A B S T R A C T

Introduction: There are two antivenoms that may be administered in Hong Kong following a bite by *Trimeresurus albolabris*: the green pit viper antivenom from the Thai Red Cross Society in Thailand and the *Agkistrodon halys* antivenom from the Shanghai Institute of Biological Products in China. Both are recommended by the Central Coordinating Committee of Accident and Emergency Services of the Hospital Authority for treating patients with a bite by *Trimeresurus albolabris*. The choice of which antivenom to use is based on physician preference. This study aimed to compare the relative efficacy of the two antivenoms.

Methods: This in-vitro experimental study was carried out by a wildlife conservation organisation and a regional hospital in Hong Kong. Human plasma from 40 adult health care worker volunteers was collected. The *Trimeresurus albolabris* venom was added to human plasma and the mixture was assayed after incubation with each antivenom (green pit viper and *Agkistrodon halys*). Fibrinogen level and clotting time in both antivenom groups were studied.

Results: The mean fibrinogen level was elevated from 0 g/L to 2.86 g/L and 1.11 g/L after the addition of green pit viper antivenom and *Agkistrodon halys* antivenom, respectively. When mean clotting time was measured, the value was 6.70 minutes in the control, prolonged to more than 360 minutes by green pit viper antivenom and to 19.06 minutes by *Agkistrodon halys* antivenom.

Conclusions: Green pit viper antivenom was superior to *Agkistrodon halys* antivenom in neutralisation of the thrombin-like and hypofibrinogenaemic activities of *Trimeresurus albolabris* venom.
Introduction
Snakebite is an important medical emergency in Hong Kong. The consequences are potentially serious, especially if not treated quickly and appropriately. In 2014, 121 cases were recorded by the Clinical Data Analysis and Reporting System of the Hospital Authority in Hong Kong. *Trimeresurus albolabris*, also known locally as the white-lipped pit viper or bamboo snake, accounts for 95% of all human envenomation cases.1 Its bite can cause potentially life-threatening bleeding.2 In Hong Kong, death following a *T albolabris* bite, although rare, has occurred. The last reported case occurred in 1986 when an aged woman died of cerebral haemorrhage.3 Nonetheless non-lethal coagulopathy is common. In a local case series (n=21), laboratory coagulation abnormalities were frequent (hypofibrinogenaemia in 48% of cases, prolonged prothrombin time [PT] in 19%, and prolonged activated partial thromboplastin time [aPTT] in 14%) and sometimes accompanied by bleeding (skin bruising in one patient, both gastrointestinal haemorrhage and haematuria in another).4

*Trimeresurus albolabris* venom has a thrombin-like effect in vitro but causes a defibrination syndrome in vivo. The snake venom’s thrombin-like enzymes are responsible for the formation of friable and loose fibrin clots, hypofibrinogenaemia, and defibrination syndrome.5 We studied the thrombin-

like effect and defibrinating activity of *T albolabris* venom by assessing the clotting time and fibrinogen level, respectively, in human plasma.

There are two antivenoms available in Hong Kong for *T albolabris* bite, the green pit viper antivenom (GPVA; raised against *T albolabris*) from the Thai Red Cross Society in Thailand and the *A hékys* antivenin (AHA; raised against *A hékys*) from the Shanghai Institute of Biological Products in China. Both are recommended by the Central Coordinating Committee of Accident and Emergency Services of the Hospital Authority in treating patients with *T albolabris* bite.6 Reports on their relative efficacy in reversing coagulopathy in humans are scarce. A case report described prompt reversal of coagulopathy that was refractory to two ampoules of AHA given 3 days apart by five vials of GPVA.7 Conclusions can hardly be drawn in this case, however, about whether the failure of AHA was due to the species mismatch or simply inadequate dosage. The choice of antivenom to use in a clinical setting is determined by physician preference.8 In this study, we compared the potency of GPVA and AHA against the haemotoxicity from *T albolabris* envenoming using an in-vitro human plasma model.

Methods
This study was approved by the ethics committees of the New Territories West Cluster of Hospital Authority and Kadoorie Farm and Botanic Garden (KFBG), a non-governmental organisation actively participating in the conservation of Hong Kong wildlife.

Venom
From August to November 2013, herpetologists from KFBG identified *T albolabris* for venom extraction from locally captured stray snakes. Venom was extracted by allowing the snakes to bite into a paraffin sheet over a plastic collection pot (Fig 1). The venom was extracted and stored in sterilised bottles at -70°C.

Antivenom
The GPVA (batch number TA00512) and AHA (batch number 20130401) [Fig 2] were purchased from the Thai Red Cross Society in Thailand and the Shanghai Institute of Biological Products in China, respectively. Both were F(ab’)2, in powder form, and reconstituted in 10 mL of sterile water in another vial in the same package before clinical use.

Plasma preparation
Blood was collected from 40 adult health care workers who had no history of snake bite. They had no history of any coagulopathy problems and were not prescribed any anticoagulant. The samples were
Inhibiting coagulopathy caused by a venom

sodium citrate anticoagulated, centrifuged, and stored at -70°C before use. In the following assays, each blood sample was individually tested.

**Fibrinogen assay**

For green pit vipers envenoming, the manufacturer recommends a first dose of three vials (30 mL) of GPVA. According to the clinical guidelines of our emergency department, three vials were the appropriate dose for both GPVA and AHA. As a typical adult has a blood volume of approximately 5 L or plasma of 3 L, the dilution of 30 mL antivenom to 3 L of plasma by intravenous infusion route would therefore be 1:100. The amount of venom yield per bite was 8 to 15 mg for the *T. albolabris*. Venom yields are an average range for a ‘standard’ snake of the species and the amount of venom injected during a bite. If a maximum of 15 mg of venom was injected into the circulation of an adult, the maximum concentration of venom in the circulating plasma would be around 5 μg/mL (lower in real snakebites unless intravascular inoculation occurs).

To simulate the in-vivo condition, plasma was incubated with venom at a concentration of 5 μg/mL; the antivenom-to-plasma ratio used was 1:100, that is, 10 μL of GPVA or AHA to 1000 μL plasma.

This test was performed in duplicate and the mean result was analysed. Venom was added at a concentration of 50 μg/mL to homemade phosphate buffered saline. Then 100 μL (5 μg venom) of this solution was added to 1000 μL of human plasma in plain glass test tubes and mixed for 30 seconds. The final concentration of the testing mixtures was 5 μg venom per mL plasma. Then 10 μL of GPVA or AHA was mixed with the venom/plasma mixture and incubated at 37°C for 45 minutes. The same procedures were performed in controls using 10 μL of saline instead of antivenom. The fibrinogen level was measured after 45 minutes using a Sysmex CA-7000 analyser (Siemens, Germany) with Thrombin Reagent (Clauss assay, Dade; Siemens, Germany).

**Clotting time assay**

The working venom was added at a concentration of 50 μg/mL to homemade phosphate buffered saline. An amount of 100 μL antivenom (GPVA or AHA) was added to 1000 μL of working venom solution. The samples were mixed and incubated at 37°C for 45 minutes. After incubation, one tenth or 110 μL of the antivenom/venom mixture was withdrawn and added to 1000-μL plasma. A final concentration of 5-μg venom per mL plasma mixture was added to a glass test tube and clotting time was measured. The same procedures were performed in controls with 100 μL of saline used instead of antivenom. Fibrin formation (precipitation) was carefully observed and clotting time was recorded. No fibrin clot observed after 360 minutes was recorded as no clot formation (NCF). Theoretically, NCF would indicate that all the clotting activity (thrombin-like effect) of the venom in the plasma had been completely neutralised by the neutralising antibodies in the antivenom.

**Data analysis and statistics**

Continuous variables such as fibrinogen level and clotting time were expressed as means and standard deviations. Analysis of variance (ANOVA) test and post-hoc Tukey’s honest significant difference (HSD) test were used to compare three means. All statistical analysis was performed with the Statistical Package...
Venom was harvested from a total of 46 snakes and pooled together for subsequent testing. There were two bottles containing no venom, that is, dry bite. The total weight and total volume of venom collected was 2.3791 g and 2170 µL, respectively.

**Fibrinogen assay**

As illustrated in Table 1, GPVA showed a higher neutralising capacity against venom than AHA. The measured fibrinogen in the GPVA group (mean ± standard deviation, 2.86 ± 0.52 g/L) was higher than that in the AHA group (1.11 ± 0.23 g/L), and undetectable in the control group, ie 0 g/L. The ANOVA test yielded significant variation between them. Post-hoc Tukey’s HSD test showed that differences in all pairwise comparisons were statistically significant (Table 2).

**Clotting time assay**

The ANOVA was performed for the clotting time of the three groups and yielded significant variation. Post-hoc Tukey’s HSD test showed that all pairwise comparisons were significantly different (Table 2). The mean clotting time in the AHA group was 19.06 minutes, which was significantly longer than the 6.70 minutes in the control group (Table 1). This indicated that venom in the plasma was partly neutralised by the neutralising antibodies in AHA.

The mean clotting time in the GPVA group was >360 minutes, which was significantly longer than that in the AHA group (Table 1). The fulfilment of NCF definition implied that venom in the plasma was completely neutralised by the neutralising antibodies in GPVA.

**Discussion**

Although both belong to the family Viperidae and subfamily Crotalinae, *T. albolabris* and *A. (synonym Gloydius) halys* differ with respect to genus, geographic range, venom composition, and envenoming features. The species *T. albolabris* is endemic to South-East Asia encompassing Thailand, Vietnam, and southern China, including Hong Kong. Its toxins encompass jerdonitin (a metalloproteinase), stejnobin (a fibrinogen clotting enzyme),11 and alboaggregins (the platelet agglutinants).12 They give rise to local swelling and

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**TABLE 1. The effects of green pit viper antivenom (GPVA) and *Agkistrodon halys* antivenom (AHA) on fibrinogen and clotting time assays**

<table>
<thead>
<tr>
<th></th>
<th>Mean ± standard deviation</th>
<th>F</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GPVA</td>
<td>AHA</td>
<td>Control</td>
</tr>
<tr>
<td>Fibrinogen level (g/L)</td>
<td>2.86 ± 0.52</td>
<td>1.11 ± 0.23</td>
<td>0 ± 0</td>
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<tr>
<td>Clotting time (mins)</td>
<td>&gt;360</td>
<td>19.06 ± 1.49</td>
<td>6.70 ± 0.56</td>
</tr>
</tbody>
</table>

**TABLE 2. The association between different antivenoms and testing parameters using Tukey’s honest significant difference test**

<table>
<thead>
<tr>
<th>Antivenom (I)</th>
<th>Antivenom (J)</th>
<th>Mean difference (I-J)</th>
<th>Standard error</th>
<th>P value</th>
<th>95% Confidence interval</th>
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<tr>
<td>Clotting time</td>
<td>GPVA</td>
<td>AHA</td>
<td>340.9*</td>
<td>0.205</td>
<td>&lt;0.0005</td>
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<tr>
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<td>Control</td>
<td>353.2*</td>
<td>0.205</td>
<td>&lt;0.0005</td>
<td>352.81 to 353.78</td>
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<tr>
<td></td>
<td>AHA</td>
<td>GPVA</td>
<td>-340.9*</td>
<td>0.205</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td></td>
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<td>12.35*</td>
<td>0.205</td>
<td>&lt;0.0005</td>
<td>11.87 to 12.85</td>
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<tr>
<td></td>
<td>Control</td>
<td>GPVA</td>
<td>-353.2*</td>
<td>0.205</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td></td>
<td>AHA</td>
<td>GPVA</td>
<td>-12.35*</td>
<td>0.205</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>Fibrinogen level</td>
<td>GPVA</td>
<td>AHA</td>
<td>1.264*</td>
<td>0.174</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>2.570*</td>
<td>0.174</td>
<td>&lt;0.0005</td>
<td>2.1562 to 2.9852</td>
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<td>0.174</td>
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<tr>
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<td>1.305*</td>
<td>0.148</td>
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<td>GPVA</td>
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<td>0.174</td>
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<tr>
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<td>AHA</td>
<td>GPVA</td>
<td>-1.305*</td>
<td>0.148</td>
<td>&lt;0.0005</td>
</tr>
</tbody>
</table>

Abbreviations: AHA = *Agkistrodon halys* antivenom; GPVA = green pit viper antivenom

* The mean difference is significant at the 0.05 level
coagulopathy. The species *A halys* ranges from Russia to northern and central China. Its venom contains metalloproteinase, haemotoxins, and neurotoxins.13 A bite may produce local swelling, ecchymosis, and neurotoxicity, mostly in the form of ptosis, blurred vision, and diplopia.14

Despite the differences in zoology and toxicology between *T albolabris* and *A halys*, AHA has been shown to be more effective than GPVA on a volume basis in the reduction of mouse mortality arising from *T albolabris* envenoming. In an in-vivo study, the intraperitoneal lethal dose 50 (LD₅₀) of *T albolabris* (called Cryptelytrops albolabris in the study but *T albolabris* is the latest name for the same species) was elevated from 0.14 μL to 0.36 μL and 0.52 μL by GPVA and AHA, respectively; and the effective dose 50 was 32.02 μL for GPVA and 6.98 μL for AHA.8 Nonetheless these favourable results for AHA may not be applicable to humans for several reasons. Firstly, haemotoxicity rather than death is the primary concern in *T albolabris* bite. In the above paper, the authors also pointed out the need for further study of clinically relevant toxicities other than mortality.8 Second, studies in animals revealed that the mortality and haemotoxicity outcomes might not correlate with each other. Of the six *Trimeresurus* species including *T albolabris* in Thailand, there was an inconsistent ratio between the LD₅₀ and minimum haemorrhagic dose (MHD).15 An animal study on *T albolabris* venom revealed that GPVA antivenom could neutralise a greater LD₅₀ than Habu antivenom (200 by GPVA, 106 by Habu) and likewise a greater MHD (2000 by GPVA, 750 by Habu).16 Sánchez et al17 tested the efficacy of two antivenoms against LD₅₀ and MHD of different snakes of North America. Within a single species, the relative superiority of one antivenom might apply to only one outcome, ie LD₅₀ or MHD, but not both.

We evaluated the antivenoms on a volume basis in order to simulate the way in which a patient is treated. Evaluation based on molecular weight and contents of proteins, all immunoglobulins or specific immunoglobulins towards venom antigens are alternative methods. Given that GPVA and AHA are supplied in powder form without a dosage-based weight and dissolved in liquid for administration, a volume-based result is deemed more practical for clinical dosing and drug reconstitution.

In human snakebite victims, venom is mostly deposited subcutaneously, not intravascularly. We employed a dose of venom assumed to be higher than that achievable in the plasma of most human snakebite victims for two reasons. First, the primary aim of this study was to compare the relative potency of two antivenoms, therefore a single dose of venom in both antivenom groups was more important than the dose quantity itself. Second, there were inadequate data on the usual venom concentrations, particularly the concentrations associated with coagulopathy, in the circulation of humans bitten by *T albolabris*.

In 1981, Visudhiphan et al18 reported the effect of GPVA on clotting time and fibrinogen level in human plasma exposed to green pit viper (*Trimeresurus*) venom. The venom promoted clotting and depleted fibrinogen level in a dose-dependent fashion. After incubation of the venom with plasma at a concentration of 5 μg/mL (the same concentration employed in our study), clotting time was 12 minutes at 1 hour and a drop in fibrinogen level from that of normal plasma control occurred at 45 minutes. At the same venom plasma concentration (5 μg/mL) and for the same incubation time, GPVA added to plasma in a volume ratio of 1:5 prolonged the clotting time to more than 3 times that of the saline control, and there was failure to correct the hypofibrinogenaemia in 1:20 samples.18 In contrast, we observed a marked antidotal response to GPVA. It is possible that the purity of the antivenom has improved over the intervening years.

There are limitations to our study. First, in addition to its procoagulant and fibrinolytic effects on the coagulation pathway, *T albolabris* venom also affects platelets. Of the patients in a local case series, thrombocytopenia was detected in 29% of cases, not necessarily associated with prolongation of PT or aPTT.4 Future study may consider checking for any thrombocytopenia. Second, laboratory and clinical outcomes may be disparate. In contrast with its inferior clinical performance, the Behringwerke antivenom has been proven to be more effective than the Pasteur antivenom in promoting mouse survival.19 Third, potential inconsistencies in the composition of antivenoms in various batches and venoms derived from individual snakes may affect the applicability of the results in another setting. Nevertheless given the in-vitro benefits of GPVA in antagonising coagulopathy in our study, future trials, particularly in-vivo clinical trials, should be conducted to determine its effect on other clotting parameters and the required dosage. Furthermore, fibrin formation (precipitation) and clotting time were recorded by a single observer who was not blinded to the treatment. This could have introduced information bias.

**Conclusions**

We conducted in-vitro clotting and fibrinogen assays on human plasma to assess the relative therapeutic effects of GPVA and AHA on the haemotoxicity produced by *T albolabris* envenomation. The results indicated a higher potency of GPVA than AHA in neutralisation of the thrombin-like and hypofibrinogenaemic activities of *T albolabris* venom.
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Declaration

All authors have disclosed no conflicts of interest.

References