การพัฒนาวิธีตรวจชนิดของพิษงูกลุ่มงูพิษที่ออกฤทธิ์ต่อระบบโลหิตโดยใช้แอนติบอดีที่ออกแบบให้จำเพาะต่อชนิดของพิษงู

กัญณัฏฐ์ พรหมรุ่งเรือง* จุรีพร น้อยพรหม อรวรรณ แซ่โค้ว
และ นฤมล พักมณี

ฝ่ายวิจัยและพัฒนา สถานเสาวภา สภากาชาดไทย กรุงเทพมหานคร

บทคัดย่อ

อุบัติการณ์การถูกงูพิษกัดยังเป็นปัญหาทางสาธารณสุขที่สำคัญของประเทศไทย การรักษาผู้ถูกงูพิษกัดจำเป็นต้องรู้ชนิดของงูพิษที่กัดเพื่อจะให้เซรุ่มแก้พิษงูที่จำเพาะต่อพิษของงูที่กัด ในการศึกษานี้ได้พัฒนาเทคนิค enzyme-linked immunosorbent assay (ELISA) สำหรับตรวจชนิดของพิษงูที่ออกฤทธิ์ทางระบบโลหิต (พิษงูเขียวหางไหม้ พิษงูกะปะ และพิษงูแมวเซา) โดยใช้อิมมูโนโกลบูลินที่จำเพาะต่อพิษงูแต่ละชนิด ซึ่งแยกจากพลาสมาของม้าที่ได้รับการกระตุ้นให้สร้างแอนติบอดีต่อพิษงูนั้น จากการแยกด้วย affinity chromatography ได้ความเข้มข้นของอิมมูโนโกลบูลินที่จำเพาะต่อพิษของงูเขียวหางไหม้ งูกะปะ และงูแมวเซาเท่ากับร้อยละ 4.62, 5.97 และ 3.43 ตามลำดับ โดยสามารถตรวจระดับพิษต่ำสุดได้ที่ 12.5 นาโนกรัมต่อมิลลิลิตร อย่างไรก็ตามวิธีนี้ไม่สามารถตรวจวัดระดับพิษได้กรณีที่ผู้ป่วยมีความเข้มข้นของพิษในพลาสมาต่ำมาก ๆ (น้อยกว่า 12.5 นาโนกรัมต่อมิลลิลิตร) นอกจากนี้ยังพบปฏิกิริยาข้ามกัน (cross reactivity) ภายในกลุ่มพิษงูเหล่านี้อีกด้วย

คำสำคัญ: พิษงูกลุ่มงูพิษที่ออกฤทธิ์ต่อระบบโลหิต อีไลซา แอนติบอดีที่ออกแบบจำเพาะต่อชนิดของงูพิษ

***ผู้รับผิดชอบบทความ E-mail address:** kanyanat_prom@yahoo.com
รับบทความ: 9 กันยายน 2561 แก้ไขบทความ: 26 กุมภาพันธ์ 2562 รับตีพิมพ์บทความ: 18 พฤษภาคม 2562

Development of Hematotoxic Snake Venom Detection using Species Specific Designed Antibody

Kanyanat Promruangreang*, Jureeporn Noiphrom,
Orawan Khow and Narumol Pakmanee

Research and Development Queen Saovabha Memorial Institute, The Thai Red Cross Society, Bangkok, Thailand

Abstract

Snakebite is still an important public health problem in Thailand. The current scheme of management for snakebite includes attempting to identify snake species and selecting the corresponding antivenom for therapy. In this study, an enzyme-linked immunosorbent assay (ELISA) was developed to detect hematotoxic snake (Green pit viper, Malayan pit viper and Russell's viper) venoms by using species specific immunoglobulins isolated from hyperimmune horse plasma. By affinity chromatography, the percent concentration of immunoglobulins specific to Green pit viper, Malayan pit viper and Russell's viper hematotoxic venoms were 4.62, 5.97 and 3.43, respectively. This established technique could differentiate among the three Thai hematotoxic snake venoms in serum samples with the detection limit of 12.5 ng/mL. However, the assay is not sensitive enough to detect venom concentration in snakebite patients with lower concentration of venom. Moreover, cross reactivity was observed among the three hematotoxic snake venoms.

Keywords: Hematotoxic snake venom, Enzyme-linked immunosorbent assay, Species specific immunoglobulins

***Corresponding author E-mail address:** kanyanat_prom@yahoo.com

Received: 9 September 2018 Revised: 26 February 2019 Accepted: 18 May 2019

Introduction

Snakebite is a common and frequently devastating environmental and occupational hazard, especially in rural areas of tropical developing countries. Green pit viper (*Trimeresurus albolabris* and *T. macrops*), Malayan pit viper *(Calloselasma rhodostoma)* and Russell's viper *(Daboia siamensis)* are known as venomous snakes with hematotoxicity. They are classified in the family Viperidae (called vipers) and are commonly found in every region of Thailand. The toxins, hematotoxins, cause bleeding disorders and the mechanisms of each hematotoxin are different.[1-3]

In many cases of envenoming following snakebite, the species of snakes responsible for the accidents remain unidentified; this frequently results in difficulties deciding which antivenom to be administered to the envenomed victim. The clinical diagnosis depends upon the recognition of snake types and symptoms in the patient. Laboratory diagnosis is based on the changes which occur in envenomed victims including the detection of irregularity in blood parameters, presence or absence of neurotoxic signs and changes in enzyme levels, thus making treatment with the correct antivenom more difficult. Moreover, snake venom consists of many kinds of protein and some proteins are defined in all types of snake venom (common component) thus causing cross-reactivity among species of the snakes. This is a major problem interfering with the detection of snake venom in patient, which is one of the major reasons for the development of sensitive enzyme-linked immunosorbent assay (ELISA).[4-7]

The aim of the present study was to develop an enzymatic immunoassay as a basis for immunodiagnosis in envenomation caused by hematotoxic snake venom using species specific designed antibody.

Materials and Methods

Selection of specific venom component from hematotoxic snake venom

Hematotoxic snake venom (50 ug of protein per well) was loaded onto 12.5% SDS-PAGE. The separated proteins were visualized by staining with Coomassie Brilliant Blue. The specific protein from each type of hematotoxic snake venom was selected and eluted from the gel, and further dialysed with PBS pH 7.2.

Preparation of immunoglobulins by ammonium sulfate precipitation

Saturated ammonium sulfate solution was used for immunoglobulin separation. Hyperimmune horse plasma against Green pit viper, Malayan pit viper and Russell's viper venom were obtained from the horse farm of Queen Saovabha Memorial Institute. Each immunoglobulin (30 mL) was precipitated with ammonium sulfate at 40% saturation. The mixture was stirred for 30 min and incubated overnight in cold room (4°C). The mixture was then centrifuged at 8000 rpm (Eppendorf

5810R) for 10 min and the precipitate was dissolved in 30 mL of PBS buffer (0.01 M phosphate buffer pH 7.2 containing 0.15 M NaCl). The solution was made 33% saturated with ammonium sulfate and stirred for 30 min. After centrifuging at 8000 rpm (Eppendorf 5810R) for 10 min the precipitate was dissolved in 20 mL of PBS buffer. Finally, the solution was dialyzed 3 times against PBS buffer.

Separation of specific Horse IgG by venom specific affinity chromatography

Polyacrylamide gel affinity chromatography was used to isolate immunoglobulins which recognized specific venom components of Green pit viper, Malayan pit viper and Russell's viper. The columns were prepared by dissolving 100 mg of specific part of each snake venom in 10 mL of PBS buffer pH 7.4 containing 16% acrylamide monomer and 4% N,N'-methylene bisacrylamide . The mixtures were polymerized by adding 500 µL of 0.4% ammonium persulfate and 50 µL of TEMED. The solid was granulated using 0.3 mm pore size stainless screen followed by washing 5 times with 100 mL of PBS. The sieved gel was packed into 1.5 × 7.5 cm columns and washed with Gly/HCl (0.1 M glycine, 0.154 M NaCl, pH adjusted to 2.5 with HCl) and PBS buffers. The effluents were measured at 280 nm until the baselines were closed to zero and steady. The precipitated horse IgGs were loaded onto venom polyacrylamide affinity columns containing specific components of the snake venom. The unbound fractions were eluted with PBS and absorbance measured at 280 nm until stable. The bound fractions were eluted with Gly/HCl buffer and collected.

Preparation of peroxidase-conjugated specific horse IgG

Venom specific horse IgG was labeled with peroxidase enzyme. Briefly, 10 mg of peroxidase enzyme were dissolved in 2 mL of 0.3 M sodium bicarbonate buffer pH 8.1 (freshly prepared). The solution containing 0.2 mL of 1 %DNFB (2,4-Dinitrofluorobenzene) in ethanol was added to the mixtures, gentle stirring for 1 hr at room temperature, followed by addition of 2 mL of 0.06 M sodium metaperiodate, gentle stirring for 1 hr at room temperature (the color of the mixtures was changed to yellow-green). Two millitres of 0.16 M ethylene glycol were added to the mixtures and stirred for 1 hr at room temperature. The mixtures were dialyzed in 0.01 M sodium carbonate buffer pH 9.5 at 4°C. Ten milligrams venom specific horse IgG dissolved in 1 mL of 0.01 M sodium carbonate buffer pH 9.5 were added to the mixtures, and stirred for 2-3 hr at room temperature. After that, 5 mg of sodium borohydride were added to the mixtures with stirring for 3 hr at 4 °C and the mixtures were finally dialyzed in PBS pH 7.2 at 4 °C.

The mixtures were separated by Sephadex G-200 gel filtration chromatography. The column (1.6 × 95 cm) was packed with

Sephadex G-200 (Pharmacia, Uppsala, Sweden) and eluted with PBS pH 7.2 at the flow rate of 14 mL/hr. The eluate was collected in 1 mL fractions and the absorbance was measured at 280 and 403 nm.

Analysis of peroxidase-conjugated specific horse IgG

The specific snake venom dissolved with 0.05 M carbonate buffer pH 9.6 at the final concentration of 2 μg/mL was coated on 96-well ELISA microplate (50 μL per well) and incubated at 4 °C overnight. After washing with PBST (Phosphate Buffer Saline with Tween20), 2% BSA in PBST was used as blocking reagent with incubation time of 1 hr at room temperature. After washing three times with PBS (pH 7.2), peroxidase-conjugated specific horse IgG at various dilutions (undiluted, 1:2, 1:4 and 1:8) were added and incubated for 1 hr at room temperature. After washing, the substrate OPD (o-Phenylenediamine dihydrochloride) was added and incubated for 30 min at room temperature. Finally, 0.5 M sulfuric acid was used as stopping solution and absorbance measured at 490 nm using ELISA plate reader (TECAN, Austria).

Development ELISA method for hematotoxic snake venom detection

Checkerboard titration was done by varying incubation times and concentrations of ELISA reagents; the first specific IgG coated on plate, blocking solution, venom dilutions and peroxidase-conjugated specific horse IgG. The proper steps of the assay were considered for the detection of the smallest amount of snake venom with the least background noise. Plates were coated with 50 μL per well of 0.05 M carbonate buffer pH 9.6 containing horse monovalent anti-venom (10 μg/mL) and incubated overnight at 4 °C. Washing was done three times with 200 μL/well of PBS pH 7.2. Plates were blocked with 200 μL per well of 2% BSA in PBST for 1 hr at room temperature, and washed three times with PBS. Serial dilutions of snake venom or the sample were added (50 μL per well) and incubated for 1 hr at room temperature. After washing, peroxidase-conjugated specific horse IgG was added to the plates and incubated for 1 hr at room temperature. The plates were then washed and the substrate (OPD) was added and incubated for 30 min at room temperature. The absorbance at 492 nm was recorded using an ELISA plate reader.

Results

1. Selection of specific venom component from hematotoxic snake venom

Hematotoxic snake venom (50 ug of protein per well) was loaded onto 12.5% SDS-PAGE. The specific protein from each type of hematotoxic snake was selected from the gel and was dialyzed with PBS pH 7.2. The venom components from hematotoxic snake venom are shown in Fig. 1.

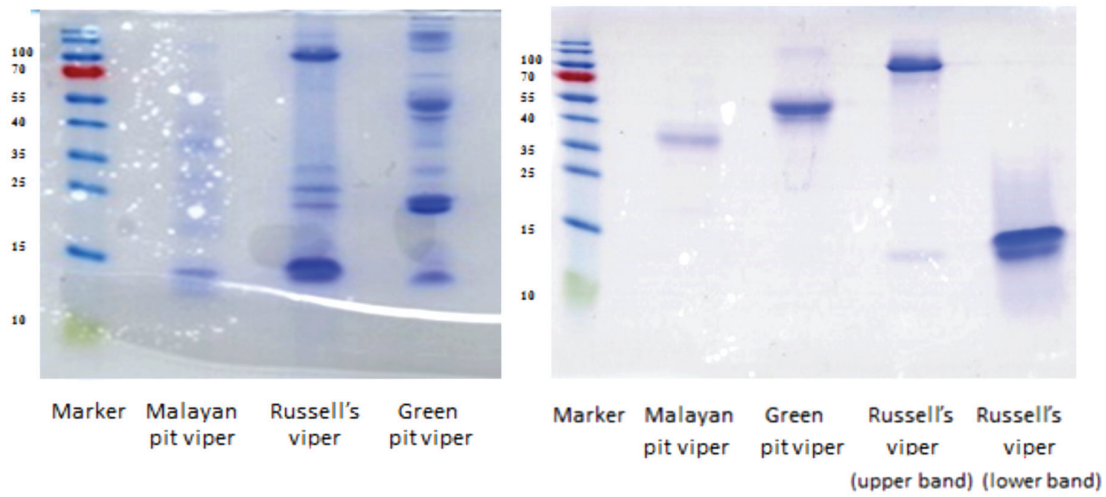

Fig. 1 Selection of specific components of hematotoxic snake venom. Crude venom was loaded onto 12.5% SDS-PAGE (Left). The specific components were selected by cutting gel and the gels were pooled (Right).

2. Preparation of specific immunoglobulin from the snake venoms

Snake venom-specific immunoglobulin was separated from horse plasma using ammonium sulfate precipitation. The concentration of Green pit viper, Malayan pit viper and Russell's viper immunoglobulins were 16.67, 19.05 and 20.39 mg/mL, respectively. One milliliter of each immunoglobulin was applied to polyacrylamide gel affinity column coupled to its specific venom component. Non-specific IgG (unbound) and specific IgG (bound) were eluted and the absorbance measured at 280 nm. Comparing to the initial concentration, percent concentration of the immunoglobulin against specific components of Green pit viper, Malayan pit viper and Russell's viper venom were 4.62, 5.97 and 3.43, respectively.

3. Preparation of peroxidase-conjugated specific horse IgG.

Venom-specific immunoglobulin was lyophilized. The immunoglobulin was labeled with peroxidase enzyme and purified by gel filtration chromatography. The filtrate obtained consisted of 3 parts: free immunoglobulin, free peroxidase and peroxidase-conjugated immunoglobulin. The pooled fractions of peroxidase-conjugated specific horse IgG were further investigated to determine the proper dilution used in ELISA. The result showed that the proper dilution of peroxidase-conjugated specific horse IgG was 1:4, data not shown.

4 Analysis by ELISA method

4.1 Separation of peroxidase-conjugated specific horse IgG by Gel filtration chromatography

Peroxidase-conjugated specific anti-horse IgG was purified by using Sephadex G-200 gel filtration column chromatography. The fractions were analyzed using ELISA by coating microplates with specific venom and the OPD was used as substrate for detecting reaction. Finally, active fractions were pooled and stored at -20 °C.

4.2 Detection of snake venom

Hematotoxic snake venoms (Green pit viper, Malayan pit viper and Russell's viper) were detected using ELISA. Horse monovalent anti-venom against Green pit viper, Malayan pit viper and Russell's viper venoms were coated on the plate overnight at 4°C. 2% BSA in PBS was used as blocking reagent. Snake venom samples at the concentration of 5 and 50 ng/mL were added and then bound to the peroxidase-conjugated specific horse IgG in the next step. Each step of washing with PBST was done three times. The reaction was expressed by OD at 492 nm using OPD as substrate. Green pit viper venom, Malayan pit viper venom and Russell's viper venom could be detected by the developed method and the results are shown in Table 1, 2 and 3 respectively.

Table 1 Specificity of ELISA for detection of Green pit viper snake venom

Concentration of snake venom (ng/mL)	OD 492 nm		
	Green pit viper snake venom	Malayan pit viper snake venom	Russell's viper snake venom
5	0.208 ± 0.011 (n=4)	0.077 ± 0.013 (n=4)	0.066 ± 0.014 (n=4)
50	0.506 ± 0.018 (n=4)	0.166 ± 0.018 (n=4)	0.146 ± 0.018 (n=4)

** coat plate with horse monovalent anti-venom against Green pit viper

Table 2 Specificity of ELISA for detection of Malayan pit viper snake venom

Concentration of snake venom (ng/mL)	OD 492 nm		
	Green pit viper snake venom	Malayan pit viper snake venom	Russell's viper snake venom
5	0.076 ± 0.017 (n=4)	0.245 ± 0.03 (n=4)	0.082 ± 0.015 (n=4)
50	0.153 ± 0.012 (n=4)	0.608 ± 0.023 (n=4)	0.194 ± 0.015 (n=4)

** coat plate with horse monovalent anti-venom against Malayan pit viper

Table 3 Specificity of ELISA for detection of Russell's viper snake venom

Concentration of snake venom (ng/mL)	OD 492 nm		
	Green pit viper snake venom	Malayan pit viper snake venom	Russell's viper snake venom
5	0.062 ± 0.021 (n=3)	0.058 ± 0.007 (n=3)	0.166 ± 0.035 (n=4)
50	0.160 ± 0.004 (n=3)	0.188 ± 0.010 (n=3)	0.542 ± 0.014 (n=4)

** coat plate with horse monovalent anti-venom against Russell's viper

4.3 Calculation of ELISA cut-off value

The absorbance value at 492 nm of ten normal human plasma in developed ELISA for Green pit viper, Malayan pit viper and Russell's viper venoms were 0.08 ± 0.0095, 0.078 ± 0.012 and 0.084 ± 0.007, mean ± SD, respectively. The cut - off value was further manipulated as 0.099, 0.102 and 0.098, mean ± 2SD, respectively (Fig. 2, 3 and 4). Sample value measured at OD492 nm ⩽ cut-off point was considered as negative result.

4.4 Limit of detection

The detection limit for the developed ELISA was 12.5 ng/mL which was venom concentration estimated from intersection at the cut-off point of each venom (Fig. 2, 3 and 4).

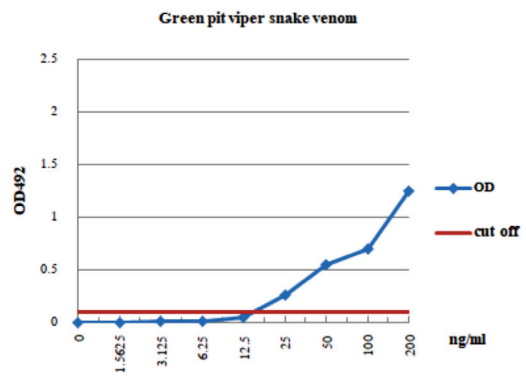

Fig. 2 Relation between concentration of Green pit viper snake venom and cut-off value at OD492 nm using developed ELISA method

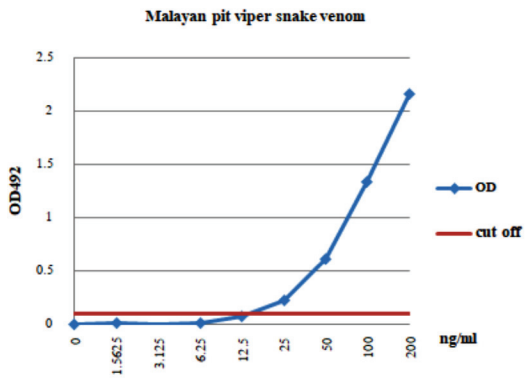

Fig. 3 Relation between concentration of Malayan pit viper snake venom and cut-off value at OD492 nm using developed ELISA method

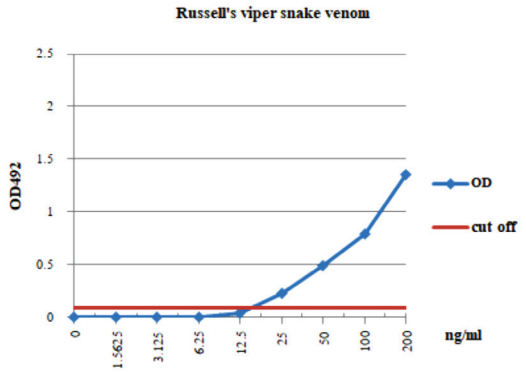

Fig. 4 Relation between concentration of Russell's viper snake venom and cut-off value at OD492 nm using developed ELISA method

Discussion

The identification of snake type directly involves the right antivenom selection in snakebite treatment. Because of many multiple proteins with the same or similar epitope consisting in snake venom, cross — reactivity among snake venom types was observed. Common antigens in heterologous venoms has been testified to be a major source for the development of venom detection.[6, 8, 9] Antibodies against specific portions are important tools for immunoassays in envenomation diagnosis. The affinity chromatography appears for removing unspecific antibodies in antiserum[7, 10]

In this study, SDS-PAGE was used to select the specific protein from Green pit viper, Malayan pit viper or Russell's viper venom, which might improve snake venom -specific immunoglobulin preparation by affinity chromatography. ELISA using the peroxidase-conjugated venom specific IgG was studied in this present work. Hematotoxic snake venoms could be detected by this developed ELISA within 4 hr after sample receiving. Due to ten normal human plasma gave the result of low standard deviation, about 8-15%, at least ten samples were used to determine the cut-off value. However, to get more accurate, more samples of normal human plasma should be further investigated. These cut-off values obtained from normal plasma without venom related to the venom detection limit. This developed ELISA could detect hematotoxic snake venoms at concentration higher than 12.5 ng/mL. Therefore, this assay is not sensitive enough to detect venom in snakebite cases with lower concentration in plasma. Although each venom could be detected, the cross reactivity among three hematotoxic snake venoms was observed, as shown in the results of venom detection at 50 ng/mL. Cross reactivity might be reduced by improving specific venom components selection, specific immunoglobulin preparation and each step of ELISA. Moreover, the test in known samples in plasma and real samples of snakebite cases should be done for more effective data.

Conclusion

This established technique could differentiate among the three Thai hematotoxic snake venoms in serum samples with the detection limit of 12.5 ng/mL. To verify

maximum efficiency, expanded studies on all pertinent factors should be further studied in the future.

Acknowledgement

This work was supported by Queen Saovabha Memorial Institute: Thai Red Cross Society, Thailand.

References

1. Dumavibhat B, Visudhiphan S, Malasit P. Severe cases of green pit viper snake venom poisoning. J Med Assoc Thai 1989; 72: 593-96.
2. Hutton RA, Looareesuwan S, Ho M, *et al*. Arboreal green pit vipers (genus *Trimeresurus*) of South-East Asia: bites by *T. albolabris* and *T. macrops* in Thailand and a review of the literature. Trans R Soc Trop Med Hyg 1990; 84: 866-74.
3. Warrell DA. Guideline for the Management of Snakebites. New Delhi, India: World Health Organization; 2010.
4. Coulter AR, Harris RD, Sutherland SK. Enzyme immunoassay for the rapid clinical identification of snake venom. Med J 1980; 1: 433-35.
5. O'Leary MA, Isbister GK, Schneider JJ, Brown SGA, Currie BJ. Enzyme immunoassays in brown snake (*Pseudonaja spp*) envenoming: Detecting venom, antivenom and venom-antivenom complexes. Toxicon 2006; 48: 4-11.
6. Theakston RD, Laing GD. Diagnosis of Snakebite and the Importance of Immunological Tests in Venom Research. Toxins (Basel) 2014; 6: 1667-95.
7. Theakston, RD. The application of imunoassay techniques, including enzyme-linked immunosorbent assay (ELISA), to snake venom research. Toxicon 1983; 21: 341-52.
8. Bhatti AR, Wong JP, Siddiqui YM, Siddiqui S. A sensitive fluorogenic enzyme linked immunosorbent assay for the detection of *Vipera russelli* venom. Nat Toxins 1993; 1: 277-82.
9. Steuten J, Winkel K, Carroll T, *et al*. The molecular basis of crossreactivity in the Australian Snake Venom Detection Kit (SVDK). Toxicon: official journal of the international Society on Toxinology. 2007; 50: 1041-52.
10. Oh JS, Ha GW, Cho YS, *et al*. One-step immunochromatography assay kit for detecting antibodies to canine parvovirus. Clin Vaccine Immunol 2006; 13: 520-24.