DEVELOPMENT OF A DUAL TARGET RAPID DIAGNOSTIC DOT-ELISA SYSTEM FOR IDENTIFICATION OF COBRA AND VIPER SNAKE VENOM ANTIGENIN SNAKE BITE CASES

ABSTRACT
Dot-ELISA test is developed in mice models to identify cobra and viper snake species in snake bite cases. Simplicity and cost effective qualities of this test showed great promises to be utilized in the field which can be useful for farmers, plantation workers and fisherman who are working in field and become victim of snake bite cases. Dot-ELISA test can be an alternative solution to utilize for identification of snake species during the treatment of patient. Study was conducted in Malaysia, so we have considered local snake species commonly found in rain forest of Malaysia. Polyclonal antibodies were generated against *Calloselasmarhodostoma* and *Naja-najasumatrana* in mice model. Hyper immune sera was raised against specific snake venom antigen werecollected followed by immunoglobulin (Ig) purification to improve the specificity and sensitivity of the test. Purified IgG were utilized as a primary antibody. This test showedpositive hope for rapid identification of snake species in snake bite cases in future though test needs clinical trial before implementation in human.

KEYWORDSDot-ELISA, Cobra, viper, snake venom.

INTRODUCTION
Science and technology progressing day by day but, still basic need for rapid identification of correct snake species is a main challenge for clinicians in treatment of snake bite cases. In the management of snake bite cases, most of the time; identification of snake was made on the basis of configuration of bite without catching a culprit snake as it is very difficult to have a track of culprit poisonous snake. Out of the many abundant snake species, seventeen species of land snakes and more than twenty-two species of sea snakes are venomous. The seventeen venomous land snakes are represented by the subfamilies of Crotalinae and Elapinae whereas the other three are sea snakes [1].Previous studies reported approximately 5,400,000 cases of snake bites at a yearly estimate. Out of these cases, 2,500,000 envenoming occurred from which 125,000 deaths ensued [2]. South and South East Asia were identified as having the highest incidences of snake bite morbidities and mortalities [3, 4]. Literature showed that snake bites are an occupational hazard which is prevalent among farmers, plantation workers and fishermen [5]. The current management principle of snake bites is to observe the patient for any symptoms of envenoming. If the patient exhibits any symptoms of envenoming, supportive treatment will be the next step. Due to the cost and lack of supply of antivenom, it is only given as specific treatment in cases where systemic poisoning is evident and snake species is known. The most challenging job for clinician is to identify snake species. Currently, the diagnosis of snake species is made on the basis of clinical examination of species specific symptoms and laboratory report which takes time. For the effective treatment of snakebite cases, there is a need for reliable and quick serological methods for identification of snake species. Several snake venom detection kits (SVDK) have been developed over the years and these includes radioimmunoassays and plate ELISA. These SVDK are still reported as incoherent in the field of identifying snake species in a clinical setting. Rapid identification of specific snake species will support the clinician to select the correct antivenom for immediate treatment of patient. Establishment of an ELISA method has been reported previously for diagnosis of snake bite cases [6, 7].In studies of many diseases ranging from leishmaniasis to toxocariasis, many dot-ELISA systems have been developed. Studies have covered the use of Dot-ELISA in many fields like medicine, [8, 9, 10, 11, 12, 13, 14], veterinary sciences [15] and food sciences. On the basis of same principle we have developed rapid detection kit for Cobra and Viper snake venom. We have started this study with the objective to raise polyclonal antibodies against crude cobra and viper venom in experimental animals and utilize them for development of rapid diagnostic dot- ELISA test for detection of Cobra and Viper antigen. Aim of this study was to develop dot ELISA test which should be user friendly, cost effective, and able to be utilized in the field without the use of any other sophisticated laboratory instruments.

MATERIAL AND METHODS
The study protocol was approved by research and ethics committee of International Medical University (No:IMUJC211010). Snake venoms were collected in powdered form, from eight different species of cobra, krait, sea snake and viper. Hyper immune sera (HIS) was developed against the cobra and viper (*Calloselasmarhodostoma* , Cobra Venom Fraction V and *Naja-najasumatrana*) antigen in animal models. Protein estimation of all eight different species was done by using bovine serum albumin standards, ranging from 0 to 10 μg were prepared. Venom samples of 0.125mg/ml concentration using crude venom were prepared and proteins were estimated by Bradford's method. Two New Zealand white rabbits were immunized subcutaneously with 100μg of *Naja-najasumatrana* venom in

complete Freund's adjuvant (Sigma-Aldrich). Weekly pre-immunization bleeds were taken for eight weeks before the start of the immunization period. With seven day intervals, eight booster doses with 100μg of venom were given. The first six booster doses were given subcutaneously with incomplete Freund's adjuvant (Sigma-Aldrich). The final two booster doses were delivered intravenously with phosphate buffered saline (PBS). Blood samples were collected before the administration of each booster dose. Rabbits were sacrificed after the three days of last booster dose followed by blood collection. The hyper immune sera was collected and stored at 4^0C temperature [16]. Hyper immune sera from the two rabbits immunized with the *Naja-najasumatrana* venom (NSAb) were combined as one batch for processing. The same procedure was repeated for the two rabbits immunized with the venom of *Calloselasmarhodostoma* (CRAb) and the two of the cobra venom fraction V antigen (CFAb). Ammonium sulphate (Sigma-Aldrich) per liter serum was dissolved in the serum to achieve 50% ammonium sulphate saturation. The mixture was stirred for 2 hours before centrifugation at 10,000g for 15 minutes with a centrifuge (eppendorf). The supernatant was decanted and the precipitate dissolved in twice it is amount in PBS. The mixture was dialyzed against PBS using a 10K MWCO snakeskin dialysis tubing (Pierce) for 2 hours at room temperature. The PBS was changed and the mixture dialyzed for another two hours at room temperature. The mixture was dialyzed at 4^0C overnight with further change in PBS. Immunoglobulin Gamma (IgG) was purified by using DEAE-cellulose. For the separation of IgG, hyper immune sera were diluted four times with distilled water before the addition of the DEAE-cellulose. The ratio of DEAE-cellulose to serum was 2.2g to 1ml. The mixture was incubated for one hour at 3^0C, which was stirred at every 10 minutes. The mixture was then sucked dry in a Buchner funnel before washing with 0.01M phosphate buffer pH 8.0 three times. The volume of phosphate buffer used was ⅓ the weight of DEAE-cellulose used. The combined effluent was dialyzed against 0.0003M phosphate buffer pH 8.0 for 4 hours at room temperature with one buffer change. After that, another buffer change was done and the mixture was left to dialyse at 4^0C overnight. The mixture was then frozen at -80^0C and lyophilized with a freeze dryer (Labconco). The lyophilized product was stored at 4^0C. Horse radish peroxidase, HRP RZ3 (Sigma-Aldrich) enzyme was coupled with the IgG. 5mg of the lyophilized IgG was dissolved in 1ml of 0.1M phosphate buffer pH 6.8 followed by 12mg HRP. 0.05ml 1% aqueous solution glutaraldehyde was added drop wise while the solution was stirred. The mixture was left to stand for 2 hours at room temperature before dialysis against two changes of 5L PBS at 4^0C overnight. The mixture was then centrifuged at 20,000rpm at 4^0C for 30 minutes in a centrifuge. The precipitate was discarded and the supernatant kept at 4^0C.Titration of hyperimmune sera was done by plate ELISA [17]. Dot-ELISA Combs were prepared manually by attaching nitrocellulose membranes to plastic sheets. Antibodies of NSAb, CRAb and CFAb were coated accordingly and stored at 4^0C for further use [18].

RESULT AND DISCUSSION

Protein concentration of eight snake venoms (two cobra species, two krait species, one sea snake and three viper species),were determined as shown in Table 1.Titration of antibodies against its corresponding venom type was determined by plate ELISA. Corresponding venom type and HIS in different dilutions before and after processing (saturation with ammonium sulphate and purification with DEAE-cellulose) were titrated against 10μg/ml venom antigen concentration as shown in Figure 1 & 2 respectively. For the HIS before processing, the absorbance at neat HIS for NSAb was 1.147, 1.273 and 1.230 for NSAb, CRAb and CFAb respectively. At the dilution of 1: 65536, absorbance values for NSAb and CRAb drop below 0.14. For CFAb, the absorbance value was recorded above 0.14 at dilutions below 1: 131072. The titre of NSAb and CRAbwere reported 1: 32768. After saturation and purification of the HIS, the absorbance of the neat was 1.332 for NSAb, 1.309 for CRAb and 1.328 for CFAb. For all three types of antibodies, the absorbance values were reported above 0.14 with dilutions higher than before. Less than 0.14 OD was observed at 1: 262144 dilution forNSAb, 1: 131072 for CRAb and 1: 262144 for CFAb. Titre-wise, both NSAb and CFAb seem to be at 1: 131072 dilutions whereas CRAb was found to be at 1: 65536 dilutions. Specificity of antibodies was examinedagainst snake venoms of different species by plate and Dot-ELISA [19].Titration of venom antigens against corresponding raised antibodies was also determined by the plate and Dot-ELISA methods.Testing revealed system specificity of 100% and sensitivity of 94% with false negatives seen from *Naja-najasumantrana* antibodies, as shown in Figure 3. Target specific sensitivity was reported at 88% for *Naja-najasumantrana*and 100% for *Calloselasmarhodostoma.*Dot ELISA system ability to detect two snake species at a time is minimal compared to the five that the CSL system is able to detect [20]. Though, newly developed dot-ELISA system is expandable just by adding more teeth to the test strip comb to attach more species-specific antibodies. CSL system is more for snake species commonly found in Australia though Dot ELISA system is more for Malaysian snake species which can be expanded for further improvement in system for detection of other poisonous snake venoms commonly found in Malaysia. Time-wise, we used the same timeline from a standard plate ELISA protocol. The reason for this is to manipulate only one aspect of the system at a time, this being the solid state of ELISA from plate to membranes on strips. This explains the extended time required which is incomparable to the time offered by CSL and Gao[21]as shown in Table 2. The dot-ELISA system developed was able to identify snake species using venom as an antigen.

CONCLUSION

Dot-ELISA system offers a practical approach to detect cobra and viper snake venom species without the use of any equipment ability of test to detect more than one snake species at a time. The dot-ELISA test kit revealed high specificity and sensitivity with its test results. The flexibility of this system to be miniaturised in size will be one of the areas which can be expanded as per the need of country specific snake species. We were unable to continue further research on this area because of time and financial constrain which can be further studied to reduce the time of test. For further research, we would like to suggest use of flow cassettes to improve the sensitivity and specificity of the system with reduced time.

Tables &Figures:

Table 1: Protein concentrations of venoms of different snake species.

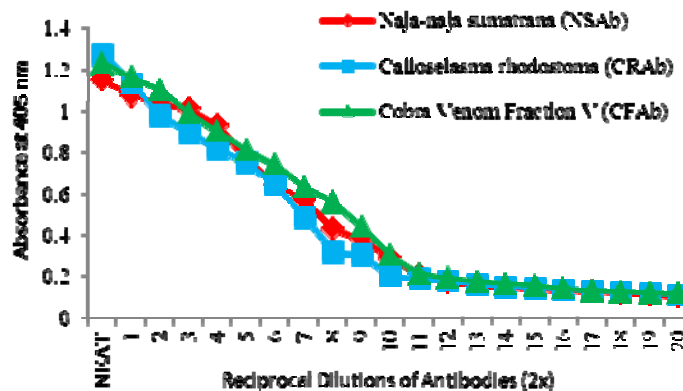| Snake Species	Protein Concentration (mg/ml)
Bungaruscandidus	0.65
Bungarusfasciatus	0.72
Calloselasmarhodostoma	0.55
Cobra Venom Fraction V	0.86
Enhydrinaschistosa	0.76
Naja-najasumatrana	0.68
Trimeresuspurpureomaculatus	0.68
Trimeresussumatranus	0.77

Figure 1: Titration of hyper immune sera to venom antigen by plate ELISA before IgG purification
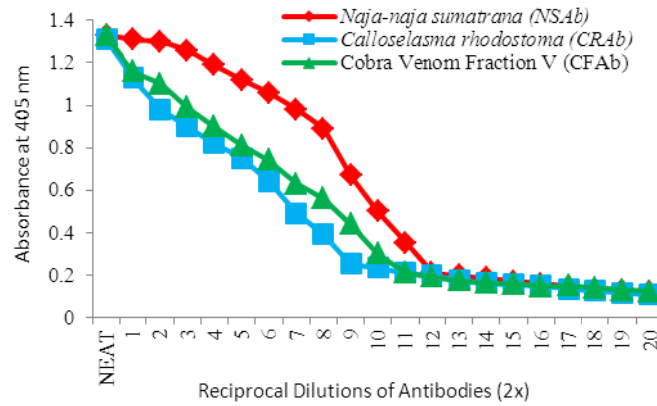
Figure 2: Titration of hyper immune sera to venom antigen by plate ELISA after IgG purification
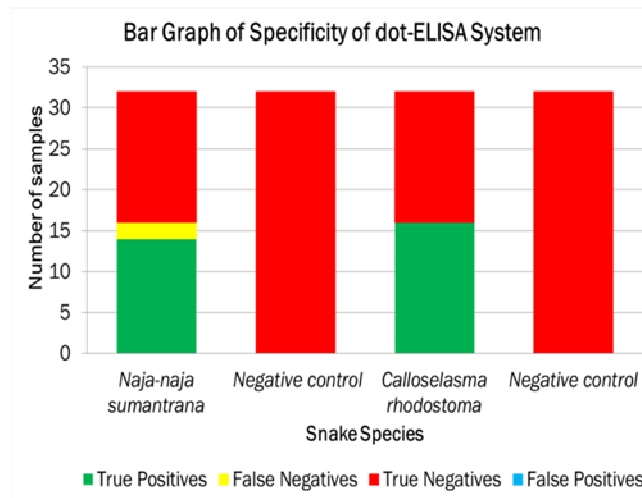

Figure 3: Testing of dot-ELISA system in identification of snake venom antigen.

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Table 2: Comparison of newly developed dot ELISA with prior studies.

Study	Dhaliwal (1983)	CSL Australia (2007)	Gao (2008)	Current Study (2011)
Type of Kit	Microplate ELISA	Microplate ELISA	Nanotechnology	dot-ELISA
Duration required	35 – 45 minutes	15-25 minutes	30 minutes	32 – 35 minutes
Equipment required	Spectrophoto-meter	-	UV microscope	-
Species detectable	1	5	1	3

REFERENCES

1. Tan NH (2010).The management of snakebites in Malaysia.
   http://www.tanngethong.com/Management%20of%20snakebites/management_of_snakebites_in_mala.htm
2. Chippaux JP.(1988). Snake bites:appraisal of the global situation. Bulletin of World Health Organisation. 76:515-524.
3. Kasturiratne A, Wickremasinghe AR, de Silva N, GunawardenaNK,Pathmeswaran A, et al. The global burden of snakebite: A literature analysisandmodelling based on regional estimates of envenoming and deaths. PLoSMed 5.2008; e218.
4. Swaroop S, Grab B. Snakebite mortality in the world. Bull World HealthOrgan. 1954;10:35-76.
5. Jamaiah I, Rohela M, Roshalina R, Undan RC. Prevalence of snake bites inKangar District Hospital, Perlis, west Malaysia: a retrospective study(January 1999-December 2000). Southeast Asian J Trop Med Public Health.2004 Dec;35(4):962-5.
6. AmbuS, Lim BL.(1988). A study of snake-bites in peninsular Malaysia with special reference to perlis& Kedah from 1979-1983.Tropical Biomedicine.;5:65-76.
7. Alirol E, Sharma SK, Bawaskar HS, KuchU,Chappuis F. (2010). Snake bite in South Asia:a review. PloSNeglectedTropicalDisesess. ;4(1):e603.
8. Selvanayagam ZE, Gnanavendhan SG, Ganesh KA, Rajagopal D, Rao PV .(1999).ELISA for the detection of venoms from four medically important snakes of India. Toxicon.;37(5):757-70.
9. Kashyap RS, Kainthla RP, Biswas SK, Agarwal N, Chandak NH, Purohit HJ. (2003). Rapid diagnosis of tuberculous meningitis using the Simple Dot ELISA method.Medical Science Monitoring ,9(11):MT123-6.
10. Camargo ED, Nakamura PM, Vaz AJ, da Silva MV, Chieffi PP, de Melo EO.(1992). Standardization of dot-ELISA for the serological diagnosis of toxocariasis and comparison of the assay with ELISA.Review Journal of the São Paulo Institute of Tropical Medicine34(1):55-60.
11. Pappas MG, Hajkowski R, Hockmeyer WT.(1983). Dot enzyme-linked immunosorbent assay (Dot-ELISA): a micro technique for the rapid diagnosis of visceral leishmaniasis. Journal of Immunological Methods. 11;64(1-2):205-14.
12. Tellez-Giron E, Ramos MC, Dufour L, Alvarez P, Montante M.(1987). Detection of Cysticercuscellulosae antigens in cerebrospinal fluid by dot enzyme-linked immunosorbent assay (Dot-ELISA) and standard ELISA.American Journal of Tropical Medicine and Hygiene.;37(1):169-73.
13. Roldan W, Cornejo W, Espinoza Y. (2006). Evaluation of the dot enzyme-linkedimmunosorbent assay in comparison with standard ELISA for the immunodiagnosis of human toxocariasis.Memórias do Institution OswaldoCruz ,101(1):71-4.

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INTERNATIONAL JOURNAL OF SCIENCE AND TECHNOLOGY

14. Allan JC, Mencos F, Garcia-Noval J, Sarti E, Flisser A, Wang Y.(1993). Dipstick dot ELISA for the detection of Taeniacoproantigens in humans. Parasitology. ; 107 (1):79-85.

15. Mancianti F, Pedonese F, Poli A. (1996). Evaluation of dot enzyme-linked immunosorbent assay (dot-ELISA) for the serodiagnosis of canineleishmaniosis as compared with indirect immunofluorescence assay.Veterinary Parasitology.;65(1-2):1-9.

16. Theakston RD, Lloyd-Jones MJ, Reid HA.(1977). Micro-ELISA for detecting and assaying snake venom and venom-antibody.Lancet. 24;2(8039):639-41.

17. Engvall E, Perlmann P.(1997). Enzyme-linked immunosorbent assay (ELSA).Quantitative assay of immunoglobulin G. Immunochemistry. 8(9):871-4.

18. Sharma R, Tuteja U, Khushiramani R, Shukla J, Batra HV.(2007). Application of rapid dot-ELISA for antibody detection of leptospirosis.Journal of Medical Microbiology.873-874.

19. Sikarwar A. S., Ambu S., T .H. Wong. "Evaluation of Newly Developed Dot-ELISATest for Identification of *Naja-najasumantrana*and*Calloselasmarhodostoma*Venom Antigens".2012, World Academy of Science Engineering and Technology, 68.

20. Warrell DA. (1999). WHO/SEARO Guidelines for the clinical management of snakebites in the Southeast Asian region. SE Journal of Tropical Med ical Public Health. 30(1):1-85.

21. Hauck, T. S., S. Giri, et al. (2010). Nanotechnology diagnostics for infectious diseases prevalent in developing countries. Advanced Drug Delivery Reviews 62(4-5): 438-448.)

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