ORIGINAL ARTICLE

Green pit viper antivenom from Thailand and *Agkistrodon halys* antivenom from China compared in treating *Cryptelytrops albolabris* envenomation of mice

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Objective To compare the relative efficacy of the green pit viper antivenom from Thailand and *Agkistrodon halys* antivenom from China.

Design In-vivo experimental study.

Setting A wildlife conservation organisation, a university, a poison information centre, and a regional hospital in Hong Kong.

Main outcome measures Pre- and post-antivenom lethal dose 50 (LD_{50}) of the *Cryptelytrops albolabris* venom, median effective dose (ED_{50}) of green pit viper antivenom and *Agkistrodon halys* antivenom against a lethal dose of the venom.

Subjects Adult mice.

Results The intraperitoneal LD_{50} of the venom from locally caught *Cryptelytrops albolabris* was 0.14 µL. After post-exposure treatment with 10 µL of antivenom, it was elevated to 0.36 µL and 0.52 µL by the green pit viper antivenom and the *Agkistrodon halys* antivenom, respectively. The ED_{50} was 32.02 µL for green pit viper antivenom and 6.98 µL for *Agkistrodon halys* antivenom. Both green pit viper antivenom and *Agkistrodon halys* antivenom ameliorated the lethality of *Cryptelytrops albolabris* venom in mice.

Conclusion The overall superior neutralisation capacity of *Agkistrodon halys* antivenom over green pit viper antivenom may be related to the geographic proximity of the venoms used for antivenom preparation. The results point towards the need for further comparison of the two antivenoms on protein or immunoglobulin weight basis, and with respect to non-lethal clinically significant toxicities.

Key words
Antivenins; Cross reactions; Crotalid venoms; Neutralization; Snake bites

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New knowledge added by this study

- *Agkistrodon halys* antivenom (AHA) possesses para-specific activity against the venom of the local *Cryptelytrops albolabris*.
- On volume basis, *Agkistrodon halys* is generally more potent than green pit viper antivenom (GPVA) in mortality studies on mice.

Implications for clinical practice or policy

- The two antivenoms should also be compared with respect to protein and immunoglobulin contents.
- Besides the specific GPVA, AHA may be effective in humans envenomed by *Cryptelytrops albolabris* bites.
- To confirm the clinical applicability of the AHA, studies on parameters other than mortality and determination of the appropriate doses are indicated.

Introduction

In Hong Kong, a city in southern China, snakebite is quite a common emergency. In 2009, 139 cases were recorded.[1] Of all the instances of snakebite, 95% were due to the white-lipped pit viper (*Cryptelytrops albolabris*).[2] For many years, the Hong Kong Hospital Authority has been importing antivenom as an antidote for *C albolabris* bites from two sources. They are the green pit viper antivenom (GPVA) from the Thai Red Cross Society and the *Agkistrodon halys* antivenom (AHA) from the Shanghai Institute

of Biological Products. Both GPVA and AHA are on the recommendation list in the clinical guidelines of the Hong Kong Poison Information Centre and the Central Coordinating Committee of Accident and Emergency Services of the Hospital Authority.[3,4] Over the past years, physicians in Hong Kong have been utilising both antivenoms for conditions such as coagulopathy or severe local reaction. Owing to paucity of data, however, selecting between them and the doses to use in individual cases were not based on any clear understanding of their relative efficacy. Reports on a limited number of local cases showed that both antivenoms were successful in reversing prolonged prothrombin times and to a lesser extent thrombocytopaenia.[5] However, in one patient the coagulopathy was not corrected after one ampoule of AHA but to improve after five vials of GPVA.[5,6] These clinical observations raised question as to whether the AHA failure was due to species mismatch or simply inadequate dose.

In this study, we therefore compared the efficacies of GPVA and AHA in a mouse model of *C albolabris* envenomation, using volume-based dosing (as employed in clinical practice).

比較應用分別來自泰國竹葉青蛇和中國蝮蛇的抗毒血清來處理老鼠中白唇竹葉青蛇毒液的療效

目的 比較分別來自泰國竹葉青蛇和中國蝮蛇的抗毒血清的相對效用。

設計 體內實驗研究。

安排 香港的一所野生動物保護組織、一所大學、一所中毒資訊中心和一所分區醫院。

主要結果測量 白唇竹葉青蛇毒液注射半數致死量（LD_{50}）的前後值、泰國竹葉青蛇抗毒血清和中國蝮蛇抗毒血清的半有效劑量（ED_{50}）。

實驗對象 成鼠。

結果 本地捕捉的白唇竹葉青蛇毒液成鼠腹膜注射半數致死量（LD_{50}）為0.14 μL。注射兩種抗毒血清10 μL後，注射泰國竹葉青蛇抗毒血清的升至0.36 μL，注射中國蝮蛇抗毒血清的則升至0.52 μL；而兩者的ED_{50}分別為32.02 μL和6.98 μL。泰國竹葉青蛇和中國蝮蛇的抗毒血清皆可用於對抗白唇竹葉青蛇毒素。

結論 可能由於地理上與白唇竹葉青蛇較接近，總括來說，中國蝮蛇的抗毒血清比泰國竹葉青蛇的抗毒血清有較佳的抗毒中和力。本研究顯示要進一步比較兩種抗毒血清，需要從兩者的蛋白或免疫球蛋白量著手，並且探討兩種抗毒血清非致命性的臨床毒性。

Methods

This study was approved by the ethics committees of the Chinese University of Hong Kong and Kadoorie Farm and Botanic Garden (KFBG), a non-government organisation actively participating in the wildlife conservation of Hong Kong.

Venom

In the summer of 2008, herpetologists of KFBG identified *C albolabris* for venom extraction from locally captured stray snakes. A total of 34 snakes were collected; 15 were adults, 11 were subadults, 6 were juveniles, and for 2 data were unavailable. Venom was extracted by allowing the snakes to bite into paraffin sheet over a plastic pot (Fig 1). The venoms from all the snakes were pooled, lyophilised and stored under 4°C in the dark. The protein content of the venom was determined using a standard BCA (bicinchoninic acid) protein quantification kit according to the instructions of the manufacturer (Sigma-Aldrich, St Louis, US). The protein content was determined to be 236 μg/μL. The same batch of venom was used throughout the study.

Antivenom

The GPVA in powder form was purchased from the Thai Red Cross Society in Thailand. When used in a clinical setting, the powder was reconstituted in 10 mL of water in another vial in the same package. The AHA was purchased from Shanghai Institute of Biological Products in China. It consists of 10 mL of liquid in an ampoule and is administered undiluted to the patient. In both antivenoms, the dosage-based weight was not available.

Dose-lethality study

Nine groups (10 each) of adult C57 mice (average weight, 25 g; range, 22-27 g) were injected intraperitoneally with saline (control), or one of eight different doses of venom (0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1 and 2 μL, diluted to a final injected volume of 100 μL). The dose-lethality curve was obtained and the dose that killed 50% of animals (LD_{50}) within 48 hours was determined by Probit analysis.[7]

FIG 1. Milking a *Cryptelytrops albolabris* for venom collection

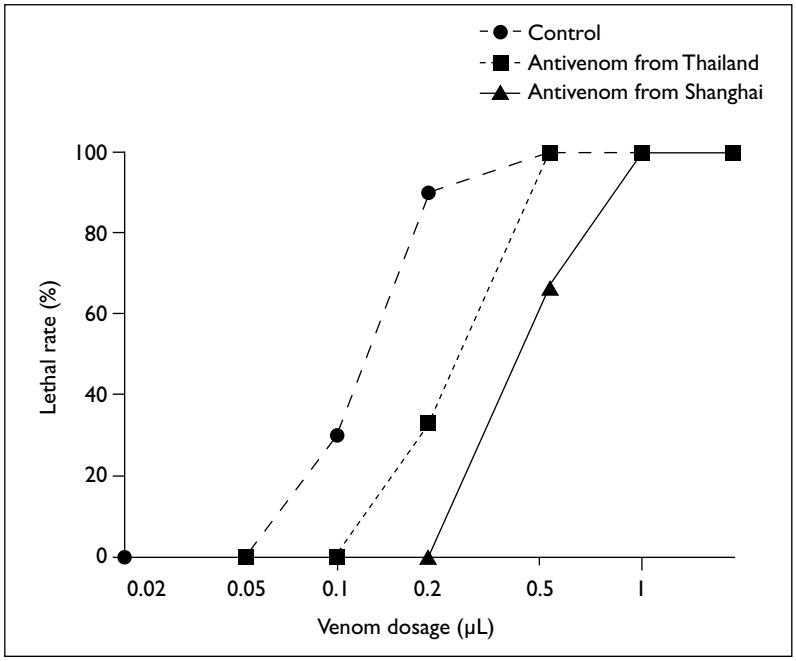

FIG 2. The effect of antivenoms from Thailand (green pit viper antivenom) and Shanghai (*Agkistrodon halys* antivenom) on the lethal rate after various doses of *Cryptelytrops albolabris* venom

Antivenom neutralisation studies

The effect on LD_{50} of a fixed dose of antivenom was studied as follows. Each antivenom was prepared according to the manufacturers' instruction. Based on the minimum recommended dose, we prepared 20 mL samples of both GPVA and AHA as detailed in the clinical guidelines of our hospital cluster and normally used to treat human subjects.[8] The potencies of 10 μL of each of the two antivenoms were then compared. Six groups consisting of six adult C57 mice were used. To assess the efficacy of the antivenom in neutralising the lethal activity of the venom as in a clinical scenario in which antivenom is administered after snake venom exposure, 5 minutes after envenoming with the venom at increasing doses (0.05, 0.1, 0.2, 0.5, 1 and 2 μL), 10 μL of antivenom was injected intraperitoneally. The dose-lethality curves and the LD_{50} for each type of antivenom (GPVA and AHA) were then determined.

The median effective dose (ED_{50}) of the two antivenoms was determined by intraperitoneal administration of incremental doses to groups of mice 5 minutes after the exposure to a lethal dose of venom.

Results

Lethality study

Preliminary trials indicated that if the experimental animal survived the first 48 hours after envenoming, it could survive up to the last day of experiment, namely the seventh day. Therefore, 48 hours was used as the cut-off point in determining lethality in subsequent experiments. In the dose-lethality study, it was found that the mice were able to tolerate up to 0.05 μL of the venom (0% lethality). Injection of 0.1 μL of venom started to cause animal deaths within 48 hours (30% lethality) and 0.5 μL (protein content 118 μg) killed all tested animals. Accordingly a dose-lethality curve was plotted as shown in Figure 2. The LD_{50}, determined by Probit analysis, was 0.14 μL (95% confidence interval [CI], 0.11-0.18 μL), which amounted to 0.005 μL/g or 1.18 μg/g.

Antivenom neutralisation studies

As shown in the dose-lethality curves (Fig 2), both antivenoms were able to reduce the lethality. For example, no lethality was recorded in animals treated with either antivenom in response to 0.1 μL of venom (a dose that killed 30% of control mice). The 90% lethal rate following 0.2 μL of venom injection was also significantly reduced to 30% by GPVA and 0% by AHA. After treatment with GPVA, the venom LD_{50} was 0.36 μL (95% CI, 0.24-0.67 μL); without any antivenom treatment the LD_{50} was 0.14 μL. The LD_{50} in the group treated with AHA was even higher, being 0.52 μL (95% CI, 0.37-0.82 μL).

Greater potency of AHA compared to GPVA was noted when we determined the ED_{50} of antivenin against a lethal dose of venom (0.3 μL, 2.2 LD_{50}). The ED_{50} of AHA was 6.98 μL (95% CI, 4.0-17.4 μL), which was several folds below that of the GPVA (32.02 μL; 95% CI, 24.0-42.0 μL).

Discussion

In our antivenom neutralisation studies, AHA was clearly more potent than GPVA in terms of its ED_{50} and reduction of lethality from the venom dose originally giving rise to 90% lethality. When the LD_{50} were compared, their CIs overlapped, and that after GPVA treatment it was only slightly outside the CI range of the AHA group. Thus, our study might not have enough statistical power to examine the individual effects of these two antivenoms, though the results indicated a trend in favour of AHA over GPVA.

The GPVA from Thailand and AHA from China were generated against different venomous snake species. Thus for GPVA, the F(ab')2 antivenom produced against *C albolabris*[9] is recommended on its insert to be given to patients of green pit viper bites. Regarding the green pit viper snakes in Thailand, *C albolabris* and *Trimeresurus macrops* are common in inflicting bites and *C albolabris* is the most widely distributed species.[10] The specifications of AHA, which is also a F(ab')2 antivenom, claimed effectiveness for bites not only by *A halys*, but also *Trimeresurus stejnegeri* and *Trimeresurus mucrosquamatus*.

Information was lacking as to whether the latter two snake species were employed in the immunisation protocol. Since *C albolabris* and *A halys* were the major targets of GPVA and AHA respectively, the main targets of these antivenoms differed in terms of species, genus, geographical distribution of the snakes, and clinical symptomatology of their bites. For example, *C albolabris* of genus *Cryptelytrops* inhabits South-East Asia, including the southern part of China. Its bite produces coagulopathy and local swelling. Whereas, *A halys* belongs to the genus *Agkistrodon* (synonym *Gloydius*) and is found in the region stretching from western Russia to central China. Bites by *A halys* have the potential to cause neurotoxicity in addition to the coagulopathy and local swelling (in common with *C albolabris* bites).[11] For these reasons, it was surprising that in our study AHA appeared to be more potent than GVPA activity against *C albolabris* envenoming. The interplay of many factors influencing the biochemical and clinical impact of the venom from particular snake species could explain such effects. Among them are species specificity, species cross-reactivity, geographical location, and the active ingredients in each antivenom.[12]

The non-identical toxicities of many different snake species, even from the same family, imply that venom composition is species dependent. The varying extent of neurotoxicity and cytotoxicity across the multiple cobra species is an example illustrating this phenomenon. It is therefore reasonable to expect that antivenom is more effective for the species it is developed against. The effectiveness of homologous viper antivenom has been described in numerous studies. In mice, the monovalent GPVA manufactured by Thai Red Cross Society against *C albolabris* is about four-fold more potent against *C albolabris* than several common Southeast Asian snakes under the *Trimeresurus* genus (when using survival as the end-point).[13] In comparison with Habu antivenom, this antivenom is derived from *Trimeresurus flavoviridis* and was more effective inhibiting lethality and haemorrhage induced by *C albolabris* venom in a mouse study. The species-specific action of snake antivenom thereby suggested was also substantiated in the same trial by the superior results of the Habu antivenom over GPVA in terms of activity against *T flavoviridis*.[9]

However, snake venom structure is not strictly species-specific. Protein similarity over a range of snake species exists, possibly as a result of divergent evolution or other factors. Venoms from unrelated snakes often contain many common enzymes. Antibodies raised against specific toxins from a single snake species have been detected to cross-react with proteins of close molecular weight from the other species within the same genus and even a differing genus.[14] Among Asian vipers, cross-species protein resemblance was revealed by sequence analysis of the amino acid of *C albolabris* venom, which recovered proteins linked to jerdonitin from *Trimeresurus jerdonii*, and stejaggregin-A, stejnobin and stejnihagin-A from *T stejnegeri*.[15] Consistent with our study results, cross-protection against *T mucrosquamatus* by AHA leading to improved survival has been previously proven using another mouse model,[16] and there have also been reports of a stronger reaction between the heterologous venom and antivenom. Immunoblotting assays have revealed that instead of their specific antivenoms, the highest reactivity index for the venoms of *Crotalus adamanteus* and *Crotalus horridus horridus* was obtained with anti-*Crotalus viridis viridis* and anti-*Crotalus atrox*, respectively.[17] A study on mice administered a mixture of antivenom and venom also showed that for the protection against *T purpureomaculatus*–induced lethality, a larger injection volume was required for the homologous antivenom than the antivenoms against the other *Trimeresurus* species.[13]

Besides species factor, geographical location may affect the venom characteristics. Russell's viper (*Daboia russelli*) in Sri Lanka has been described to cause clinical envenoming features distinct from those encountered in India. The antivenom from India is relatively ineffective in clearing the venom antigenemia. To enhance the therapeutic efficacy, treatment with antivenom produced from indigenous snakes is considered a better option.[18] Besides, there is laboratory evidence of intraspecies diversity of venom composition from different regions. It has been documented that venoms from *Bothrops atrox* in Columbia contained predominantly PI-metalloproteinase and K49-PLA2 while that in Brazil, Ecuador and Peru were mainly comprised of PIII-metalloproteinase. Whether this variation is a reflection of the varied habitats and ecologies of a widespread, highly adaptable species or an indication that this species is a composite of several subspecies remains controversial.[19]

As shown by our study results, AHA appears more potent than GPVA on volume basis but it may be preferable to compare the two antivenoms according to their protein or to be more accurate the immunoglobulin content, as it is the immunoglobulin that is the active component in the antivenom. In the antivenom comparison study carried out by Laing et al,[20] FUNDED antivenom contained 40 mg/mL protein (70% gamma-globulin), whilst Vital Brazil antivenom contained 71 mg/mL (73% gamma-globulin), and Butantan antivenom contained 100 mg/mL (84% gamma-globulin). When measuring the ED_{50} based on dose volumes, all three antivenoms were equally potent. However, when ED_{50} was expressed as mg protein and immunoglobulin, FUNDED antivenom emerged as the most effective.[20]

These snake-related factors indicate that venom from different species may share common immunological properties and geographical location is a factor in addition to species in determining venom profile. Common antigens and geographic proximity may explain the efficacy of AHA in our study. Although *C albolabris* was not recruited in Yi's study[16] that showed survival benefit of AHA in *T mucrosquamatus* envenoming, the cohabitation of *T mucrosquamatus* with *C albolabris* in southern China favours the possession of common antigens by them. It is therefore plausible that the geographic proximity overpowers the species advantage of GPVA from Thailand in the antagonism against our local *C albolabris* bite. In relation to *C albolabris*, factors such as geographical variability of venom phenotype are yet to be proven.

The possible advantage of AHA over GPVA suggested in our study was based on dosing used for envenomed humans, but should not be extrapolated to human without considering other issues. First, superiority was demonstrated in mortality reduction and not other clinically relevant venom toxicities. Snake antivenom has been reported to inhibit mortality and haemorrhage unequally in mice.[9] Actually, in human the principal clinical envenoming effect of concern of *C albolabris* venom is haemorrhage rather than death. Second, the correlation between clinical efficacy and laboratory results after antivenom use may not be consistent. The efficacy of the Pasteur antivenom for humans bitten by *Echis ocellatus* was poor based on a mouse assay.[21] Third, we cannot rule out inconsistency of the composition of antivenoms in various batches and venoms derived from individual snakes.

Apart from demonstrating a possible outcome difference between GPVA and AHA, our study lays the foundation for further research on the two antivenoms. Such research could address doses based on immunoglobulin content, and clinical settings involving humans. Similar principles and processes should be adopted for the evaluation of other antivenoms. Notably, it is important for any antivenom applied in clinical practice to be tested against the venom of local snakes to ascertain efficacy. Understanding that this may be a logistically demanding process means that adopting antivenom raised against specific species remains the reasonable approach until relevant data become available.

Conclusion

Our results indicate possibly greater potency of AHA than GVPA (after volume-based dosing) in reducing lethality in mice after the exposure to our local *C albolabris* venom. The reasons may be related to: the preparation of AHA from geographically closer snake venoms (despite the absence of *C albolabris*), and the immunoglobulin content in the antivenoms. Further studies should be considered to evaluate the efficacy of these two antivenoms based on immunoglobulin content dosing and exploring clinically relevant toxicities other than lethality.

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References

1. Hospital Authority, Hong Kong. Clinical Data Analysis and Reporting System. http://cdars.home. Accessed 3 Mar 2010.
2. Ng WS, Cheung WL. Snake bites in Hong Kong (*T. albolabris* and other species): clinical features and management. Hong Kong J Emerg Med 1998;5:71-6.
3. Poison Information Centre, Hospital Authority, Hong Kong. Mechanism for the emergency supply of antidotes in HA hospitals version 4. 2009. HA website: http://ha.home/hkpic. Accessed 3 Mar 2010.
4. Management of snakebite. Accident and emergency clinical guidelines number 9. Hong Kong: Central Coordinating Committee of Accident and Emergency Services, Hospital Authority; 2008.
5. Fung HT, Lam KK, Kam CW. Efficacy and safety of snake antivenom therapy: experience of a regional hospital. Hong Kong J Emerg Med 2006;13:70-8.
6. Yang JY, Hui H, Lee AC. Severe coagulopathy associated with white-lipped green pit viper bite. Hong Kong Med J 2007;13:392-5.
7. Finney PJ. Probit analysis, 3rd ed. Cambridge: Cambridge University Press; 1971: 333.
8. Management guidelines for snakebite. Hong Kong: Accident and Emergency Department, New Territories West Cluster, Hospital Authority; 2007.
9. Pakmanee N, Khow O, Wongtongkam N, Omori-Satoh T, Sitprija V. Efficacy and cross reactivity of Thai green pit viper antivenom among venoms of *Trimeresurus* species in Thailand and Japan. J Nat Toxins 1998;7:173-83.

10. 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.
11. *Agkistrodon halys* bites treated with specific antivenin: discussion on 665 cases [in Chinese]. Zhonghua Wai Ke Za Zhi 1980;18:568-9.
12. Glenn JL, Straight R. Mojave rattlesnake *Crotalus scutulatus scutulatus* venom: variation in toxicity with geographical origin. Toxicon 1978;16:81-4.
13. Tan NH, Choy SK, Chin KM, Ponnudurai G. Cross-reactivity of monovalent and polyvalent *Trimeresurus* antivenoms with venoms from various species of *Trimeresurus* (lanced-headed pit viper) snake. Toxicon 1994;32:849-53.
14. Berger BJ, Bhatti AR. Snake venom components and their cross-reactivity: a review. Biochem Cell Biol 1989;67:597-601.
15. Soogarun S, Sangvanich P, Chowbumroongkait M, et al. Analysis of green pit viper (*Trimeresurus albolabris*) venom protein by LC/MS-MS. J Biochem Mol Toxicol 2008;22:225-9.
16. Yi Q. The antivenin of *Agkistrodon acutus* and *Agkistrodon halys* neutralizing *Trimeresurus mucrosquamatus* venom in vitro and in vivo. Acad J Guangzhou Med Coll 1994;22:74-8.
17. Ownby CL, Colberg TR. Comparison of the immunogenicity and antigenic composition of several venoms of snakes in the family of Crotalidae. Toxicon 1990;28:189-99.
18. Ariaratnam CA, Sjöström,L, Raziek Z, et al. An open, randomized comparative trial of two antivenoms for the treatment of envenoming by Sri Lankan Russell's viper (*Daboia russelii russelii*). Trans R Soc Trop Med Hyg 2001;95:74-80.
19. Núñez V, Cid P, Sanz L, et al. Snake venomics and antivenomics of *Bothrops atrox* venoms from Columbia and the Amazon regions of Brazil, Perú and Ecuador suggest the occurrence of geographic variation of venom phenotype by a trend towards paedomorphism. J Proteomics 2009;73:57-78.
20. Laing GD, Theakston RD, Leite RP, da Silva WD, Warrell DA. Comparison of the potency of three Brazilian *Bothrops* antivenoms using in vivo rodent and in vitro assays. BIASG (Butantan Institute Antivenom Study Group). Toxicon 1992;30:1219-25.
21. Warrell DA, Warrell MJ, Edgar W, Prentice CR, Mathison J, Mathison J. Comparison of Pasteur and Behringwerke antivenoms in envenoming by the carpet viper (*Echis carinatus*). Br Med J 1980;280:607-9.