear for the dilution range from 1:32 to 1:32768 (Fig. 1).

3.2.3 Accuracy and Precision

The accuracy of ELISA is explained in terms of fiducial limits at 95% level. The assay was performed three times using the standard reference serum and mean absorbance calculated. Results of ELISA showed no significant variation since mean absorbance values were found to be well within the upper and lower fiducial limits (Table 3).

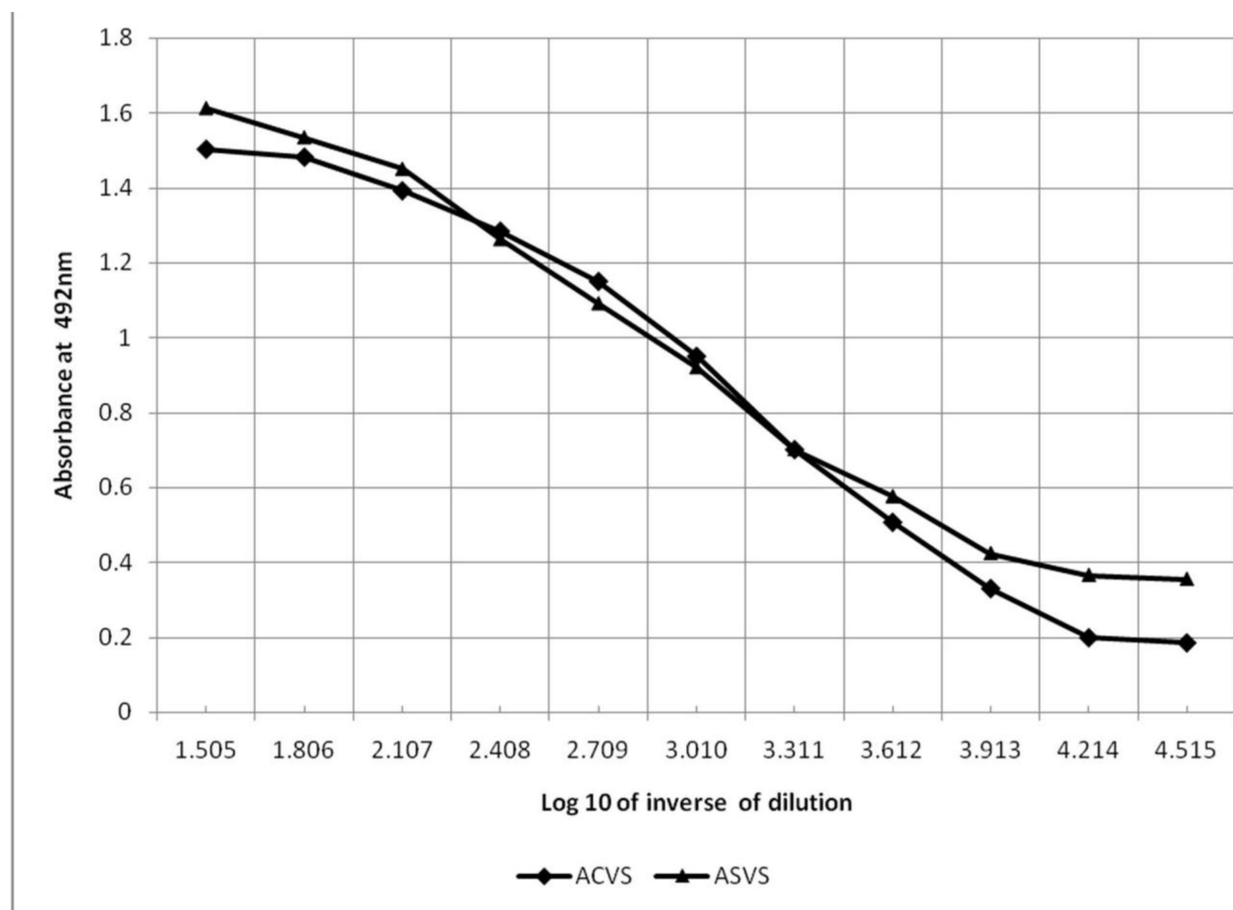

Figure 1. Figure showing results for titration of Anti Cobra Venom Serum (ACVS) and Anti Snake venom Serum (ASVS) using indirect-ELISA assay and the results of validation of linearity of the assay in standardization of ELISA.

3.3 Intra-assay precision (Reproducibility)

Intra-assay precision was established by calculating coefficient of variation (C.V.) and limit of variation (L.V.). The CV of the two batches of ASVS (6 vials each) was found to be 0.72% & 0.51% while the LV was 0.81% and 0.63% respectively indicating nearly less than 1% variation in the results (Table 4).

3.4 Inter-assay precision (Repeatability)

For ELISA inter-assay precision, six batches of ASVS were titrated on three different time periods, i.e. day 1, 3 and 5. Less than 1% variation was observed on all three days. The average CV and LV were calculated to be 0.19% and 0.21%, respectively, for the inter-assay precision (Table 5).

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Table 4. Validation of intra-assay precision of indirect-ELISA assay.

ASVS Batch No.	ASVS Batch (Vial No.)	Inverse of titre of ASVS	Mean of Inverse of titre	SD	CV (%)	LV (%)
A	1	1850	1850	13.23	0.72	0.81
	2	1865				
	3	1840				
	4	1870				
	5	1835				
	6	1840				
B	1	1850	1852	9.43	0.51	0.63
	2	1840				
	3	1855				
	4	1860				
	5	1840				
	6	1865				

ASVS – Anti snake venom serum (polyvalent).

CV – Coefficient of variation.

SD – Standard deviation.

Limit of variation (LV) is defined as = ± % highest individual deviation from the Mean value and was calculated as: LV(%) = [(mean of inverse of titre – lowest titre) / mean of inverse of titre] X 100 .

Table 5. Validation of inter-assay precision of the indirect-ELISA assay.

ASVS Batch No.	Inverse of titre of ASVS			Mean	SD	CV (%)	LV (%)
	1st Day	3rd Day	5th Day				
A	1860	1850	1855	1855	4.08	0.22	0.27
B	1830	1835	1830	1832	2.36	0.13	0.09
C	1860	1855	1855	1857	2.36	0.13	0.09
D	1840	1855	1850	1848	6.24	0.34	0.45
E	1835	1840	1830	1835	4.08	0.22	0.27
F	1855	1855	1860	1857	2.36	0.13	0.09

The average CV for the batches tested = 0.19%

Limit of variation (LV) is defined as = ± % highest individual deviation from the Mean value and was calculated as: LV(%) = [(mean of inverse of titre – lowest titre) / mean of inverse of titre] X 100 .

The average LV for the batches tested = 0.21%

3.5 Robustness

Robustness of the assay was determined by variation of time and temperature on antigen coating and effect of storage time on ELISA performance. Maximum titer was obtained when plate was incubated at 37^0C for 2 hours, followed by incubation at 4^oC overnight. It has been speculated that storage of ELISA plate for longer periods after antigen coating may affect the test results. To validate this, assay was performed on

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four different storage time intervals i.e. day 0, 10, 20 and 30 after antigen coating. A significant difference in the ELISA titre was observed on day 10 onwards as compared to freshly coated plates ($p \leq 0.0021$) (Table 6).

Table 6. Validation of robustness of indirect-ELISA assay.

			Mean $\pm$ SD[c]	CV
Parameter –I[a]	i.	37^0C for 2 hours and 4^0C overnight	1863 $\pm$ 14.63	0.79
	ii.	37^0C for 3 hours and 4^0C overnight	1753 $\pm$ 57.49	3.28
	iii.	4^0C overnight	1501 $\pm$ 101.04	6.7
	iv.	37^0C for 3 hours	1565 $\pm$ 119.10	7.6
Parameter –II[b]	i.	0th Day	1860.00 $\pm$ 17.56	0.94
	ii.	10th Day	1816.70 $\pm$ 15.72	0.87
	iii.	20th Day	1797.70 $\pm$ 12.47	0.69
	iv.	30th Day	1751.30 $\pm$ 15.29	0.87

a Effect of incubation temperature and time period for antigen coating on ELISA assay.

b Effect of storage of antigen coated plates at 2-4 oC for different lengths of time.

c Mean and standard deviation of n=6 titres.

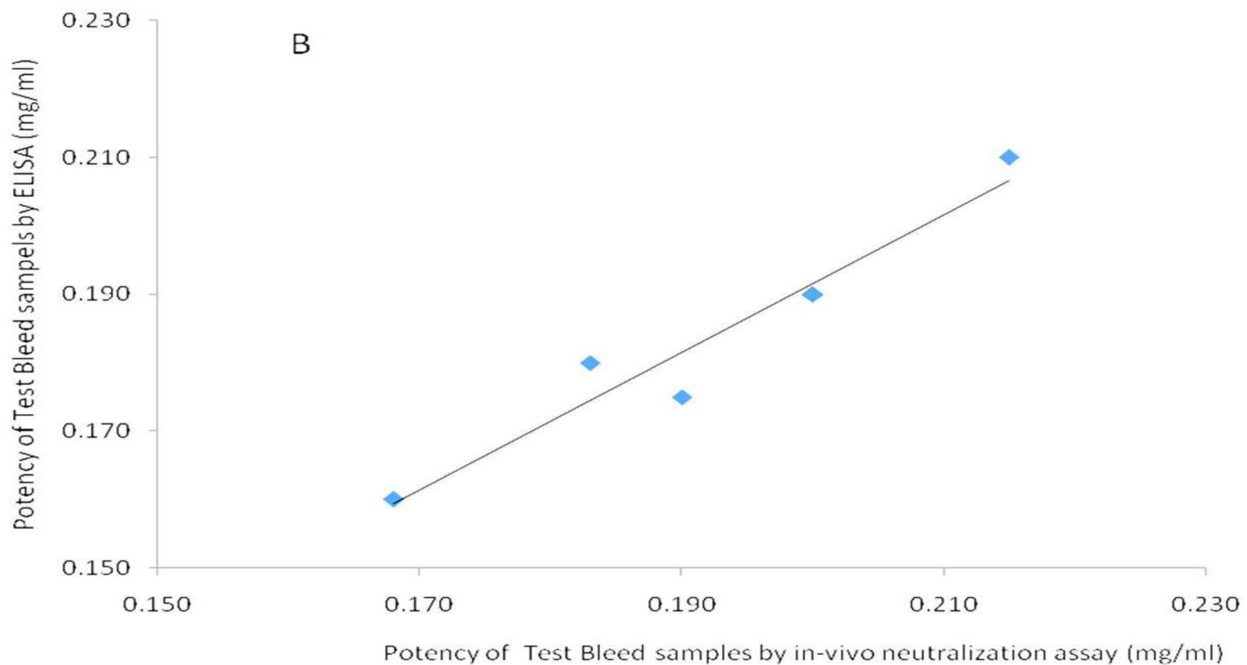

Figure 2. Representative figure showing the results of correlation between ELISA and *in-vivo* neutralization assay for potency estimation of test bleed serum samples.

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3.6 Comparison of potency testing by ELISA or *in vivo* neutralization assay

Results of both the assays showed a high degree of correlation when findings of ELISA were compared with the observations of *in vivo* neutralization assay (Table 7). Significant regression values were observed for each antiserum tested, purified ASVS (r = 0.969); test Bleed (r = 0.968) and hyper immune plasma (r = 0.934) (Figure 2).

Table 7: Antisnake venom serum, ELISA, Cobra venom, Neutralization assayAntisnake venom serum, ELISA, Cobra venom, Neutralization assayTable 7: Test of equivalency for determination of anti-cobra antibodies using *in-vivo* neutralization assay and ELISA.

	Inverse of antibody titre by ELISA	**Potency of antiserum***		**Coefficient of correlation**
		ELISA Assay (mg/ml)	*In-vivo* Assay (mg/ml)	
ASVS	1849.1	0.678	0.680	0.969
	1914.5	0.702	0.710	
	1838.2	0.674	0.690	
	2015.5	0.739	0.740	
	1922.7	0.705	0.720	
	1838.2	0.674	0.680	
Test Bleed	458.2	0.168	0.160	0.968
	499.1	0.183	0.180	
	545.5	0.200	0.190	
	518.2	0.190	0.175	
	586.4	0.215	0.210	
	545.5	0.200	0.190	
Hyper immune Plasma	621.8	0.228	0.230	0.934
	600.0	0.220	0.220	
	518.2	0.190	0.200	
	545.5	0.200	0.200	
	572.7	0.210	0.220	
	545.5	0.200	0.210	

*Potency of antiserum in mg of cobra venom neutralized by 1ml of antiserum.

Calculation of potency using ELISA was done as follows:

Potency = {Inverse of titre of test sera / Inverse of titre of standard sera} x Potency of Standard sera.

The inverse of titre of standard sera (ACVS) =1800; Potency of Standard sera (ACVS) = 0.66 mg/ml.

ACVS = Anti Cobra Venom Serum (monovalent).

ASVS = Anti Snake Venom Serum (polyvalent).

4. Discussion

Treatment of snakebite cases with a specific and potent ASVS is the only and effective therapy available till date [1]. However, the production and quality testing of ASVS is a complex procedure and has not fully evolved, yet. Although extensive work has been done to address this problem but determination of antivenom efficacy still relies on *in vivo* neutralization test only [1, 4]. Development of *in vitro* alternatives for the potency estimation of ASVS is need of hour to replace the conventional *in vivo* test. The main objective remains to reduce

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the suffering of animals used and/or to refine the *in vivo* test in a way so that pain and distress to test animals can be reduced/eliminated [24]. Further, *in vitro* test offers high sensitivity and specificity; are less complicated, economical and less laborious.

In the present study, an attempt was made to standardize ELISA assay to measure the potency of ASVS. Futher , the assay was validated on the basis of different variables such as antigen–antibody concentration, antigen-coating conditions and incubation time as recommended [20, 22]. The ELISA method has been reported to deliver satisfactory results for the detection of antibodies in serum against different snake venoms, previously [12, 13, 25-28]. However, none of these studies evaluated the ELISA in detail with an approach to adopt in routine practice. In the present study, ELISA method was used for the quantitation of anti-venom antibodies in purified ASVS, equine hyper-immune plasma and test bleed samples. Checkerboard titration was adopted for determination of optimal antigen antibody concentration and it was observed 0.00625 μg of venom antigen was optimum for antibody at a titre of 1:128. Antigen coating at 37°C for 2 hrs followed by storage at 4°C gave the optimal results. Further ELISA assay was validated on various statistical parameters such as sensitivity/specificity, linearity, accuracy/precision and robustness. The assay was found to be sensitive and specific enough with significant reproducibility indicating that ELISA assay can be adopted easily for the potency estimation of ASVS. Results of ELISA assay were also compared with in vivo neutralization assay. A high degree of correlation was observed between both the methods suggesting ELISA as a competent alternative. Similar results have also been documented earlier where good degree of corroboration was observed within results of ELISA and *in vivo* neutralization assay [13].

The production of ASVS is a multistep process involving a series of immunization steps targeted to achieve the desired antibody titre in test bleeds, followed by final bleeding; enzyme digestion of plasma and fractionation of $F(ab')_2$ fragments to final product. At each stage of production, the potency of ASVS is required to be determined. Presently, *in vivo* neutralization assay has been the only choice, which in turn become expansive, cumbersome and time consuming with inherent variability and lack of reproducibility [13]. The purpose of the present study was to develop and standardize an alternate/adjunct to present *in vivo* assay to reduce animal burden. The results suggest that ELISA can be opted as a pre-screen test for potency testing at different stages of ASVS production. The application of ELISA seems important especially during early stage of immunization where segregation of non- or poor responders is required since ELISA can detect even very low amount of antibody, which is otherwise difficult to quantify in the initial stages by conventional *in vivo* assay. This is ethically important as not to unnecessarily induct a poor responder animal into production activity and continue undesired immunization for a longer period. It will definitely reduce the time and cost factor applicable to animal issue.

5. Conclusion

The need of present scientific fraternity is 3R's (Reduce, Refine and Replace) to satisfy the animal issues involved. Scientific communities world-wide are working to develop newer *in vitro* methods as an alternative to currently used *in vivo* biological methods. In these considerations, results of this study suggest that ELISA may be used satisfactorily for estimation of ASVS potency at initial stages whereas *in vivo* neutralization test can be applied at the final stages of antivenom production. However, more controlled study will throw more light on this aspect and will pave the path towards adoption of this method.

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