ISSN 0975-6299 Vol 2/Issue 1/ Jan-Mar 2011

International Journal of Pharma and Bio Sciences

RESEARCH ARTICLE BIOTECHNOLOGY

STANDARDIZATION OF ELISA AS A TEST FOR ESTIMATION OF ASVS IN EQUINE SERA

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ABSTRACT

Snakebite is a serious medical problem worldwide especially in the tropics and subtropics. Administration of anti-snake venom serum (ASVS) is the only effective treatment for snakebites. Snake venom is used to make antivenom serum from horse as the host. Several methods have been suggested as alternatives to the mouse bioassay for venom toxicity and to determine the potency of polyvalent anti-snake venom serum. The present study is done by using ELISA as an alternative to animal assay in screening the equine sera for the presence of antibodies. In the present study, Cobra venom concentrations ranging from 1ng to 100ng / 100 µl / well were used initially for coating. From this 1ng of Cobra venom was found to be sufficiently enough for estimating ASVS. ASVS dilutions upto 1:128 were used and dilutions exceeding this range were found to have no detectable Antivenom. There was a reduction in the O.D values when the coating time was reduced, but still the O.D values between Neat and negative controls are discriminatory. This protocol if standardized will minimize the time duration of the test.

KEY WORDS

ELISA, venom, Anti-snake venom serum. Bioassay, Antibodies

INTRODUCTION

Snake envenomation (considered as a subcutaneous or intradermal injection of venom into the prey or human victims) constitutes a medical hazard in most regions of the world. Snakebite is a serious medical problem worldwide especially in the tropics and subtropics. Snakebite is regarded as a significant medical problem in parts of Asia and Africa. Over the years many medications have been tried to treat snake bite victims (**Robert Morris., 2002**). Snake envenomation is complex involving the direct action of venom components on the tissue and release of various endogenous mediators. Snake venoms are known to cause different metabolic disorders by altering the cellular and enzymatic activities in animals. (**B.V.Lipps., 1999).** The neurotoxin of cobra venom blocks nerve transmission at the site of the neuromuscular junction. (**Su CJ., 1960**).Neurotoxin exerts their toxicity by binding to the acetylcholine receptor (AchR) and block neuromuscular transmission. (**Lee et al., 1972**).

In addition to neurotoxins, snake venom from cobras usually contains another group small basic protein called cardiotoxin or cytotoxin. Despite their different modes of pharmacological action, neurotoxins and cardiotoxins share a high degree of homology (Book-Venoms). ASVS is produced from healthy horses. Nonlethal dose of venom is injected intramuscularly. The dose of venom is increased and administered over a period of time to enhance immunity. The horse serum, which contains antibodies to the venom, is harvested and concentrated. (**Gutieruz et al., 1989).**

Administration of anti-snake venom serum (ASVS) is the only effective treatment for snakebites. History of ASVS dates back to1987. Snake venom is used to make antivenom serum from horse as the host. The use of ELISA to determine antisnake venom potency of horse immune sera should provide benefits of costs and reproducibility compared to in vivo assays. For the indirect ELISA method, 0.016 micrograms/well of Bothrops jararaca or Crotalus durissus terrificus venom were used to coat the plates and 100 microliters/well of each sample of antibothropic or anticrotalic venom sera were used at 1:10,000 dilution. (**Barbosa CF, Rodrigues RJ, Olortegui CC, Sanchez EF, Heneine LG., 1995).**

Potency of ASVS has been tested by using immunodiffusion, passive haemagglutination, counter current immuno electrophoresis and Radio immunoassay (RIA). None of these methods was totally satisfactory for use in tropical countries where snakebites are a major public health problem due to their cost, insensitivity or instability of reagents.

ELISA **[Selvanayagam Z.E** et al.(1999). helps in the differential diagnosis of patients bitten by snakes of different species, which can produce similar clinical features. Refined ELISA techniques have allowed the evaluation of the potency of antivenom. Therefore in the present study, use of ELISA as an alternative to animal assay in screening the equine sera for the presence of antibodies was evaluated.The potency of horse therapeutic polyvalent antibothropic antivenom was assessed by ELISA. They indicated that the fractions of purified antigens would be suitable for the *invitro assay.* **(Heneine et al 1998**).

MATERIALS AND METHODS

Materials

1% Cobra venom was received from the Department of Antitoxin (DAT), King Institute of Preventive Medicine Chennai. Polyvalent ASVS used for neutralization of cobra venom. Rabbit Anti-Horse IgG peroxidase conjugate (Product no. A 6917) was obtained from SIGMA-ALDRICH, Missouri, USA.

Nunc Immuno U16 maxisorp L-469264 strips were used for ELISA.

Carbonate Buffer (0.04 M),Phosphate Buffered Saline (PBS),PBS- Tween solution Blocking solution (Bovine serum Albumin 1%) Tetramethyl benzidine(TMB) solution ,Stop solution (0.1M Phosphoric acid solution) were prepared using standard procedure

Preparation of venom concentrations:

Stock solution: → (A)
1% venom = 1000mg (or) 1g/ 100ml

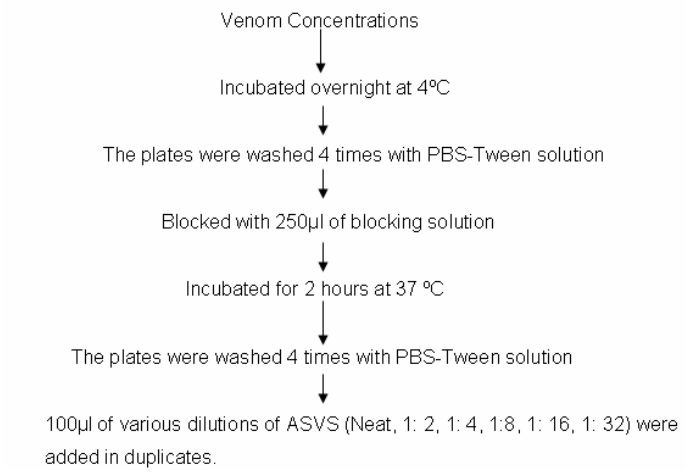
Working standard: → (B)
1µl of 1% venom + 10 ml of carbonate buffer (Concentration- 100ng/100µl)

Preparation of ASVS dilutions:

Neat ASVS → (I)
From (I) 1 ml + 1ml of PBS = 1:2 dilution → (II).
From (II) 1ml + 1ml of PBS = 1:4 dilution → (III).
From (III) 1 ml + 1ml of PBS = 1:8 dilution → (IV).
From (IV) 1ml + 1ml of PBS = 1:16 dilutions → (V).
From (V) 1 ml + 1ml of PBS = 1:32 dilutions → (VI).
From (VI) 1 ml + 1ml of PBS = 1:64 dilutions → (VII).
From (VII) 1 ml + 1ml of PBS = 1:128 dilutions → (VIII).
From (I) 10µl + 990 µl of PBS = 1:100 dilutions. → (IX).
From (IX) 500µl + 500 µl of PBS = 1:200 dilutions. → (X).
From (X) 500µl + 500 µl of PBS = 1:400 dilutions. → (XI).

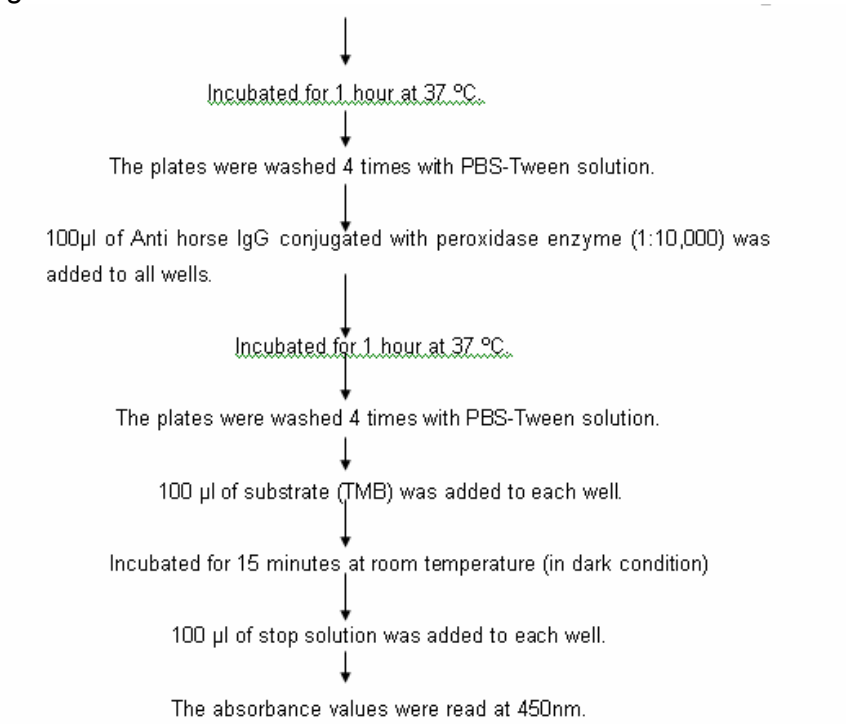
Methods

Experiment 1:



100µl of various dilutions of ASVS (Neat, 1: 2, 1: 4, 1:8, 1: 16, 1: 32) were added in duplicates.

1. 100µl of Neat ASVS was added from A1 to A10.
2. 100µl of 1:2 dilutions of ASVS was added from B1 to B10.
3. 100µl of 1:4 dilutions of ASVS was added from C1 to C10.
4. 100µl of 1:8 dilutions of ASVS was added from D1 to D10.
5. 100µl of 1:16 dilution of ASVS was added from E1 to E10.
6. 100µl of 1:32 dilution of ASVS was added from F1 to F10.
7. 100µl of substrate control was added from G1 to G10.
8. 100µl of negative control was added from H1 to H10.



Experiment 2:

U16 strips were coated with 1ng and 10ng of cobra venom in duplicates.

- Venom concentration:
  1ng / 100µl & 10 ng / 100 µl of venom with carbonate buffer were coated from A1 to E2 & A3 to E4 respectively.

***Experiment* 3:**

U16 strips were coated with 1ng of cobra venom in duplicates.

- Venom concentration:
  1ng / 100µl of venom with carbonate buffer was coated from A1 to E2

Experiment 4 and 5:

U16 strips were coated with 1ng of cobra venom in duplicates.

- Venom concentration:
  1ng / 100µl of venom with carbonate buffer was coated from A1 to H2
  as depicted in table 4.

RESULTS

Determination of ASVS concentrations in various dilutions by ELISA:

- 1ng, 10ng, 25ng, 50ng and 100ng Venom concentrations were used.

Table: 1

Determination of ASVS dilutions for various venom concentrations were carried out:

	1 ng		10ng		25 ng		50 ng		100 ng	
	1	2	3	4	5	6	7	8	9	10
A Neat	0.560	0.561	0.750	0.748	0.849	0.847	0.745	0.747	0.725	0.723
B 1:2	0.543	0.545	0.721	0.720	0.813	0.811	0.698	0.696	0.682	0.683
C 1:4	0.507	0.509	0.686	0.683	0.776	0.776	0.632	0.635	0.617	0.620
D 1:8	0.481	0.483	0.641	0.645	0.745	0.748	0.601	0.600	0.591	0.593
E 1:16	0.463	0.462	0.617	0.618	0.709	0.712	0.579	0.580	0.565	0.563
F 1:32	0.441	0.443	0.594	0.597	0.691	0.689	0.550	0.552	0.531	0.534
G SC	0.053	0.053	0.055	0.054	0.053	0.052	0.053	0.051	0.056	0.056
H NC	0.061	0.061	0.059	0.028	0.057	0.059	0.060	0.060	0.059	0.058

The above readings depict the Optical density values of Experiment 1, Where Neat, 1:1, 1:2, 1:4, 1:8, 1:16 and 1:32 of ASVS dilutions were used.

From the above Table it is inferred that as the dilutions of ASVS increases the O.D values decreases gradually. Though there is decrement, the differences in the titer values are not much. It is observed from above that 1ng of venom is able to give a very discriminatory O.D value between the negative control and the neat. This shows that even 1ng of Cobra venom can be effectively used in detecting the potency of ASVS. This economizes the use of venom in ELISA.

Table: 2

Determination of ASVS concentrations for 1ng and 10ng of Cobra venom

	1ng		10ng	
	1	2	3	4
A 1:32	0.445	0.447	0.591	0.589
B 1:64	0.421	0.423	0.563	0.561
C 1:128	0.401	0.404	0.537	0.539
D SC	0.053	0.051	0.054	0.051
E NC	0.057	0.056	0.058	0.057

The above readings depict the Optical density values of Experiment 2, Where 1:32, 1:64 and 1:128 of ASVS dilutions were used against 1ng and 10ng of Cobra venom.

The above table shows the same result as that of the first experiment. Here too the O.D value decreases consistently with the increase in dilution of ASVS. It is found that even 1:128 dilutions of ASVS gave a detectable O.D value. This shows that even 1ng of Cobra venom can be effectively used in detecting the potency of ASVS.

Table: 3
Determination of ASVS concentrations for 1ng of Cobra venom

	1ng	
	1	2
A 1:100	0.413	0.416
B 1:200	0.361	0.359
C 1:400	0.302	0.305
D SC	0.051	0.054
E NC	0.063	0.061

The above readings depict the Optical density values of Experiment 3, Where 1:100, 1:200 and 1:400 of ASVS dilutions were used against 1ng of Cobra venom. The above table depicts that, as the dilutions exceed 1:200 the O.D value decreases considerably. Dilutions greater than this do not have detectable antivenom implying waning antibodies.

Table: 4
Determination of ASVS concentrations for 1ng of Cobra venom

	1 ng	
	1	2
A NC	0.060	0.060
B SC	0.053	0.053
C Neat	0.563	0.561
D 1:8	0.483	0.484
E 1:16	0.461	0.463
F 1:32	0.446	0.445
G 1:64	0.421	0.420
H 1:128	0.392	0.393

Table: 5
Determination of ASVS concentrations for 1ng of Cobra venom

	1 ng	
	1	2
A NC	0.060	0.060
B SC	0.053	0.056
C Neat	0.565	0.562
D 1:8	0.486	0.484
E 1:16	0.461	0.463
F 1:32	0.445	0.445
G 1:64	0.423	0.420
H 1:128	0.391	0.390

The above readings depict the Optical density values of Experiments 4 & 5, Where Neat, 1:8, 1:16, 1:32, 1:64 and 1:128 of ASVS dilutions were used against 1ng of Cobra venom. Reproducible results were obtained in the above two experiments with respect to various dilutions of ASVS as evidenced by calculation of mean and standard deviation.

U16 strips were coated with various concentrations of cobra venom in duplicates.

DISCUSSION

ELISA for detection of ASVS against cobra venom in equine sera is proved to be simple, sensitive and reproducible. ELISA method is economical, as it requires minimum concentration of venom and Antivenom serum. ELISA is rapid as the time taken for assessing the potency of ASVS against Cobra venom is minimized. Dilutions 1:1, 1:8, 1:16, 1:32, 1:64 and 1:128 of ASVS showed antibodies against cobra venom. Further assessment of ELISA has to be done to formulate the assay as a kit for routine use.

CONCLUSION

Standardization of ELISA as a test for estimation of ASVS in the sera produced from Horses was carried out in the present study. Cobra venom concentrations ranging from 1ng to 100ng / 100 µl / well were used initially for coating. From this 1ng of Cobra venom was found to be sufficiently enough for estimating ASVS. ASVS dilutions upto 1:128 were used and dilutions exceeding this range were found to have no detectable Antivenom.

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