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Original Article

Experimental Manufacture of Equine Antivenom againt Yamakagashi (*Rhabdophis tigrinus*)

Kazunori Morokuma*, Norihiro Kobori, Tadashi Fukuda[1], Tetsuya Uchida[1], Atsushi Sakai[2], Michihisa Toriba[2], Kunio Ohkuma, Kiyoto Nakai[3], Takeshi Kurata[1,4], and Motohide Takahashi[1]

Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN), Kumamoto 860-8568;
[1]National Institute of Infectious Diseases, Tokyo 208-0011;
[2]Japan Snake Institute, Gunma 379-2301;
[3]Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Tokyo 100-8916; and
[4]Toyama Institute of Health, Toyama 939-0363, Japan

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SUMMARY: Yamakagashi, *Rhabdophis tigrinus*, is a natricine snake widely distributed in eastern Asia. Severe bite cases, some with fatal outcomes, occur regularly in Japan. Because previous production of *R. tigrinus* antivenom in rabbits and goats was quite effective, we considered the experimental manufacture of a new antivenom against *R. tigrinus* in horses. This new antivenom could be used in emergency treatment of snakebite victims. Two horses were immunized with venom extracted from about 500 snakes. After an adequate increase of the antivenom titer, serum was collected and subjected to standard purification procedures for the manufacture of equine antivenoms. The purified immunoglobulin fraction was freeze-dried in 1,369 vials under optimum conditions for therapeutic use. This antivenom proved to be very potent in neutralizing the coagulant and hemorrhagic activities of the snake venom. In cases of severe bites, this antivenom was used and recognized as effective even after the occurrence of severe symptoms.

INTRODUCTION

Yamakagashi, *Rhabdophis tigrinus*, is a natricine snake widely distributed in eastern Asia including most parts of China, the mountainous areas of Taiwan, Korea, southern Primorsky in Russia, and Japan. In Japan, this species is very common, mainly in paddy fields on the major islands, except Hokkaido. Because this snake has no grooved fangs, envenomation does not occur in most bites; therefore, this snake has long been considered non-venomous. In recent years, however, several cases of fatal coagulopathy following *R. tigrinus* bites have been reported (1,2). *R. tigrinus* venom shows strong coagulant activity on plasma, with prothrombin activating effects and weak thrombin-like effects. These results suggest that the extensive hemorrhage induced by envenomation is due to hypofibrinogenemia induced by the coagulant activity of the venom (3,4). Antivenoms produced by immunization of rabbits and goats have been used successfully to treat emergency cases of *R. tigrinus* bites (5–11). Since the current lots of these antivenom products are in low supply, we prepared a new lot of *R. tigrinus* antivenom by immunizing horses. Several methods have been proposed to collect venom from colubrid snakes (12). However, the posterior-most teeth of *R. tigrinus* are ungrooved, and this makes it difficult to collect sufficient venom to immunize two horses. Therefore, we collected the venom for immunization from the excised venom glands of many snakes. Goats and horses were the preferred candidate species for immunization, because a large quantity of plasma for antivenom production could be obtained from these species. Horses were selected for immunization because all antitoxins manufactured in Japan are of equine origin, and hence, there is much clinical expertise in the manufacture of equine antitoxins; in addition, horses have no known zoonoses. Furthermore, the experimental method developed in this trial can be applied in other countries with *R. tigrinus* and/or related species.

*Corresponding author: Mailing address: Chemo-Sero-Therapeutic Research Institute (KAKETSUKEN), 1-6-1 Okubo, Kumamoto-shi, Kumamoto 860-8568, Japan. Tel: +81-96-344-1211, Fax: +81-96-345-1345, E-mail: morokuma@kaketsuken.or.jp

MATERIALS AND METHODS

Collection and preparation of *R. tigrinus* venom: Venom was extracted from excised Duvernoy's glands, following methods described previously (3,5,9,13). About 500 heads of *R. tigrinus* were collected in Honshu and Kyushu, Japan, and Duvernoy's glands, weighing about 90 g in total, were excised. The glands were frozen at −80°C and homogenized in purified water. The homogenate was centrifuged at 10,000 × *g* for 20 min and the supernatant was harvested. Then the precipitate was resuspended in purified water. After the extraction procedure was repeated 2–3 times, the supernatants were combined for use as the venom extract. The extract was purified by freeze-drying, reconstituted with purified water, and centrifuged to remove insoluble mucus material. Then the purified extract was freeze-dried again, yielding 11 g of purified venom for use as the live venom for immunization.

The venom was detoxified with formalin by adding 0.1 g of L-lysine hydrochloride to 100 mL of 5 mg/mL live venom solution. Then, two 0.5-mL aliquot of formalin (37% formaldehyde) were added to the mixture at 7-day intervals, and the mixture was maintained at 37°C for 2 weeks. The excess formalin was removed by dialysis. The absence of residual toxicity was confirmed by intracutaneous injection into rabbits and by intravenous injection into mice. The venom was coupled with liposomes (45 mg lipid/mL) composed of dipalmitoylphosphatidylcholine, dipalmitoylphosphatidylethanolamine, cholesterol, and dimyristoylphosphatidylglycerol by generating glutaraldehyde cross-links between the amino groups of the liposomes and the venom (liposome-treated venom toxoid) (14). Briefly, 0.5 mL of the 2.5% glutaraldehyde solution was added to a 2.5-mL suspension of 5 mg live venom in 90 mg of liposomes, and the mixture was stirred at 37°C for 30 min. To block excess aldehyde groups, 0.5 mL of 3 M glycine-NaOH (pH, 7.2) was added and the mixture was left overnight at 4°C. Then the mixture was applied to a CL-4B column to remove the non-liposome-bound fraction, and the liposome-bound fraction (liposome-treated venom toxoid) was isolated for use in immunization. Detoxification was confirmed by the same procedure as that specified for formalin-detoxified venom.

Immunization: Two Thoroughbred horses were immunized with the *R. tigrinus* venom toxoid, one by the method currently recommended for manufacturing Habu (*Protobothrops flavoviridis*) and Mamushi (*Gloydius blomhoffii*) antivenom, and the other by the method reported for liposome-treated antigens (15).

(i) Immunization according to the current procedure (Animal 1313): Formalin-detoxified *R. tigrinus* venom (5 mg/mL) was mixed at a 1:1 ratio with incomplete Freund's adjuvant to prepare an immunizing solution (2.5 mg/mL). The horse was immunized by subcutaneous injection of 2 primary doses of 10 mL of the detoxified venom-adjuvant mixture at a 1-week interval. After 1.5 months, the animal was given 3 subcutaneous booster doses of 5 mL of the detoxified venom without the adjuvant (5 mg/mL) at weekly intervals. Twenty days later, the horse was given, 8 escalating subcutaneous secondary booster doses of 1, 5, 10, 20, 50, 250, and 500 mg (×2) of the live venom without the adjuvant. The doses were modified according to the health of the horse and the increase of the blood antivenom titer. After the titer increased in the 4th month of immunization, four 5-L aliquots of blood were collected at weekly intervals during the 5th month. About 9 L of serum was separated from the 20 L of collected blood.

(ii) Immunization with a liposome-treated antigen (Animal 1319): The horse was immunized with 3 primary doses of 10 mL of liposome-treated *R. tigrinus* venom toxoid by subcutaneous injection at weekly intervals. After 1 month, the animal was subcutaneously injected with 7 escalating booster doses of 1, 10, 50, 250, and 500 mg (×2), and 1,000 mg of the live venom without the adjuvant. The doses were modified according to the health of the horse and the increase of the blood antivenom titer. The antivenom titer gradually increased from the first month of immunization and showed a rapid rise during the 3rd month. Five 5-L aliquots of blood were collected at weekly intervals during the 4th and 5th months. About 9.5 L of serum was separated from the 25 L of blood thus collected.

Neutralization test (determination of the antivenom titer): (i) Determination of antihemorrhagic activity (by

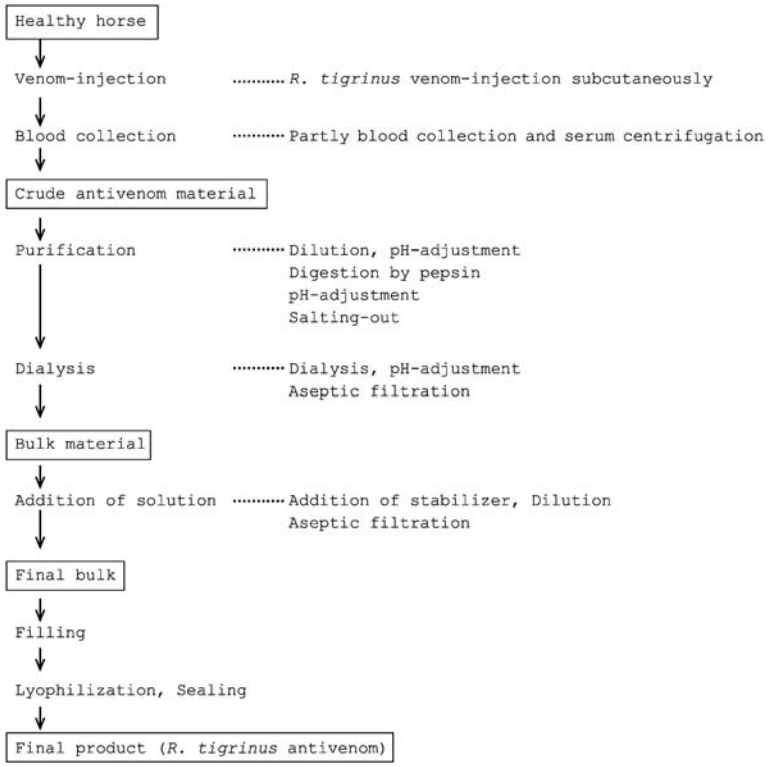

Fig. 1. Procedure of purification of *Rhabdophis tigrinus* antivenom.

intracutaneous injection into rabbits): Ten minimum hemolytic doses (MHD; 1 MHD is defined as the intracutaneous dose inducing an ecchymosis with a diameter of about 10 mm in rabbits) of *R. tigrinus* venom was mixed at a 1:1 ratio with 2-fold serial dilutions of the antivenom. Each mixture was allowed to stand at room temperature for 1 h, and then a 0.2-mL aliquot was administered to a rabbit by intracutaneous injection. After 18–24 h, the injection site was examined. The dilution that neutralized the hemorrhagic activity of *R. tigrinus* venom was used to calculate the neutralizing antibody titer of the antivenom.

(ii) Determination of anticoagulant activity: A 0.05-mL aliquot of the diluted antivenom was mixed with 0.05 mL of various concentrations of *R. tigrinus* venom and incubated at 37°C for 30 min. To each mixture, we added 0.1 mL of $CaCl_2$ solution and 0.1 mL of normal rat plasma. Mixtures of various concentrations of *R. tigrinus* venom with $CaCl_2$ solution and normal rat plasma served as controls. A dose-response curve was prepared using the clotting times determined in the test and control systems and the concentrations of the venom solutions. The neutralizing antibody titer of the antivenom was calculated as the dose of venom inducing coagulation after 20 s (4,9,16).

Purification of *R. tigrinus* antivenom: As shown in Fig. 1, *R. tigrinus* antivenom was purified according to the procedure currently adopted for other equine antivenoms to ensure that the quality of this antivenom was equivalent to that of other marketed antivenom products.

RESULTS

Response to immunization with *R. tigrinus* venom: Serial serum samples obtained from the 2 immunized horses were monitored for antivenom titer in terms of antihemorrhagic activity after intracutaneous administration to rabbits and anticoagulant activity.

In Animal 1313 (immunized by the current procedure), the serum antivenom titer slowly increased after 5 injections of formalin-detoxified venom and showed a marked increase after booster immunization with escalating doses of live venom (Fig. 2). In Animal 1319 (immunized by the liposome-treated antigen method), the serum antivenom titer showed a rapid increase after the 5th booster dose of live venom that followed 3 primary doses of liposome-treated venom toxoid (Fig. 3).

Purification of *R. tigrinus* antivenom: Serum isolated from 9 blood samples collected partly from the 2 horses was combined. Distilled water was added to 18.5 L of crude serum to adjust the protein concentration to 3% (pH 4.2). Pepsin was added to the diluted serum at a final concentration of 0.1%, and the mixture was allowed to stand overnight at 37°C. After adjusting the pH to 4.5 and addition of ammonium sulfate at a final concentration of 15%, the serum was inactivated by heating at 56°C for 1 h. After centrifugation, the supernatant was collected and adjusted to a pH of 7.0, and ammonium sulfate was added at a final concentration of 20%. Then the precipitate was extracted with distilled water, and the extract was dialyzed against 0.85% saline. Sodium glutamate (2%) and NaCl (0.85%) were added to the extract to obtain 7.1 L of immunoglobulin. Subsequently,

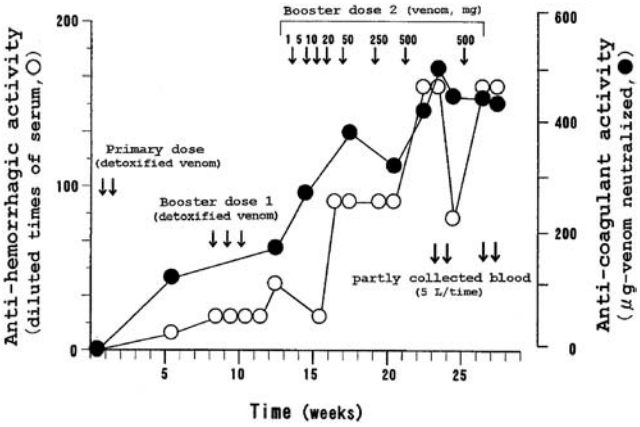

Fig. 2. Change of *Rhabdophis tigrinus* antivenom-titer in horse immunized by conventional method (Animal 1313).

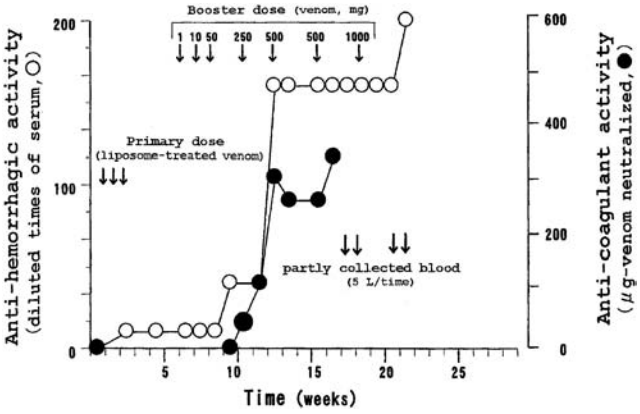

Fig. 3. Change of *Rhabdophis tigrinus* antivenom-titer in horse immunized by liposome-treated venom (Animal 1319).

5-mL aliquots of this extract were added to 20 mL vials and freeze-dried to prepare 1,369 vials of *R. tigrinus* antivenom (Lot# 0001).

Testing of *R. tigrinus* antivenom: (i) Assessment of purity: (i–i) Cellulose acetate membrane electrophoresis (Fig. 4): The purity of the final *R. tigrinus* antivenom product was determined by cellulose acetate membrane electrophoresis. Analysis of the crude serum revealed peaks of albumin and globulin, whereas the purified antivenom only showed peaks of immunoglobulins (γ-globulin and T-globulin) with no evidence of albumin.

(i–ii) Immunoelectrophoresis (Fig. 5): The purity of *R. tigrinus* antivenom was determined by immunoelectrophoresis using goat anti-(horse whole serum) antiserum. The crude serum was almost completely composed of horse plasma proteins that reacted with the goat antiserum. In contrast, the final antivenom product was of high purity and only showed bands for immunoglobulins (γ-globulin and T-globulin) with no albumin band.

(i–iii) Determination of potency: (i–iii–i) Determination of antihemorrhagic activity (by intracutaneous administration to rabbits): Each milliliter of a 120-fold dilution of *R. tigrinus* antivenom reconstituted with 5 mL of distilled water could neutralize 22.4 μg of *R. tigrinus* venom. This indicates that each vial of the Freeze-dried *R. tigrinus* Antivenom, Equine (Lot# 0001) has the abil-

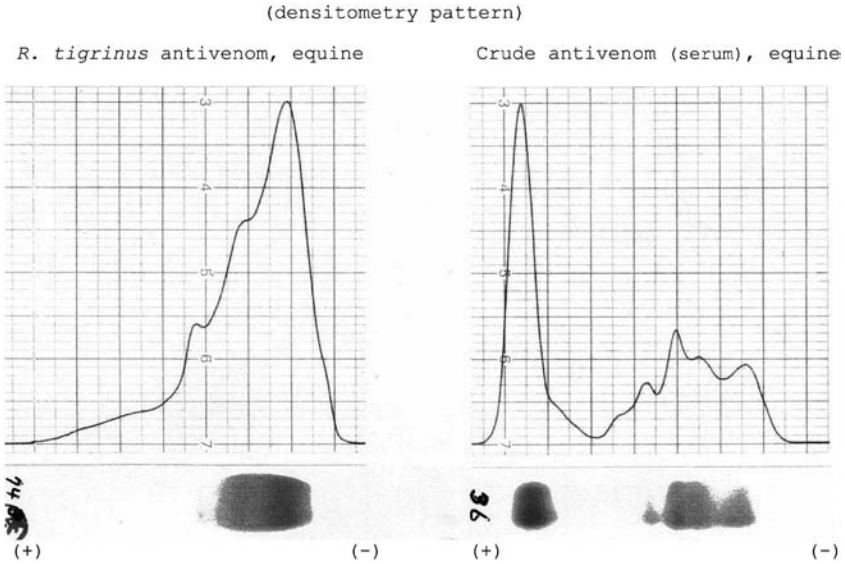

Fig. 4. Cellulose acetate membrane electrophoresis of *Rhabdophis tigrinus* antivenom. The purity analysis of the crude serum revealed the peaks of albumin and globulin, while the purified antivenom only showed immunoglobulins peaks (γ-globulin and T-globulin) without the evidence of albumin.

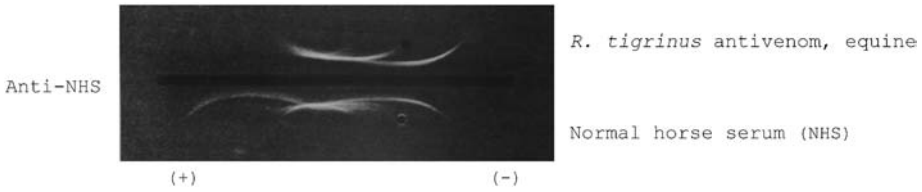

Fig. 5. Immunoelectrophoresis of *Rhabdophis tigrinus* antivenom.

Table 1. Property of freeze-dried anti-Yamakagashi, equine antivenom

Item	Results (Lot# 0001)[1)]
Moisture content	pass (0.32%)
pH	pass (7.10)
Protein content	pass (30.8 mg/mL)
Sterility	pass (No organisms)
Test for freedom from abnormal toxicity	pass (Normal)
Pyrogen test	pass (0.39°C [2 rabbits])
Potency test	
Anticoagulant activity	Each vial neutralized 4 mg of venom
Antihemorrhagic activity	Each vial neutralized 13 mg of venom

[1)]: Performed at the National Institute of Infections Diseases, Tokyo, Japan.

ity to neutralize the hemorrhagic activity of about 13 mg of *R. tigrinus* venom (Table 1).

(i–iii–ii) Determination of anticoagulant activity: Each milliliter of *R. tigrinus* antivenom reconstituted with 10 mL of distilled water was able to neutralize 431 μg of *R. tigrinus* venom. This indicates that each vial of the Freeze-dried *R. tigrinus* Antivenom, Equine (Lot# 0001) is able to neutralize the coagulant activity of about 4 mg of *R. tigrinus* venom (Table 1).

(i–iv) Other tests: The final *R. tigrinus* antivenom product was also tested to define its general pharmaceutical properties (moisture content, pH, protein content, sterility, test for freedom from abnormal toxicity, and pyrogenicity) as specified for other freeze-dried equine antivenom products in the Minimum Requirements for Biological Products of Japan (19). The Freeze-dried *R. tigrinus* Antivenom, Equine (Lot# 0001) was shown to be equivalent in quality to the marketed equine antivenom products (Table 1).

DISCUSSION

Antitoxins for some snake venoms and marine animal poisons are not commercially available in Japan because the incidence of envenomation is too low to motivate pharmaceutical manufacturers to develop such products despite the potential fatal effect of a bite/sting (17). Although *R. tigrinus* has a wide distribution, severe bites by this sneak are recorded only in Japan. The development and manufacture of an antivenom in horses will be helpful in emergency treatment of snakebite victims. *R. tigrinus* antivenom has been experimentally manufactured for emergency use by immunizing rabbits and goats, and this antivenom has been successfully used to treat *R. tigrinus* bites (5–11). However, these antivenoms were not made by a manufacturer of biological products but by regional health laboratories without any special equipment. Therefore, there were concerns about the sterility and safety of these products, which were manufactured without strict quality control or quality assurance, even though all of them proved to be

very effective in controlling coagulopathy after *R. tigrinus* envenomation. At the time of manufacture of the Freeze-dried *R. tigrinus* Goat Antivenom in 1987 (Lot# 3; Japan Snake Institute), it was confirmed that the protein nitrogen (PN) content was 4.226 mg/mL and that each milliliter neutralized 2,028 μg of *R. tigrinus* venom (specific activity, 480 μg-venom/mg PN). The potency of this product was determined in 2000 (13 years after manufacture) and was found to be about half the initial potency, because each milliliter of antivenom could only neutralize 1,110 μg of *R. tigrinus* venom (specific activity, 263 μg-venom/mg PN).

The antivenom reported here was very effective for treating a *R. tigrinus* bite in a 5-year-old boy, who was given the product with the consent of his parents and physician in July 2001 (the year after manufacture) (18). The patient was bitten on his left index finger by a snake and immediately developed disseminated intravascular coagulopathy (DIC). The bite showed bleeding with surrounding ecchymoses, and the finger became swollen. On the following day, epistaxis occurred. After failure of initial empirical treatment with equine Mamushi (*G. blomhoffii*) antivenom, the patient was treated by plasma exchange and equine Yamakagashi (*R. tigrinus*) antivenom after abnormalities of coagulation were detected. Six hours after receiving the *R. tigrinus* antivenom, all laboratory data were normalized and his coagulopathy improved.

In the second case in which the present antivenom was used, a 14-year-old boy was bitten on his right middle finger by *R. tigrinus* while handling the snake in a field in August 2005 (Satake, personal communication). About 12 h after the bite, the boy showed continuous bleeding at the wound site and had a tendency to develop DIC. The area around the bite showed ecchymoses in addition to the bleeding, and the finger became swollen. One vial of the antivenom was administered to the patient 24 h after the bite, and 30 min later, the continuous bleeding stopped. At 3 h after receiving the antivenom, all laboratory data were normalized, except the value of fibrinogen, which improved the next morning. In both cases, no side effects were detected.

The *R. tigrinus* antivenom produced by immunized goats was also shown to be effective against snakebite, even when administered after coagulopathy had developed (6,7). While diphtheria, gas gangrene, and botulism need to be treated promptly with horse antitoxins for the specific exotoxins involved in these diseases, *R. tigrinus* antivenom is effective for controlling bleeding complications and coagulopathy caused by snake venom even if administered after symptoms have developed. In all cases of successful treatment with the *R. tigrinus* antivenom, including the two recent cases, the antivenom was administered within 3 days after the bite. In a case of unsuccessful treatment, the antivenom was administered 5 days after the bite. In August 2006, a fatal case of a *R. tigrinus* bite occurred. In this case, the doctor who first treated the patient was unaware of the availability of the *R. tigrinus* antivenom, and the third hospital contacted us much too late. By the time we were contacted by the hospital staff, the patient had cerebral hemorrhage that was already spreading (Karume, personal communication). In such cases, early use of the antivenom might be effective.

The quality of the new antivenom was evaluated according to the procedures specified for "Freeze-dried Habu (*Protobothrops flavoviridis*) Antivenom, Equine" and "Freeze-dried Mamushi (*G. blomhoffii*) Antivenom, Equine" in the Minimum Requirements for Biological Products of Japan (19). *R. tigrinus* venom and *R. tigrinus* antivenom have previously been assayed in rabbits by intracutaneous administration or in mice by administration into the tail vein (in vivo tests), and in vitro tests of precoagulant activity have been done (4,9,13,16). The precision, reproducibility, and sensitivity of these three assays were evaluated. Quantification of the lethality of *R. tigrinus* venom for mice after administration into the tail vein produced variable data at low doses. This caused great variability in the results of neutralization tests performed with a mixture of the venom and antivenom. Therefore, the immune response of horses immunized with the *R. tigrinus* venom was monitored by determining the in vitro anticoagulant activity of the antivenom and its in vivo antihemorrhagic activity in rabbits after intracutaneous injection. The two horses were immunized with different forms of venom antigens, i.e., a conventional formalin-treated toxoid and a liposome-conjugated toxoid. Both methods elicited increased anticoagulant and antihemorrhagic activities. It was thought that both methods were effective. In particular, the development of antihemorrhagic activity is rapidly increased in liposome-treated venom immunization. However, because there are few examples at this time, we could not judge which method is more effective.

The antivenom reported here is the only one for the venom of the genus *Rhabdophis*. Severe cases of *R. tigrinus* bite have not been reported on the Asian continent; this might be bacaue the species is considered nonvenomous, as it has historically been considered in Japan. For example, in China, an epidemiological study of venomous snakebites is still in progress, and there is the possibility that some severe bite cases involving this species may occur. Our study will be useful in the treatment and the development of a new antivenom in such a case. The venom of *Rhabdophis subminiatus*, a species related to *R. tigrinus* and widely distributed in southeast Asia, is known to cause symptoms similar to *R. tigrinus* venom (20). The antivenom developed in this study may also be effective in treating patients with severe bites by *R. subminiatus*.

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Conflict of interest None to declare.

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