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Evaluation of Newly Developed Dot-ELISA Test for Identification of *Naja-naja sumantrana* and *Calloselasma rhodostoma* Venom Antigens

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***Abstract*—**Snake bite cases in Malaysia most often involve the species *Naja-naja and Calloselasma rhodostoma*. In keeping with the need for a rapid snake venom detection kit in a clinical setting, plate and dot-ELISA test for the venoms of *Naja-naja sumatrana, Calloselasma rhodostoma* and the cobra venom fraction V antigen was developed. Polyclonal antibodies were raised and further used to prepare the reagents for the dot-ELISA test kit which was tested in mice, rabbit and virtual human models. The newly developed dot-ELISA kit was able to detect a minimum venom concentration of 244ng/ml with cross reactivity of one antibody type. The dot-ELISA system was sensitive and specific for all three snake venom types in all tested animal models. The lowest minimum venom concentration detectable was in the rabbit model, 244ng/ml of the cobra venom fraction V antigen. The highest minimum venom concentration was in mice, 1953ng/ml against a multitude of venoms. The developed dot-ELISA system for the detection of three snake venom types was successful with a sensitivity of 95.8% and specificity of 97.9%.

***Keywords*—**ELISA, Venom, SVDK, *Naja-naja sumatrana* , *Calloselasma rhodostoma*.

I. Introduction

SNAKE bite cases are very common in rural communities, which have taken a front seat in the medical world, with its recognition by WHO as a significant disease [1]. Though now a days lot of research is going on diagnostics, still it is a challenge to get the actual statistical data of epidemiology of snake bites and mortalities, mostly due to lack of reporting [2-5]. We have developed the plate ELISA and dot-ELISA test for identification of *Naja-naja sumantrana* and *Calloselasma rhodostoma* venom antigens. Enzyme linked immunosorbent assay is one of the most widely used method for detection of venom from snake bite cases. In 2009, WHO took a major step in disease control and prevention in the South East Asian Region by recognizing snake bites as a neglected tropical disease [1] . Most of snake bite cases often involves only a handful of snake species. Like *Calloselasma rhodostoma*, *Naja-naja*, *Trimeresus purpureomaculatus* and *Trimeresus wagleri* [6]. In order to test the reproducibility of the ELISA and dot ELISA results, triplicate testing of 20 samples were carried out. The raw data showed little inconsistencies, suggesting a possible reproducibility; the statistical significance of which would be clarified using SPSS.

II. Material and Methods

A. Animals

Six New Zealand female white rabbits, ten to sixteen weeks old, weighing 2.5 to 3.0 kg were used for experiment.

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Six Balb/C mice aging from fourteen to eighteen weeks and weighing eighteen to twenty g were obtained from the Institute of Medical Research, Malaysia.

B. Venoms

Venoms from different species of snakes : *Bungarus candidus, Bungarus fasciatus,Calloselasma rhodostoma,* Cobra venom fraction V, *Enhydrina schistose, Naja-naja sumatrana, Trimeresus purpureomaculatus, Trimeresus sumatranus* were used in lyophilized powder form.

C. Plate ELISA

Specificity of *Naja-naja Sumantrana* antibody was tested by plate ELISA by using modified Engvall method [7] at 405nm absorbance. The same was carried out for *Calloselasma rhodostoma* antibody and Cobra venom fraction V antibodies.

D. Dot -ELISA

Nitrocellulose membranes (Sigma-Aldrich) were attached to plastic sheets with acetone-based glue. The plastic sheets were then cut to size to fit a flat-bottom 96-well Falcon ELISA microplate, resulting in plastic combs of a maximum of 12 teeth, each tooth with an attached nitrocellulose membrane and one for each well. 96 combs of three teeth each were prepared as described. Microcapillary tubes were used to coat the first tooth of each comb with *Naja –naja Sumantrana* antibody. The second and third teeth were coated with *Calloselasma rhodostoma* antibody and cobra fraction antibody respectively. The combs were left to dry at room temperature before incubation in 5% defatted milk powder in PBS at 4^0C overnight. The combs were rinsed with PBS, dried and stored at 4^0C.

Separately, three combs of 12 teeth each were prepared as described. On the first comb, the first eight teeth were coated with *Naja-naja Sumantrana* antibody. The first eight teeth on the second comb were coated with *Calloselasma rhodostoma* antibody and cobra fraction antibody on the first eight teeth of the third comb. Another two combs of 12 teeth each was also prepared the same way, having all teeth coated *with Naja-naja Sumantrana* antibody. Two more sets of similar combs were prepared with *Calloselasma rhodostoma* antibody and cobra fraction antibody respectively. Three sets of the above were made.

III. Result

Using Bradford's method, the protein concentrations of venoms of two cobra species, *Calloselasma rhodostoma*, *Enhydrina schistosa*, two *Bungarus* species and two *Trimeresus* species were determined. Specificity of the antibodies obtained was tested against eight venoms of different snake species by the plate ELISA and Dot-ELISA

methods. *Calloselasma rhodostoma* antibody showed cross reactivity with the *Naja-naja sumatrana* venom with an absorbance of 0.192. *Naja-naja sumatrana* antibody and Calloselasma *rhodostoma* antibody doesn't showed any cross reactivity, both having absorbance values below 0.14 as shown in figure 1.

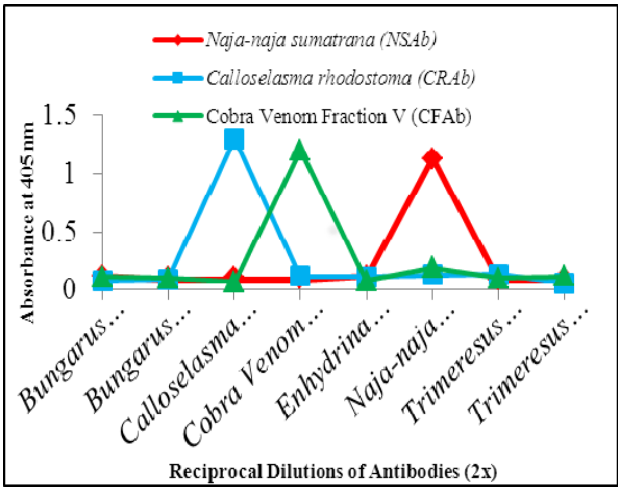

Fig. 1 Specificity of antibodies to venom antigen by plate ELISA

The resulting reactions on the specificity of newly developed dot-ELISA test for *Naja-naja sumantrana* have been shown in Figure 2.The comb of cobra fraction V antibody also showed only one colour change at one tooth, corresponding to the cobra venom fraction V. There was no colour change on the tooth incubated in the sera with *Naja-naja sumatrana* venom. There is no cross reactivity was found for cobra fraction antibody on the dot-ELISA. As earlier, no cross reactivity indications for the combs of *Naja-naja sumatrana* antibody and *Calloselasma rhodostoma* antibody each show only one tooth with a colour change.

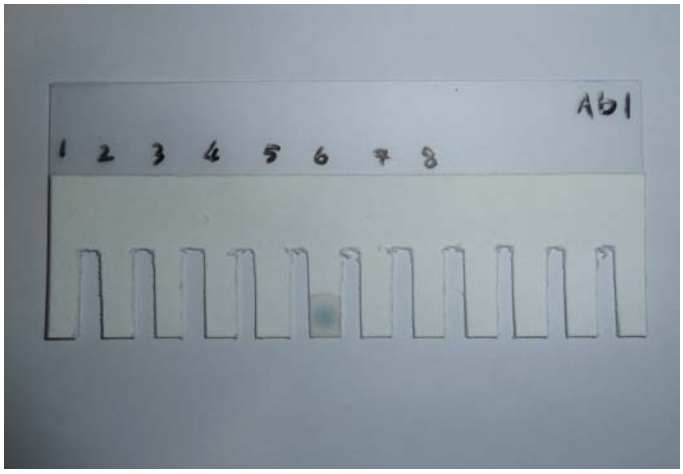

Fig. 2 Specificity of Naja-naja Sumantrana antibody to *Naja-naja sumatrana* venom by dot-ELISA where 1=*Bungarus candidus*, 2=*Bungarus fasciatus*, 3=*Calloselasma rhodostoma*, 4=Cobra venom fraction V, 5=*Enhydrina schistose*, 6=*Naja-naja Sumantarana*, 7=*Trimeresus purpureomaculatus* and 8= *Trimeresus sumantaranus*

IV. Discussion

In the present work, three venom types were tested. The three venoms are from *Naja-naja sumatrana* and *Calloselasma rhodostoma* and the cobra venom fraction V antigen. The hyper immune sera raised against *Naja-naja sumatrana* venom before processing, had a moderate sensitivity of 1: 32768 in plate ELISA. The increase for all were mainly contributed by the protocols of processing the hyper immune sera by means of precipitating the gamma globulin fraction of the sera by saturated ammonium sulphate and separating with diethyl amino ethyl cellulose. The increase in the antibody titer was not as prominent due to the choice of retaining more IgG in terms of yield rather than quality of the purified product. Although, the two methods focused on yielding higher levels of IgG, not taking into effect its antigen specificity. As for the criteria for a positive result, the absorbance cut off value was set at 0.14 O.D [8, 9]. Any value below that was considered negative and not taken into account. The final antibody titer was translated as the optimum concentration of primary antibodies to coat the dot-ELISA combs with.

Before the application of a new dot-ELISA system, a standardization of the plate ELISA had to be established with the same reagents. Standardization was done with the plate ELISA. At that stage, the amount of reagents to be used was determined, together with the incubation times and other environmental factors, one central dynamic being temperature. Triplicate repeats of 20 samples were carried out to test the reproducibility of the plate ELISA results. The test results were analyzed using the Statistical Package for Social Sciences (SPSS) statistical program and a paired t-test was carried out. At a 95% confidence interval, the p-values between the three triplicate groups were more than 0.05. This demarcates that there is insufficient evidence to reject that there is no difference of means between the groups, leading to a statistical conclusion that supports the reproducibility of the plate ELISA results. After standardization of the plate ELISA, the same criteria were applied to the dot-ELISA system.

To represent the reality of a proper out-of-laboratory setting, the developed dot-ELISA kit was to be tested out on real time animal models. Using a mouse model, a snake bite was to be simulated by means of inoculating a calculated amount of snake venom into the mouse. The kit would then be tested to determine till which hour after the snake bite was the venom still detectable in the blood. Serum was tested for rabbit, mouse and human samples with a control using PBS in place of serum. The control using PBS showed positive results from a minimum venom concentration of 1953ng/ml for *Naja-naja sumatrana* and *Calloselasma rhodostoma* venom and 977ng/ml for the cobra venom fraction V antigen.

V. Conclusion

There are three venom types *Naja-naja sumatrana*, *Calloselasma rhodostoma* and cobra venom fraction V antigen were tested. Polyclonal antibodies reactive to the three venoms were raised and characterized for sensitivity and specificity with the purpose of utilizing them for rapid and reliable immunoassays for identification of the venoms and

antigen. The rapidity and efficiency of the newly developed dot-ELISA system is merit to its use as a detection kit for snake venom. Simple and easy, dot-ELISA based snake venom detection kit could possibly replace the time and resource consuming conventional plate ELISA in snake venom detection. The flexibility of the dot-ELISA system to be miniaturized or expanded to incorporate more snake venom types holds much promise. Direct detection of venom from clinical samples of blood would require further probing with this snake venom detection kit.

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