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Original Article

Development of Enzyme-Linked Immunosorbent Assay and Lateral Flow Strip Assay for *Trimeresurus albolabris* Venom Detection

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Abstract

Background: Snakebite symptoms (eg, neurological signs, local swelling, nonclotting blood) can overlap among different snake types. Accurate venom identification is crucial for selecting the appropriate antivenom against hemotoxic, neurotoxic, or cytotoxic effects. In Thailand, the common snakes *Daboia siamensis, Calloselasma rhodostoma*, and *Trimeresurus albolabris* possess hemotoxic venoms, which can cause symptoms such as pain, swelling, bruising, and bleeding. Although enzyme-linked immunosorbent assay (ELISA) is widely employed for snake venom detection due to its high sensitivity, it is time-consuming. It requires a well-equipped laboratory and specialized skills, whereas the later flow strip assay (LFA) is easy to use and significantly reduces the time required; however, it is typically used for qualitative detection. However, both ELISA and LFA are valuable for snakebite diagnosis. Enhancing the sensitivity, accuracy, and reliability of these assays, particularly for low-abundance targets, remains a critical objective.

Objectives: To develop sandwich ELISA and LFA for detecting *T. albolabris* venom and to enhance the specificity of horse immunoglobulin G (HIgG) against *T. albolabris* venom for use in ELISA and LFA, thereby reducing the likelihood of cross-reactivity in detection.

Methods: Specific HIgG against *T. albolabris* venom was purified using an affinity column. The cross-reactivity of snake venoms was demonstrated through Western blotting. Snake venom detection was quantified by ELISA and visually assessed using LFA.

Results: The sandwich ELISA assay for *T. albolabris* venom detection yielded a coefficient of determination greater than 0.99, a limit of detection at 11.37 ng/mL, and a limit of quantification at 34.45 ng/mL, without any cross-reaction with the venom of *C. rhodostoma* and *D. siamensis*. The LFA can detect *T. albolabris* venom at 25 ng/mL, showing no cross-reaction and no positive test in the test line for either *C. rhodostoma* or *D. siamensis* venom.

Conclusions: The developed sandwich ELISA assay and the LFA could distinguish *T. albolabris* venom from *C. rhodostoma* and *D. siamensis* venom.

Keywords: *Trimeresurus albolabris*, Lateral flow strip assay, ELISA, Snake venoms

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Introduction

Each year, venomous snakebites result in the deaths of approximately 81 410 to 137 880 people worldwide.[1] Snakebites remain a significant public health concern in many tropical and subtropical countries. Southeast Asia is one of the regions most affected by

numerous venomous snakes.[2] In 2007, approximately 700-18 000 people died of venomous snakes in 8 countries of Southeast Asia, including Cambodia, Indonesia, Laos, Malaysia, Myanmar, the Philippines, Thailand, and Vietnam.[3] Thailand is located in the tropics, with lowlands, forests, and mountains, which makes it a place where snakes are abundant.

The effects of snake venoms are broadly classified into hemotoxic, neurotoxic, or myotoxic categories. Snake venoms can cause symptoms such as neurological signs, nonclotting blood/spontaneous systemic bleeding, local swelling, and tissue damage. Sometimes, it leads to permanent disability and limb amputation.[1] Identifying snake species is crucial for clinicians to select an appropriate antivenom and treatment.[5] A doctor will diagnose a patient bitten by an unknown venomous snake based on the expression of the patient's symptoms and laboratory tests.[4] *Daboia siamensis, Calloselasma rhodostoma, and Trimeresurus albolabris* are commonly found in Thailand, and their venoms affect the blood system, classified as hemotoxic venoms. Pain, swelling, blistering, bruising, nausea, vomiting, and bleeding are common symptoms following a bite from these snakes. Due to their overlapping symptoms, it may cause diagnostic confusion; however, a *D. siamensis* bite can be confirmed by testing Factor V and X levels. Besides, a bite by *T. albolabris*, known as the White-lipped Pit Viper, which is found throughout Thailand, might be mistakenly considered a cobra, *Naja kaouthia*, bite because of their similar swelling and inflammation around the wound,[6-8] in the case that neurological symptoms have not appeared. Therefore, it is beneficial to test for snake type confirmation.

Immunological tests for detecting snake venom have been identified as important clinical applications. Snake venom detection by enzyme-linked immunosorbent assay (ELISA) is currently in use worldwide. It provides specificity and sensitivity to detect and identify differentiated envenomation even with small venom quantities. Although the ELISA reagent is inexpensive and stable, it is time-consuming and requires a well-equipped laboratory and skills.[9] The same or similar proteins in snake venoms were found among the closely related snake species, referred to as cross-reactions, in the ELISA tests for snake venom.[10] The indirect ELISA test used to diagnose snakebites for *Bungarus multicinctus* and *Naja atra* (neurotoxic snake venoms) demonstrated strong cross-reactivity with the venoms of *Trimeresurus stejnegeri* and *Protobothrops mucrosquamatus*, which are hemotoxic snake venoms. Conversely, ELISA tests for *T. stejnegeri* and *P. mucrosquamatus* showed slight cross-reactivity with the venoms of *B. multicinctus* and *N. atra*.[11] Cross-reactivity tests with ingroup and outgroup samples are crucial for validating the specificity of ELISA. It is essential to ensure that antibodies target the correct antigen, thereby minimizing false positives and enhancing the test's reliability. In case of *T. albolabris* venom detection, *N. kaouthia*, a neurotoxic snake venom with distinct toxin profiles and mechanisms compared to hemotoxic snake venom, can serve as an outgroup sample, whereas *C. rhodostoma* and *D. siamensis* venoms are considered to be ingroup samples. These snake venoms can be utilized to evaluate whether the assay can detect *T. albolabris* venom without cross-reactivity, thereby enhancing the test's accuracy and reliability. To reduce or eliminate cross-reactivity and improve the discrimination capability of ELISA for the accurate identification of snake species, protein purification using immobilized venom proteins that cross-react with the relevant antibodies through affinity columns may be helpful.[12] Although the ELISA test has several advantages and is used for various applications, the later flow strip assay (LFA) is easier to use as a point-of-care test. Indeed, LFA requires minimal personal training to interpret results, with lower infrastructure needs in healthcare and the ability to provide quick outcomes for immediate decision-making, compared to ELISA.

Unfortunately, the complexities of snake venom have resulted in fewer efforts in Thailand to develop sensitive assays for its detection. Therefore, this study aimed to develop a sandwich ELISA and LFA for detecting *T. albolabris* venom. Moreover, affinity column chromatography was employed to enhance the specificity of HIgG against *T. albolabris* venom for use in ELISA and LFA, thereby reducing the likelihood of cross-reactivity in detection.

Methods

Snake Venoms, Hyperimmune Horse Plasma, and Normal Human Serum

The freeze-dried powder of *D. siamensis*, *C. rhodostoma*, *T. albolabris*, and *N. kaouthia* venom was obtained from the Queen Saovabha Memorial Institute (QSMI) Snake Farm, Thai Red Cross Society, Bangkok, Thailand (stored at 4 °C). Hyperimmune horse plasma against *T. albolabris* venom obtained from the horse farm, QSMI, Thai Red Cross Society, Prachuap Khiri Khan, Thailand. The plasma was stored at -20 °C before use. Pooled normal human serum was purchased from Sigma, USA (collected from healthy human donors) and stored at -20 °C before use.

Monovalent Antivenom for *Trimeresurus albolabris* Venom (Green Pit Viper Antivenom)

Monovalent antivenom against *T. albolabris* venom was obtained from QSMI. It was prepared from equine serum. Each 1 mL contains specific immunoglobulin that can neutralize 0.7 mg of Green Pit Viper venom.

Horse Immunoglobulin G (HIgG)

To obtain HIgG, 50 mL of hyperimmune horse plasma against *T. albolabris* venom was precipitated with 35% ammonium sulfate at 4 °C for 30 minutes. Then, it was centrifuged at 5000 rpm for 30 minutes and dialyzed overnight against 2 liters of binding buffer (10 mM Tris-HCl, pH 7.5) at 4 °C.

Preparation of an Affinity Column for Purifying HIgG Against *Trimeresurus albolabris* Venom

CNBr-activated Sepharose 4B was used to purify the specific antibody, as it enables the highly selective and efficient isolation of the target antibody from complex mixtures, such as serum or horse plasma, by covalently binding it to the Sepharose beads. As such, 1 g of CNBr-activated Sepharose 4B (per venom sample) (Cytiva, USA) medium was weighed and washed with 200 mL of 1.0 mM HCl (pH 3.0) using a sintered glass filter (porosity G3) and then washed with coupling buffer (0.1 M $NaHCO_3$, pH 8.3). Each of *D. siamensis*, *C. rhodostoma*, and *T. albolabris* venom (freeze-dried powder) was weighed at 10 mg separately and dissolved in 5 mL of coupling buffer. Each dissolved venom was added to swollen Sepharose 4B medium and incubated overnight at 4 °C. Then, each venom medium was washed several times with a coupling buffer on a sintered glass filter. Blocking any remaining active groups, each venom medium was transferred to 0.1 M Tris-HCl buffer, pH 8.0, and left to stand for 2 hours at room temperature. Washing the medium with at least 3 cycles of alternating pH by at least 5 medium volumes of each buffer on a sintered glass filter. Each cycle consisted of a wash with 20 mL of 0.1 M acetic acid/sodium acetate, pH 4.0, containing 0.5 M NaCl, followed by a wash with 20 mL of 0.1 M Tris-HCl, pH 8, containing 0.5 M NaCl. Then, each medium venom was packed into a column and equilibrated with a binding buffer (10 mM Tris-HCl, pH 7.5).

Purification of Specific HIgG Against *Trimeresurus albolabris* Venom

The 10-mg HIgG in 10 mM Tris-HCl at pH 7.5 was administered into the *C. rhodostoma* venom Sepharose 4B column, as the first column, that had been equilibrated with binding buffer and washed with binding buffer (2.5 column volumes [CV]). Then, the column was eluted with a stepwise gradient of 0.1 M glycine, pH 2.7, from 0% to 100% (4 CV). Eluted fractions were pooled and dialyzed overnight with 2 liters of binding buffer. HIgG obtained from the first column was concentrated and passed through the second column, the *D. siamensis* venom Sepharose 4B column, followed by the third, *T. albolabris* venom Sepharose 4B column. The purification method steps in the second and third columns were identical to those in the first. The specific HIgG for *T. albolabris* venom was dialyzed in phosphate-buffered saline (PBS), pH 7.3. Before use, the specific HIgG for *T. albolabris* venom was stored at -20 °C. The AKTA pure (GE, USA) system performed all purification steps at a flow rate of 0.5 mL/min. The purified specific HIgG to *T. albolabris* venom was displayed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Western Blot Analysis Between Antivenom (QSMI) and HIgG to *Trimeresurus albolabris* Venom

Accordingly, 20 µg of each snake venom (*D. siamensis*, *C. rhodostoma*, and *T. albolabris*) was processed using 12% SDS-PAGE. After electrophoresis, proteins were transferred from the gel onto polyvinylidene difluoride (PVDF) blotting membranes (GE, USA) using electroblotting. The membrane was blocked from nonspecific binding sites by incubating it with a blocking buffer (3% BSA) for 1 hour. Then, the monovalent antivenom (QSMI) or the specific HIgG at the final concentration of 0.03 mg/mL in 3% BSA buffer was added to the membrane and incubated overnight at 4 °C. The membrane was washed several times with Tris-buffered saline containing 0.1% Tween 20 (TBST) to remove unbound antibody. The anti-horse IgG conjugated to HRP (Sigma, USA) was diluted 1:1000 in 3% BSA buffer and added, followed by incubation for 1 hour at room temperature. The membrane was washed with TBST, and the substrate solution, consisting of 4-chloro-1-naphthol (Sigma, USA) and hydrogen peroxide (H_2O_2), was applied to the blotting membrane to develop the target bands.

Conjugation of HIgG Against *Trimeresurus albolabris* Venom With Horseradish Peroxidase (HRP)

The processing was followed by the HOOK™ HRP PLUS labeling kit (G-Biosciences, USA). Briefly, 1 mg/ml of HIgG against *T. albolabris* venom was coupled with HRP in HOOK™ HRP PLUS vial using the conjugation reagent given by the kit. Then, 5 M sodium cyanoborohydride was added to the vial and incubated at room temperature for 15 minutes. After that, the quenching buffer was added to the vial, and the mixture was incubated with shaking for 15 minutes. The HIgG solution from the previous step was entered into the SpinOUT™ GT-600 column to remove sodium cyanoborohydride. To collect the conjugate of HIgG against *T. albolabris* venom, the column was eluted with PBS, pH 7.3. The HRP-conjugated HIgG against *T. albolabris* venom was aliquoted and stored at -20 °C before use.

Sandwich ELISA Assays for *Trimeresurus albolabris* Venom Detection

The specific HIgG for *T. albolabris* venom detection (2 µg/mL, 50 µL) was diluted in coating buffer (0.1 M sodium carbonate, pH 9.5) and placed on 96-well polystyrene microplates (Costar™ 96-Well, USA). The plates were then incubated for 3 hours at 37 °C.

After that, the wells were washed 3 times with PBS, pH 7.3, containing 0.05% Tween 20 (PBST), using a microplate washer (Hydro Flex, Tecan, USA). The wells were then blocked with 1% BSA in PBS for 30 minutes at 37 °C. Then, the wells were rewashed. To establish a standard concentration curve, 2-fold serial dilutions of *T. albolabris* venom in human serum, ranging from 7.8 to 500 ng/mL, were prepared in 50 µL volumes and added to the wells. After incubation at room temperature for 30 minutes, the plate was washed and supplemented with 50 µL of HRP-HIgG to *T. albolabris* venom (1:5000 in PBS, pH 7.3), followed by incubation at 37 °C for 30 minutes. Next, the plate was washed, and the substrate of peroxidase, O-phenylenediamine (OPD), and 35% H_2O_2 (100 µL) were added. The mixture was incubated in the dark at room temperature for 30 minutes. Finally, 50 µL of 3 M H_2SO_4, serving as a stopping reagent, was added to the wells to terminate the OPD reaction. The specific bound complex was detected by optical density (OD) measurement at 492 nm, with a reference at 620 nm, using a microplate reader (Sunrise, Tecan, USA). The determination of the limit of detection (LOD) was calculated based on the standard deviation of the response (Sy) of the curve and the slope of the calibration curve (S) at levels approximating the LOD, as follows: LOD = 3.3 × (Sy/S). The limit of quantification (LOQ) was calculated based on the standard deviation of the response (SD) and the slope of the calibration curve (S) according to the formula: LOQ = 10 × (Sy/S), where the standard deviation of the response was determined based on the standard deviation of the y-intercepts of regression lines. The spiking experiment assessed known concentrations of *T. albolabris* venom at 31.25, 62.5, 125, and 250 ng/mL to determine whether variations in the diluent of the standard curve influenced analyte detection. To investigate the cross-reaction of the specific HIgG against *T. albolabris* venom, not only *D. siamensis* and *C. rhodostoma*, which served as ingroup samples, but also *N. kaouthia*, which served as an outgroup sample, was utilized at high concentrations of 250, 500, and 1000 ng/mL.

Colloidal Gold-Labeled HIgG to *Trimeresurus albolabris* Venom Preparation

The 40 nm gold nanoparticle solution (OD = 1) from Serve Science Company, Bangkok, Thailand, was adjusted to a pH of 8.0 with 0.2 M sodium carbonate. The activated gold solution was mixed in a 1:1 (v/v) ratio with the HIgG to *T. albolabris* venom at a concentration of 2 mg/mL in PBS, pH 7.3. The reaction was incubated for 10 minutes at room temperature with gentle shaking or rotation to promote the binding of the antibody to the gold particles. After conjugation, 0.5% BSA was added to block any unreacted sites on the nanoparticle surface, and the mixture was incubated for an additional 15 minutes to prevent nonspecific binding. Subsequently, the gold solution was centrifuged at 10 000 rpm and 4 °C for 30 minutes, and the gold pellets were collected. The gold pellets were then suspended in PBST containing 1% BSA, and this step was repeated once. Finally, the gold-labeled HIgG pellets were suspended in 1 mL of storage buffer (20 mM sodium carbonate, pH 8.0, 2% sucrose, 5% trehalose, 0.1% sodium azide) and stored at 4 °C until use. The method was modified based on Kumar et al.[12]

Lateral Flow Strip for *Trimeresurus albolabris* Venom Detection

The strips consisted of nitrocellulose membranes (Unisart CN140), sample pads (Ahstrom 8964), conjugate pads (GF33 Glass Fiber), and absorbent pads (Ahlstrom 222). Before assembly, the conjugate pads were saturated with 10 OD of HIgG against *T. albolabris* venom-conjugated colloidal gold, sprayed (10 µL/cm) using a sprayer machine

(XYZ3060, BioDot, USA), and allowed them to dry for an hour at 37 °C. In the test line, 1 mg/mL of HIgG against *T. albolabris* venom in PBS, pH 7.3, was sprayed (1 µL/cm) on nitrocellulose membranes. For the control line, 1 mg/mL of an anti-horse IgG whole molecule (Sigma, USA) in PBS, pH 7.3, was also sprayed (1 µL/cm). The membrane was dried at 37 °C for 1 hour before assembly. The nitrocellulose membranes, conjugated pads, sample pads, and absorbent pads were pasted onto the cardboard, with each pad overlapping the adjacent ones. A cutter machine (CM5000, BioDot, USA) cut the assembled strips into pieces measuring 0.4 x 6.0 cm each. Sensitivity and specificity of the lateral flow strips were tested. The spiked *T. albolabris* venom in pooled human serum, at concentrations of 12.5, 25, 50, and 100 ng/mL, was applied to the sample pads (n = 5) in a volume of 60 µL. The results were demonstrated within 15 minutes at room temperature. The lateral flow strip for *T. albolabris* venom detection was also examined for reaction with the venom of *D. siamensis*, *C. rhodostoma*, as ingroup samples, and *N. kaouthia* venom as an outgroup sample (n = 5). The intensity of the test line and control line was measured using a RapidScan ST5 (USA) without a measurement unit.

Protein Concentration

In all experiments, the Qubit Protein kit (Thermo Fisher Scientific, USA) was used for measuring protein concentration.

Statistical Analysis

Data were presented as mean (SE) and analyzed using one-way analysis of variance, followed by Bonferroni's multiple comparisons tests with PRIMER of Biostatistics software, version 6.0. Differences were considered significant at $P < .05$.

Results

Western Blot Analysis Between Antivenom (QSMI) and HIgG to *Trimeresurus albolabris* Venom

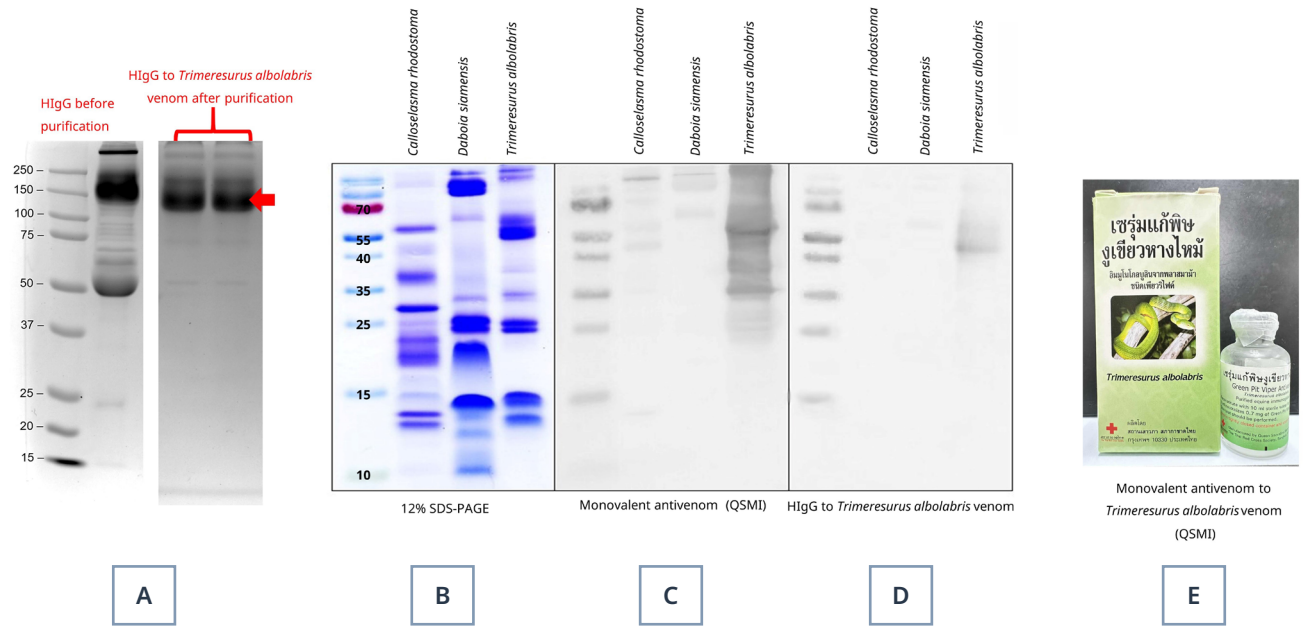
Specificity evaluation of the purified HIgG to *T. albolabris* venom was determined by Western blot analysis compared to monovalent antivenom, and the cross-reaction among hemotoxic snake venoms was considered, as well. As such, HIgG against *T. albolabris* venom was more specific to *T. albolabris* venom (Figure 1D) than monovalent antivenom (QSMI), which showed a high cross-reaction in both *C. rhodostoma* and *D. siamensis* venom (Figure 1C). However, the result of purified HIgG against *T. albolabris* venom showed slightly protein bands of *D. siamensis* venom at high molecular weights above 55 kDa. In contrast, the *C. rhodostoma* venom lane was nearly invisible (Figure 1D). The Western blot analysis revealed that the purified specific HIgG against *T. albolabris* venom exhibited less cross-reaction with *C. rhodostoma* and *D. siamensis* venom than the monovalent antivenom (QSMI).

Sandwich ELISA Assays for *Trimeresurus albolabris* Venom Detection

The conjugated HRP-HIgG against *T. albolabris* venom (a capture antibody) and OPD with 35% H_2O_2 (a detection antibody) were used to determine the sensitivity and specificity of the ELISA assay. Parallelly, pooled human serum was serially diluted to test for the presence of *T. albolabris* venom, cross-reactivity to *C. rhodostoma* and *D. siamensis* venom, and to generate a standard curve. For *T. albolabris* venom detection, LOD was 11.37 ng/mL, and LOQ was 34.45 ng/mL, with a coefficient (R^2) value greater than 0.99 (Figure 2A), with

the potential to identify and quantify *T. albolabris* venom in pooled human serum. Notably, the LOQ was passed for the standard criteria (identification, precision, and trueness), supporting the relative accuracy and precision of the assay.[13] The use of *T. albolabris* venoms (31.25, 62.5, 125, and 250 ng/mL) in a spike experiment to find the percentage recovery, recovery (%) = (observed concentration at dilution/predicted spike concentration after dilution) × 100, was performed. An acceptable recovery range of 80% to 120% signifies the absence of any matrix effect,[14] whereas a recovery percentage outside this range suggests potential interference from sample components. All concentrations of *T. albolabris* venoms were in the acceptable range of the % recovery (Figure 2B). Meanwhile, the HIgG against *T. albolabris* venom showed no cross-reactivity with the venoms of *C. rhodostoma* and *D. siamensis* at concentrations ranging from 0 to 1000 ng/mL. However, slight cross-reactivity was observed at higher concentrations of *N. kaouthia* venom at 500 and 1000 ng/mL, but not at the lower concentration of 250 ng/mL (Figure 2C).

Figure 1. Purification of HIgG Against *Trimeresurus albolabris* Involved Western Blot Analysis Using Hemotoxic Snake Venoms, Alongside Monovalent Antivenom (QSMI) and HIgG Specific to *Trimeresurus albolabris* Venom



Abbreviations: HIgG, horse Immunoglobulin G; QSMI, Queen Saovabha Memorial Institute; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

A, The crude HIgG (20 µg per well) was analyzed before and after the purification of HIgG against *T. albolabris* venom, with duplicate samples (10 µg per well each) run on a 10% SDS-PAGE under nonreducing conditions.

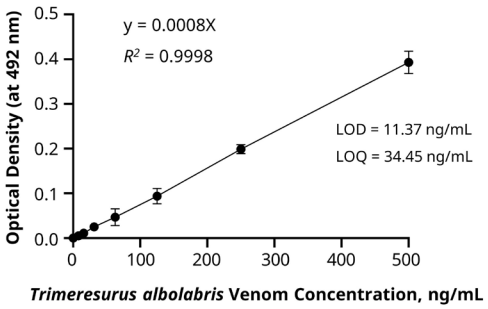
B, The Western blot analysis of crude snake venoms on 12% SDS-PAGE.

C and D, The Western blot analysis along with monovalent antivenom (QSMI) and HIgG against *T. albolabris* venom was performed using hemotoxic snake venoms (20 µg per well for each), *C. rhodostoma*, *D. siamensis*, and *T. albolabris* venoms.

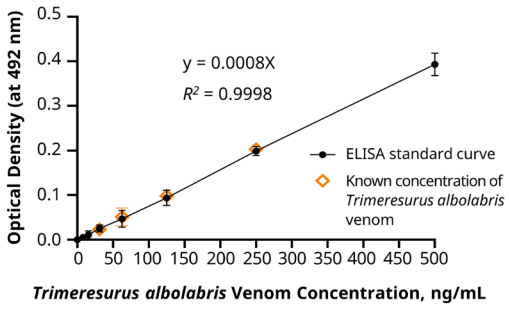
E, The commercially available green pit viper antivenom product from QSMI was displayed.

Figure 2. Development of Sandwich ELISA Assay to Detect *Trimeresurus albolabris* Venom, Including Spiking Tests With Pooled Human Serum and an Assessment of the Cross-Reactivity of HIgG With *Trimeresurus albolabris* Venom

A

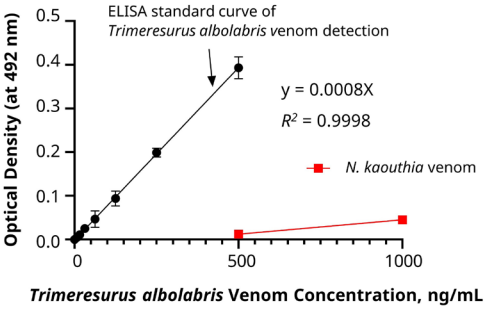


B



Concentration of *Trimeresurus albolabris* Venom on the Standard Curve, ng/mL	Spiking test of *Trimeresurus albolabris* Venom, Mean (SE), ng/mL (n = 3)	% Recovery
250	253.33 (0.011)	98.82
125	122.50 (0.012)	98.00
62.5	63.33 (0.020)	101.33
31.25	29.17 (0.005)	93.34

C



Concentration, ng/mL	Cross-Reactivity to HIgG *Trimeresurus albolabris* Antibody, Mean (SE), ng/mL (n = 3)		
	Calloselasma rhodostoma Venom	*Daboia siamensis* Venom	*Naja kaouthia* Venom
250	Under the cut-off value	Under the cut-off value	Under the cut-off value
500	Under the cut-off value	Under the cut-off value	15.00 (0.006)
1000	Under the cut-off value	Under the cut-off value	56.67 (0.010)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HIgG, horse Immunoglobulin G; LOD, limit of detection; LOQ, limit of quantification.

A, The standard curve of ELISA for *T. albolabris* venom.

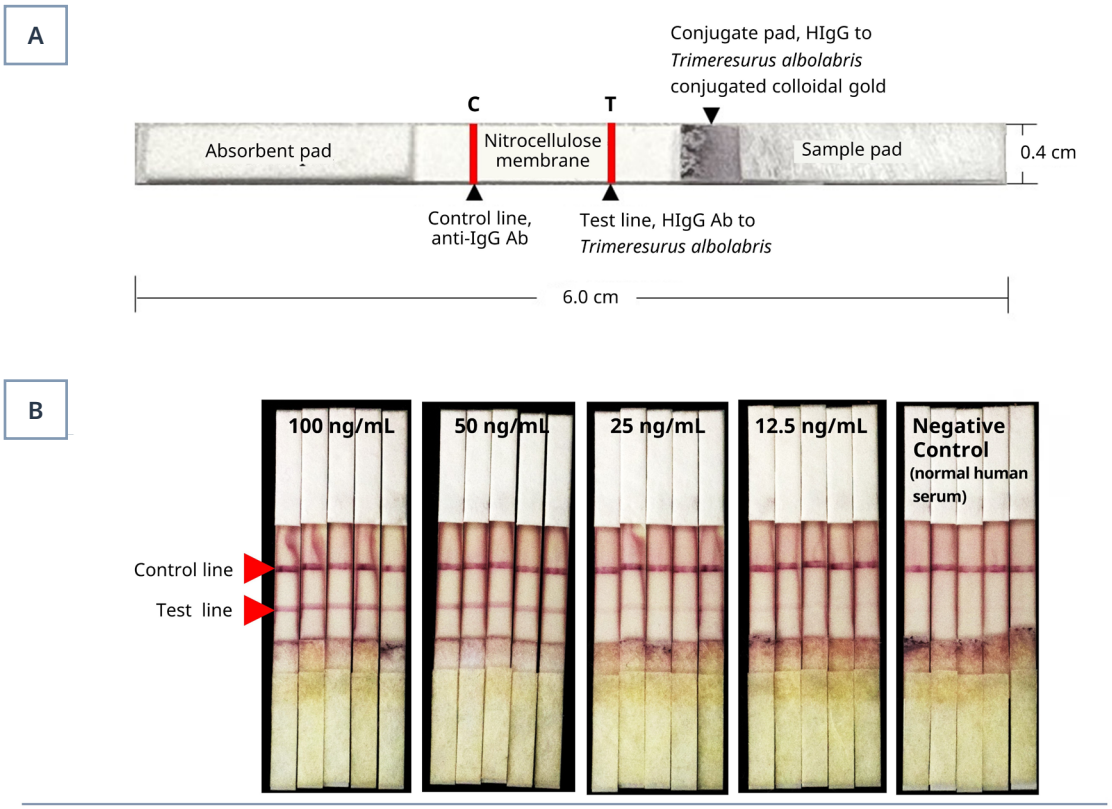
B, The *T. albolabris* venom spiking test using pooled human serum.

C, The cross-reactivity of the HIgG against *T. albolabris* venom with related snakes.

Lateral Flow Strip Test for *Trimeresurus albolabris* Venom Detection

A lateral flow strip for *T. albolabris* venom detection, which was composed of a sample pad, conjugated colloidal gold pad, absorbent pad, and nitrocellulose membrane with HIgG against *T. albolabris* venom (test line; T), and anti-IgG antibody (control line; C), was produced (Figure 3A). Sensitivity and specificity tests using various concentrations of *T. albolabris* venom in pooled human serum, along with a visual inspection of the positive test line, were clear at concentrations above 25 ng/mL but ambiguous at 12.5 ng/mL (Figure 3B). The pooled normal human serum (undiluted), serving as a negative control test, showed a low-intensity line (0-75) (Table in Figure 3B). The test line intensity for venom detection correlated well with the venom concentrations (Table in Figure 3B). To evaluate cross-reactivity, lateral flow strip tests were used to test the venom of *D. siamensis* and *C. rhodostoma*, which had hemotoxic venoms (similar to *T. albolabris*), and *N. kaouthia* (outgroup), a neurotoxic snake.

Figure 3. Development of Lateral Flow Strip Tests to Detect *Trimeresurus albolabris* Venom



Venom of *Trimeresurus albolabris*	100 ng/mL	50 ng/mL	25 ng/mL	12.5 ng/mL	Negative Control
Test line intensity, range	1544-2291	1375-1546	462-917	317-482	0-75

A, The design of a lateral flow strip for detecting *T. albolabris* venom.

B, The results of detecting *T. albolabris* venom on the strips using various venom concentrations.

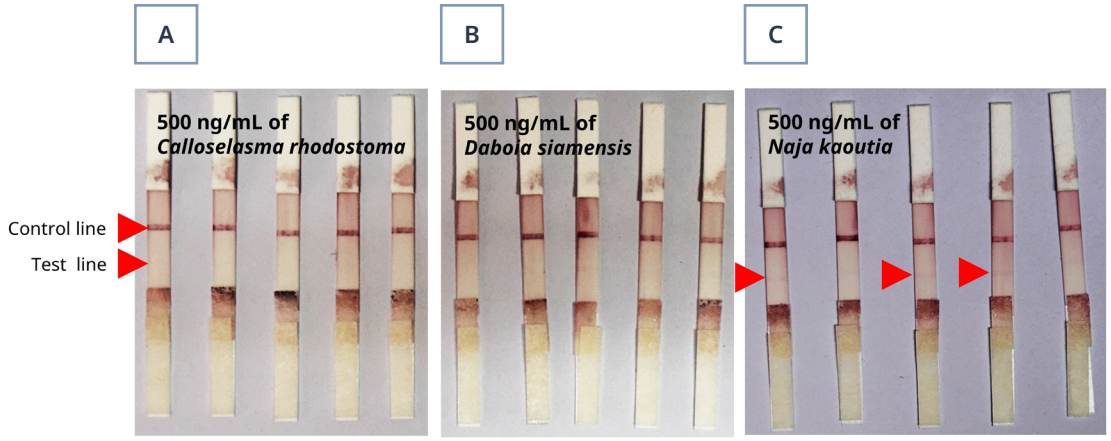
The lateral flow strip test on *C. rhodostoma* and *D. siamensis* venom yielded negative results in the test lines at concentrations of 500 ng/mL (Figure 4A and 4B), suggesting that the developed strip assay did not exhibit sufficient cross-reactivity to produce unclear results at high-dose concentrations. At a high concentration of 500 ng/mL, slight cross-reactivity was observed on the test line for *N. kaouthia* venom (Figure 4C).

When *C. rhodostoma* and *D. siamensis* venoms on the test line intensity were measured, it was found that *C. rhodostoma* venom gave intensity range values at 0-34 (Figure 4A), while *D. siamensis* venom scores were 0-87 (visually negative) (Figure 4B). At 500 ng/mL, we observed some cross-reactivity on the test line for *N. kaouthia* venom, resulting in an intensity range of 176-275 (indicated by the red arrow in Figure 4C). The visually detectable intensity surpassed 100.

Discussion

ELISA, a valuable tool for diagnosing snake envenomation by detecting the presence of specific snake venoms in blood, relies on antibodies that specifically react with venom proteins and hospital laboratory machines. Meanwhile, lateral flow strips make it easier to identify envenomation without requiring sophisticated equipment and less trained personnel for field use.

Figure 4. Evaluation of Cross-Reactivity Using Lateral Flow Strip Tests With Hemotoxic Snake Venoms



Venom (500 ng/mL)	*Calloselasma rhodostoma*	*Daboia siamensis*	*Naja kaoutia*
Test line intensity, range	0-34	0-87	176-275

A and B, The cross-reactivity detection on the lateral flow strip tests using hemotoxic snake venoms (*C. rhodostoma* and *D. siamensis*).

C, Neurotoxic snake venom (*N. kaouthia*) at 500 ng/mL.

T. albolabris bites cause local and systemic envenomation, producing painful and similar local symptoms to those of *C. rhodostoma*, *D. siamensis*, and *N. kaouthia*, including local swelling (the dominant symptom), hemorrhage formation, local damage and necrosis, which can be challenging to distinguish from those of these snake species. Although *C. rhodostoma* often causes multiple haemorrhage blebs, *D. siamensis* frequently forms blisters around the wound with necrosis, and *N. naja* is neurotoxic,[6-8] the more rapid snake identification provides a better treatment outcome. Indeed, common antigens in venom are structurally or functionally similar across multiple snake species, often leading to some degree of cross-reactivity between the venoms. Minimizing venom cross-reactivity in ELISA assays is challenging, especially for those within the same family.

To isolate the specific HIgG that recognized *T. alborabris* venom and reduced cross-reactivity, crude HIgG was purified using an affinity column bound separately with *C. rhodostoma* and *D. siamensis* venom. After purification, the specific HIgG for *T. alborabris* venom primarily comprised proteins with an approximate molecular weight of 150 kDa, with only a minimal presence of proteins of lower molecular weights (Figure 1A). Indeed, the cross-reactivity of a single-valent *T. albolabris* antivenom (QSMI) toward *C. rhodostoma* and *D. siamensis* venoms was demonstrated through Western blot analysis (Figure 1B), especially in high molecular weight fractions. With affinity purification using *C. rhodostoma* and *D. siamensis* medium columns, a better specificity of anti-*T. albolabris* HIgG was shown by Western blot (Figure 1C). Although the cross-reactivity limits the use of antibodies for snake identification, it was beneficial for neutralizing venoms from several types of snakes. For example, anti-VIPMYN (Fab2H) fraction from *Crotalus durissus* and *Bothrops asper* (North American snakes) was effective in neutralizing the hemorrhagic activity of 8 venoms (*Agkistrodon piscivorus piscivorus*, *B. asper*, *Crotalus adamanteus*, *C. durissus durissus*, *C. horridus atricaudatus*, *C. h. horridus*, *C. atrox*, and *C. molossus molossus*).[15] However, cross-reactivity may not cover all fractions of the venoms, such as anti-FabO from *C. atrox*, *C. adamanteus*, *C. scutulatus scutulatus*, and *A. p. piscivorus* neutralized only the gelatinase activity of *A. p. piscivorus*, *C. d. durissus*, and *C. m. molossus* venoms.[16]

With a more specific antibody against *T. albolabris* venom, the LOD from the ELISA standard curve met the criteria for identification, precision, and accuracy above the background noise.[13] The primary purpose of LOD is to confirm the presence of a presenting analyte without precisely measuring its concentration. The LOQ, at 34.45 ng/mL, indicated that the ELISA was assayed with relative accuracy and precision at 34.45 ng/mL (Figure 2A) and could be confidently reported as a reliable value. Knowing the quantifiable range from LOQ can help guide sample preparation decisions to fall within the assay's accurate range, improving result reliability. In ELISA, measuring the LOQ sets the lowest point of accurate quantification, which is essential in sensitive research and diagnostic settings. Due to HRP enzyme labelling in this study, which gave LOQ of 34.45 ng/mL, the biotin-streptavidin might enhance sensitivity (reduced LOQ value) as indicated in the ELISA against neurotoxic (*B. multicinctus* and *N. atra*) and hemotoxic venoms (*T. stejnegeri* and *P. mucrosquamatus*) at the LOQ of 0.39 and 0.78 ng/mL, respectively.[11] The higher sensitivity of the biotin-streptavidin system over HRP-based direct conjugates is partly due to the multivalent binding ability.[17, 18] Each biotinylated antibody can bind multiple streptavidin molecules, leading to multivalent signal amplification and enabling more enzyme molecules per target site. In contrast, HRP is usually directly conjugated to the primary or secondary antibody, meaning each antibody carries only one or a limited number of HRP molecules. This single-layered approach limits the total signal that can be produced. However,

HRP is known for its simplicity, cost-effectiveness, and effectiveness in detection. It is also widely used for standard assays with acceptable sensitivity for many substrates. Therefore, the sensitivity of the developed ELISA may be enhanced by biotin-streptavidin conjugation.

Additionally, the recovery percentage (% recovery) is used to evaluate the accuracy of ELISA by relying on the known concentration of the analyte that has been spiked into the sample. Here, the recovery percentage ranged from 93.4% to 101.33% (Figure 2B), indicating that the detection was acceptable. Notably, a good recovery percentage indicates the validity and reliability of ELISA. A recovery percentage within an acceptable range (typically 80%-120%) suggests minimal interference.[14] Here, our ELISA distinguished *T. albolabris* from *C. rhodostoma* and *D. siamensis* venoms, even at the high concentration of 1000 ng/mL, without the cross-reactivity to the *T. albolabris* HIgG. The OD at 429 nm from *C. rhodostoma* and *D. siamensis* venoms was lower than the background cut-off from pooled human sera (negative result). However, there was a slight cross-reactivity with *N. kaouthia* venom (a neurotoxic snake venom) at a concentration of 500 ng/mL or higher (Figure 2C). To reduce cross-reactivity with *N. kaouthia* venom, further purification using *N. kaouthia*-based affinity columns will enhance the specificity of HIgG against *T. albolabris* venom.

Despite the high specificity and sensitivity of our sandwich ELISA assay, the waiting time may be too long for practical use. Therefore, we created a lateral flow strip (a sandwich-based immune strip) using 40 nm gold nanoparticles (the larger gold nanoparticles exhibit more potent plasmonic properties), which could be easily detected by a color change due to enhanced optical properties. Besides, gold nanoparticles are more stable in solution than the smaller particles and are easily aggregable.[19] The specificity and sensitivity of our lateral flow strip, using the diluted *T. albolabris* venoms at 12.5, 25, 50, and 100 ng/mL in pooled human serum, were tested to see if the high viscosity of the serum could cause nonspecific binding or background noise on the test strip. Although there was low noise in our lateral flow test (no visible band by serum alone), the interference was easily reduced by diluting the serum in real clinical use with several strips in the same tests.[11] However, this strip test was possibly more suitable for the undiluted serum sample because the sample pad (Ahlstrom 8964) allows liquid samples to move across the membrane with a high flow rate (80-135 seconds per 4 cm).

A report investigating the presence of green pit viper venom in the bloodstream of snakebite victims found that the average half-life of the venom was approximately 27.5 hours during the first 3 days and extended to over 50 hours between days 5 and 7 post-bite. Notably, in about 14.8% of patients, venom remained present in the bloodstream up to day 14, and this persistent antigenaemia was associated with prolonged thrombocytopenia and coagulopathy.[20] The study on Russell's viper (*D. russelii*) venom levels in the serum of snakebite victims in Burma assessed venom concentrations using an ELISA. Serum venom levels ranged from less than 10 ng/mL to 290 ng/mL before antivenom treatment. Following antivenom administration, venom levels significantly decreased; however, residual venom was still detected in some cases. For example, one patient exhibited 11.5 ng/mL of venom 66 hours after receiving antivenom. Fatal cases had serum venom levels of 95 ng/mL and 185 ng/mL.[21]

In the LFA against *B. atrox* and *L. muta* venoms, spiked plasma and urine levels were detected at 10-50 ng/mL.[22] Additionally, the LFA identified hemotoxic snake venoms (*T. stejnegeri* and *P. mucrosquamatus*) in human serum at concentrations below 50 ng/mL, within 15 minutes.[11] The developed LFA demonstrated that the lowest concentration of

T. albolabris venom that could be visually detected was 25 ng/mL (Figure 3B), showing a slight cross-reaction with a high level of *N. kaouthia* venom (Figure 4C), but no cross-reaction with *C. rhodostoma* or *D. siamensis* venoms (Figure 4A and 4B). The cross-reactivity observed in ELISA and LFA between *T. albolabris* venom and *N. kaouthia* venom may be attributed to similarities in their protein components. Both venoms contain proteins that could share homologous epitopes, regions recognized by antibodies. These shared epitopes might cause antibodies developed for one venom to bind to components of the other, resulting in false positives or reduced assay specificity.

Conclusions

The sandwich ELISA assay demonstrated its ability to detect *T. albolabris* venom, with a LOD of 11.37 ng/mL and LOQ of 34.45 ng/mL. Additionally, the 3-column purification could enhance the specificity of HIgG against *T. albolabris* venom, which exhibits no cross-reactivity with the venoms of *C. rhodostoma* or *D. siamensis* venom, both of which belong to the same group of hemotoxic snake venoms. However, a slight cross-reaction with a high concentration of *N. kaouthia* venom occurred at levels of 500 ng/mL or higher, which was not present at 250 ng/mL.

The extended processing time of ELISA presents challenges for practical applications. To address this limitation, LFA was developed for rapid venom detection, utilizing enhanced optical properties and stability. The LFA could detect *T. albolabris* venom at 25 ng/mL, distinguishing it from the venoms of *C. rhodostoma* and *D. siamensis*, without producing false-positive test lines. However, a slight cross-reaction was observed at the test line when the sample was tested with 500 ng/mL of *N. kaouthia* venom.

Further optimization of the sandwich ELISA and LFA is necessary, particularly through validation with clinical samples from patients who have received snakebites. Despite this, the study highlights the feasibility of developing ELISA and LFA for venom detection in Thailand, marking an important step toward improving diagnostic tools for snakebite management.

Additional Information

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Ethics Approval: To clarify the ethical considerations of this study, we confirm that the research was approved by the Institutional Review Board of the Queen Saovabha Memorial Institute (QSMI-ACUC-01-2024 on 31 January 2024). This approval pertains explicitly to the current research involving the use of snake venom and hyperimmune horse plasma. This study did not involve testing in animal models or human subjects. Pooled normal human serum used in this study was obtained commercially from Sigma-Aldrich (USA).

Clinical Trial Consideration: This study does not report on a clinical trial.

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