Immunization of Rabbits with Myanmar Green Pit Viper (*Trimeresurus erythrurus*) Venom

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Green pit viper (*Trimeresurus* spp.) is a medically important venomous snake in Myanmar especially in Yangon, Mandalay and Magwe Divisions. Biological assays of Myanmar green pit viper venom showed coagulation, hemorrhagic and lethal activities in animals. Both local and systemic lethal effects are seen in human victims. Development of coagulopathy and acute kidney injury would lead to shock in patients. Antivenom for Myanmar green pit viper is not available yet. The present study was done for small scale production of monospecific antibodies against Myanmar green pit viper venom in rabbits was conducted. Immunization was performed in two adult rabbits at initial dose of 50 µg/kg subcutaneously. Thereafter, stepwise increment venom dosed up to 150 µg/kg during a 3-month period. Their antibody levels were monitored by indirect ELISA during the experiment of 8 months. The efficacy of the monospecific antiserum was tested with immunodiffusion and immunoblotting. Antibodies reached its peak at 8 weeks after first immunization. Boostering at 13 weeks resulted in maintaining its peak throughout the study period. Immunodiffusion and immunoblotting analysis showed that the rabbit antiserum cross-reacted with Myanmar green pit viper venom as well as Thai green pit viper venom. This pilot study in rabbits would provide useful information for production of Myanmar green pit viper antivenom in Myanmar.

Keywords: Green pit viper, Monospecific antiserum, Rabbit immunization

INTRODUCTION

Green pit viper (*Trimeresurus* spp.) bite had an incidence of 4% bites per year in the period of 1998-2000 (data from 87 hospitals in Myanmar).[1] In addition, it accounted for 16% of the snake bite cases admitted to Yangon General Hospital between January 1999 to April 2001 and 64% of them were bitten in Bahan Township.[2] The green snakes contributed the second most cases (62/965 cases) after Russell's viper bites in a prospective study (February 2016 to January 2017) at Mandalay General Hospital.[3]

Seven species are known to be inhabited in Myanmar. *T. erythrurus* is responsible for most bites[4]. The patients have painful local swelling at the site of bite. Massive local swelling, blisters, necrosis and bleeding from the wound are seen in severe case. Development of coagulopathy leads to haemorrhagic hypovolemic shock and death in patient.[5, 6] On rare occasions, envenomation by *Trimeresurus* spp. cause acute kidney injury (AKI).[7]

Biological assays of Myanmar green pit viper venom (MGPV) showed that it possesses coagulation, hemorrhagic and lethal activities on laboratory mice.[8] Since antivenom for Myanmar green pit viper is not available yet,

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and the current study was conducted for small-scale production of monospecific antibodies against Myanmar green pit viper (*Trimeresurus erythrurus*) venom in rabbits. The result from this study will support estimated immunization dose, immunization schedule for development of monospecific antivenom production for Myanmar green pit viper in horses or sheep.

MATERIALS AND METHODS

Venoms and antivenom

Myanmar green pit vipers were kept and collected their venom and skin from Zoological Garden, Yangon, Myanmar. The shed skin were used for species identification. The venom collected was freeze-dried at Department of Medical Research (DMR), Yangon, Myanmar. The pooled venom was used for raising antibodies in laboratory animals. Thai green pit viper venom (TGPV) and antivenom (Anti-TGPV) were purchased from the Snake Farm, Queen Saovabha Memorial Institute (QSMI), Bangkok, Thailand. Myanmar Russell's viper and Myanmar cobra venoms were purchased from Pharmaceutical Factory (Insein), Myanmar Pharmaceutical Enterprise, Yangon.

Animals and reagents

Japanese White rabbits and albino mice (ICR strains) were supplied by Laboratory Animals Services Division, DMR, Yangon, Myanmar. The animals were handled according to the Guide to the Care and Use of Experimental Animals, Canadian Council on Animal Care 1980. Ninety-six well flat bottom microtiter plates (Kartell, Italy), goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (Invitrogen, US), Tween 20 (BDH, England), dihyrochloride o-phenylenediamine (Sigma-Aldrich, USA) were purchased from the respective manufacturers. All other chemicals and reagents utilized were of analytical grade (Sigma-Aldrich, USA).

Determination of protein concentration

The protein concentration of venom and antivenom were determined by a modified Lowry's method.[9]

Determination of lethality of the venom

Venom from 14 snakes (78-100 cm in total length) were pooled to determined the lethality. The median lethal dose (LD_{50}) of the venom was assessed by injection of MGPV in 0.2 ml of physiological saline into tail vein of 22±2 g ICR-strain mice.[10] Six groups of mice injected different venom doses were used for test groups. A control group was injected with saline. Five mice were used for each group and their lethality was observed after 24 hours of injection. The LD_{50} was calculated by the Spearman-Karber method.[11] Lethal dose (LD_{50}) of venom was defined as the amount of venom causing death in 50% of injected mice.

Immunization of rabbits

Two females of Japanese White rabbits weighed 2.0 ± 0.2 kg were immunized 5 times to raise the antibodies against MGPV within 3 months[12] (Table 1).

Table 1. Immunization schedule of rabbits with Myanmar green pit viper venom

Rabbit	Venom dose (µg/kg)*		
	1st (D0) & 2nd (D30) immunization	3rd (D45) & 4th (D75) immunization	Booster immunization** (D91)
1	50	100	150
2	50	100	150

*Each dose was divided into 4 parts and injected subcutaneously on the back of rabbits.
**Booster immunization with 2-week interval (1 time: 5th immunization)

The MGPV emulsified with an equal volume of Complete Freund's Adjuvant [50 µg/kg in 0.5 ml phosphate buffer saline (PBS), pH 7.4 was emulsified in 0.5 ml of CFA], was injected subcutaneously to rabbits over back. Three other subcutaneous dose; 50 µg/kg, 100 µg/kg and 100 µg/kg in 0.5 ml PBS emulsified in 0.5 ml Incomplete Freund's Adjuvant (IFA) were followed at Day 30, Day 45 and Day 75, respectively. Thereafter, the rabbits were boostered one time at Day 91 with a dose of 150 µg/kg venom with IFA. The blood was collected from marginal ear vein of rabbits with a butterfly infusion set

under aseptic condition before immunization and at 2nd, 9th, 12th, 25th, 28th and 32nd-week after the first dose of injection. The puncture site was applied with iodine solution after the blood collection and checked daily. The wound was treated with antibiotic cream if there was inflammation.

Indirect ELISA for determination of antibodies in rabbit serum

Antibody level in rabbits was monitored by indirect enzyme immune assay (EIA).[13] In 96 well microplate, 100 µl venom (1.25 µg/mL concentration) in 0.05 M carbonate buffer, pH 9.6 was coated at 37°C for 2 hours followed by overnight at 4°C. After washing two times with 200 µl of PBS-Tween 20, the plate was blocked with 1% BSA-PBS for 1 hour at 37°C. The plate was washed 3 times with PBS-Tween and 100 µl rabbit sera was added and incubated at 37°C for 1 hour. The plate was washed again in PBS-Tween and 100 µl 1:10,000 dilution of peroxidase conjugated anti-rabbit IgG was added and incubated for 1 hour at 37°C. After washing, 100 µl substrate solution (1 mg/ml dihydrochloride O-phenylenediamine and 0.03% H_2O_2 in 0.1 M citric buffer solution, pH 5.0) was added and incubated the plate in dark for 30 minutes. The reaction was then interrupted at room temperature by adding 2.5 M H_2SO_4 50 µl/wells. The reaction was read in a microplate reader (FLUOstar Omega, BMG Labtech) at 492 nm wavelength. Preimmunization rabbit serum was added to control wells. All experiments were performed in triplicates. For validation of the ELISA test, different dilutions of Anti-TGPV 1:100, 1:300, 1:1000, 1:3000, 1:10000, 1:30000, 1:100000, 1:300000 were used to react with 1.25 µg/mL TGPV and MGPV, respectively.

Imunodiffusion and immunobloting of the venom by rabbit antiserum

Antigen antibody reaction was performed in 1% agar plate. Venoms in peripheral wells were put up against the anitvenom/antiserum in center well (15 mg/mL) in a moist chamber at room temperature for 24 hours. The precipitin bands were observed with eyes.[14] For immunoblotting, snake venom proteins were separated on 8-15% gradient sodium dodecyl-sulfate polyacrylamide (SDS-PAGE) gel[15] in Eco-line (Mini) Biometra tank.

The venom proteins were transferred to polyvinylidene fluoride (PVDF) membrane in a blotting unit. The membrane was then incubated in a protein blocking solution for 30 minutes at 37°C with gentle agitation. The membrane was washed thrice (5 min/wash) with wash buffer (0.1% Tween 20, 0.9% NaCl, 20 mM Tris/HCl pH 7.5). The blot was incubated with Anti-MGPV serum 1 µg/mL in a volume of 25 ml buffer [1% bovine serum albumin (BSA), 0.05% Tween-20, 0.9% NaCl in 20 mm Tris/HCl pH 7.5] for overnight at 4°C. Blots were then washed again and incubated with the secondary antibody conjugate (anti-rabbit IgG-peroxidase antibodies) solution (1:1000) for 4 hours at room temperature. The blot was washed in the HRP-DAB (Horse Reddish Peroxidase and Diaminobenzidine) systems to develop blue colour.[16] The control blot used were Anti-TGPV (1 µg/ml) as primary anti-body solution and anti-horse IgG-peroxidase antibodies solution (1:1000) as secondary antibody solution.

Ethical consideration

Proposal of this study was approved by Institutional Review Board, DMR, Myanmar with approval number of Ethics/DMR/2019/ Exemption-11 on 6th November 2019.

Funding

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RESULTS AND DISCUSSION

Median lethal dose (LD_{50}) of Myanmar green pit viper (MGPV) venom

The LD_{50} of the pooled venom was 62.09 µg/ mouse (Fiducial limit, 29.72-129.72 µg/ mouse).

Immunization of rabbits and determination of antibodies in rabbit serum with ELISA test

For validation of ELISA test, both MGPV and TGPV (1.25 µg/mL) were coated on the well and interacted with various dilutions of

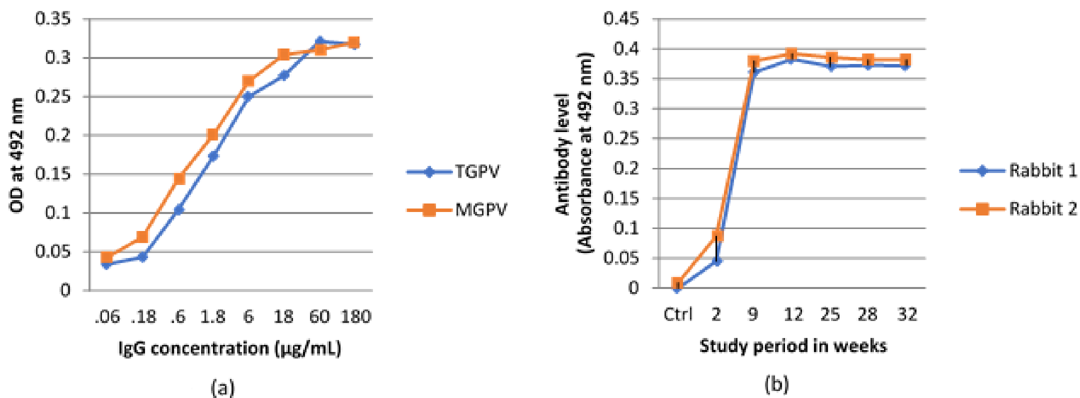


Fig. 1. (a) Validation of linearity of the assay in standardization of ELISA using anti-TGPV against MGPV and TGPV (b) Monospecific antibody levels in the both rabbits during study period

Anti-TGPV solution. The absorbance results for both venom was not significantly different. Linearity was found from 1:100,000 to 1:1,000 dilution (Fig. 1a). From this finding, dilution for control and test rabbit sera were diluted 1:10,000 for ELISA test.

The rabbits' immune system responded to MGPV. The antibodies against the venom started to rise in 2 weeks after the 1st immunization and reached to highest level after the 2nd and the 3rd immunization. This high level is continuously maintained upto eight months (Fig. 1b). The present study showed that 3 months immunization schedule with subcutaneous route induced high and sustainable level of antibodies in rabbits as 6 months immunization schedule.[12]

Immunodiffusion

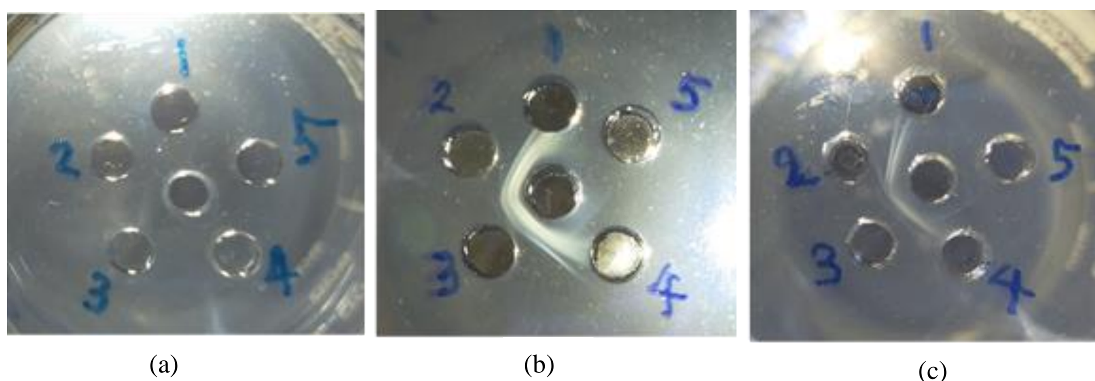
Immunodiffusion reactions of rabbit control serum, rabbit antiserum and Anti-TGPV with venoms were performed. Both rabbit antiserum and Anti-TGPV gave precipitin bands with MGPV and TGPV while a small fade precipitin band with MRV. No precipitin band was detected between the anti-serums and MCV (Fig. 2b, 2c). No immunodiffusion reaction was detected between the rabbit control serum and all venoms (Fig. 2a). It showed that the rabbit antiserum contained antibodies against various components of *Trimeresurus* venom. It is also worthy to note that the rabbit antiserum formed equally strong precipitin lines to both homologous and heterologous green pit viper venoms than the Anti-TGPV did. This might be due to pharmacodynamics of the two different anti-sera, i.e., whole IgG form of rabbit antiserum and $F(ab')_2$ of Anti-TGPV behaved differently in the body. Whole IgG molecules have higher affinity interactions between each antibody-toxin pair than $F(ab')_2$ fragments although both formats give multivalent immune-complexes.[17]

Immunoblotting

The immunoblots showed that the rabbit antiserum raised against the MGPV can recognized more proteins of MGPV and TGPV than those of MRV and MCV (Fig. 3b). The anti-TGPV also reacted more proteins from TGPV and MGPV venoms than those from MRV and MC venoms (Fig. 3c). More proteins were recognized by rabbit antiserum (whole IgG) than horse antiserum $F(ab')_2$ might be due to specific binding capability of immunoglobulins raised from two different species, i.e., rabbit and horse against toxins. Individual antivenom anti-body may recognize similar toxins with different affinities.[18] It showed the genus specific immunological cross-reactivity against the venoms of genus *Trimeresurus*.

Conclusion

In conclusion, a monospecific rabbit antiserum against Myanmar green pit viper venom was successfully raised. The low dose, short-course immunization schedule gave



(a) (b) (c)

Fig. 2. (a) Immunodiffusion of 50 µL of control rabbit serum (preimmunization) (130 mg/ml) with same volume of normal saline (1), MGPV (2), TGPV (3), MRV (4) and MCV (5) venoms; (b) Immunodiffusion of 50 µL of rabbit antiserum (150 mg/ml) with same volume of normal saline (1), MGPV (2), TGPV (3), MRV (4) and MCV (5) venoms; (c) Immunodiffusion of 50 µL of Anti-TGPV (18 mg/ml) with same volume of normal saline (1), MGPV (2), TGPV (3), MRV (4) and MCV (5) venoms

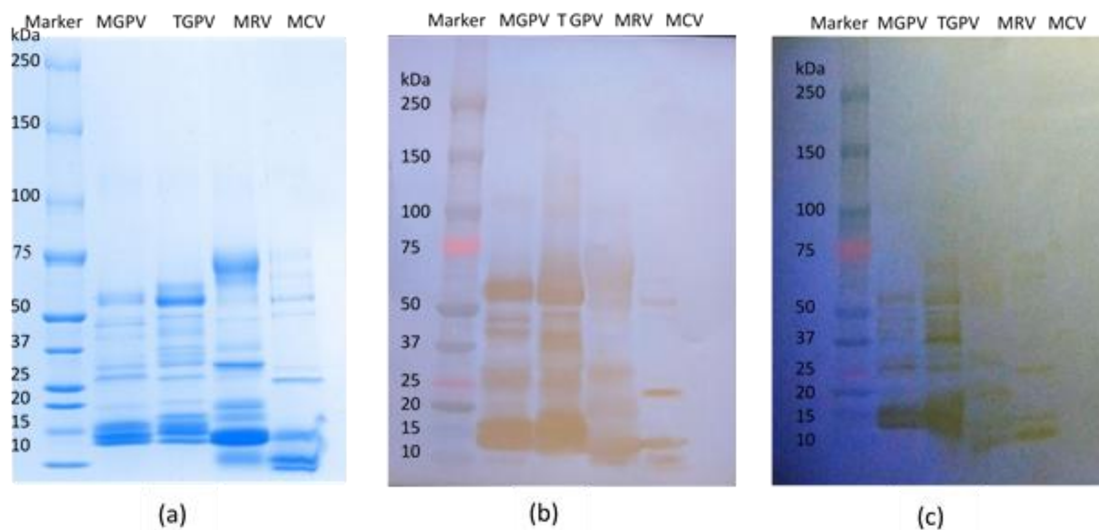


Fig. 3 (a) SDS-PAGE of different venoms [MGPV=Myanmar green pit viper venom (*T. erythrurus*), TGPV=Thai green pit viper venom (*T. albolabris*), MRV=Myanmar Russell's viper venom, MCV=Myanmar cobra venom (*Naja kaouthia*)]; (b) Immunoblot-detection of rabbit antiserum raised against MGPV to proteins in 4 different venoms; (c) Immunoblot-detection of Anti-TGPV to proteins in 4 different venoms

high, sustainable antibody level. The current 3-month immunization schedule raised high antibody level which was sustainably maintained for the 8-month duration. The antiserum well-recognized the proteins in the homologous venom as well as to heterologous venom in immunodiffusion and immunoblot test. The results obtained from the present study provided useful information for production of Myanmar green pit viper antivenom in horses or sheep for effective clinical use.

Competing interests

The authors declared that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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REFERENCES

1. Aye Aye Myint, Tun Pe & Tin Zar Maw. An epidemiological study of snakebite and venomous snake survey in Myanmar. In: *Management of Snakebite and Research*. WHO, Regional Office for Southeast Asia, New Delhi, 2002; 12-16.
2. Tun Pe, Tin Myint, Aung Htut, Than Htut, Aye Aye Myint & Nu Nu Aung. Envenoming by Chinese krait (*Bungarus multicinctus*) and banded krait (*B. fasciatus*) in Myanmar. *Transactions of The Royal Society of Tropical Medicine and Hygiene* 1997; 91: 686-8.
3. White J, Alfred S, Bates D, M ahmood MA, Warrell D, Cumming R, *et al*. Twelve month prospective study of snakebite in a major teaching hospital in Mandalay, Myanmar; Myanmar Snakebite Project (MSP). *Toxicon X* 2019; 1:100002.
4. Leviton AE, Guinevere OUW, Koo MS, Zug GR, Lucas RS & Vindum JV. The Dangerously Venomous Snakes of Myanmar . Illustrated Checklist with Keys. *Proceedings of The California Academy of Sciences* 2003; 54: 407-462.
5. Tun Pe, Aye Aye Myint & Nu Nu Aung. Green pit viper (*Trimeresurus erythrurus*) bites in Myanmar: Clinical features, venom antigen levels and development of natural venom antibodies. *Myanmar Health Sciences Research Journal* 2000; 12:1-6.
6. Tun Pe & Tin Tin Aung. Compartmental syndrome following a green pit viper (*Trimeresurus erythrurus*) bite. *Myanmar Health Sciences Research Journal* 2006; 18: 31-40.
7. Thein MM, Rogers CA, White J, Mahmood MA, Weinstein SA, Nwe MT, *et al*. Characteristics and significance of "Green snake" bites in Myanmar, especially by the pit vipers *Trimeresurus albolabris* and *Trimeresurus erythrurus*. *Toxicon* 2021; 203: 66-73.
8. Khin Than Yee, Lwin Zar Maw, Aung Myat Kyaw, Khow O, Aye Win Oo, Tin Ko Ko Oo, *et al*. Evaluation of the cross-neutralization capacity of Thai green pit viper antivenom against venom of Myanmar green pit viper. *Toxicon* 2020; 177: 41-45.
9. Miller G. Protein determination for large numbers of smples. *Analytical Chemistry* 1959; 31: 964.
10. WHO. Essential preclinical assays to measure antivenom neutralization of venom-induced lethality. In: *WHO Guidelines for the Production, Control and Regulation of Snake Antivenom Immunoglobulins*. WHO, Geneva, Switzerland, 2016; 85-87.
11. WHO. Determination of Median Lethal Dose (LD50). In: *Progress in the characterization of venoms and standardization of antivenoms*. WHO, Geneva, 1981; 23-24.
12. Chanhome L, Puempunpanich S, Omori-Satoh T, Chaiyabutr N & Sitprija V. Immunization of rabbits with *Bungarus candidus* venom. *Journal of Natural Toxins* 2002; 11: 353-356.
13. Rial A, Morais V, Rossi S & Massaldi H. A new ELISA for determination of potency in snake antivenoms. *Toxicon* 2006; 48: 462-466.
14. Ouchterlony O & Nilsson L. Immunodiffusion and immunoelectrophoresis. In: *Handbook of Experimental Immunology*, Oxford: Blackwell Scientific Publications; 1978.
15. Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-685.
16. Pluskal M, Przekop M, Kavonian M, Vecoli C & Hicks D. Immunobilon™ PVDF transfer membrane: a new membrane substrate for Western blotting of proteins. *Biotechniques* 1986; 4: 272-282.
17. Gutiérrez JM, León G & Lomonte B. Pharmacokinetic-Pharmacodynamic Relationships of Immunoglobulin Therapy for Envenomation. *Clinical Pharmacokinetics* 2003; 42: 721-741.
18. Laustsen AH, María Gutiérrez J, Knudsen C, Johansen KH, Bermúdez-Méndez E, Cerni FA, *et al*. Pros and cons of different therapeutic antibody formats for recombinant antivenom development. *Toxicon* 2018; 146: 151-175.