分布：アメリカ大陸，ヨーロッパの一部

注：本種が現在問題の吸虫である．Bray et al. (2008)は，この属名である *Ochetosoma* Braun, 1901 はユムシ動物の *Ochetosoma* Leuckart and Rüppell, 1828 のホモニムで使用できないとして，次に古い *Renifer* Pratt, 1902 を復活させた．しかし，Leuckart and Rüppell (1828)が記載した名称は *Ochetostoma* であり，これらがホモニムというのはの誤認である．また Bray et al. (2008)は，科名として Reniferidae Pratt, 1902 を使っているが，国際動物命名規約（条 40）に従い，ここでは Ochetosomatidae を採用した．本属は北米で種分化が進んでおり，現在シマヘビで見つかっている種も，北米からの移入種と考えられる．

Dicrocoeliidae Looss, 1899

Paradistomum megareceptaculum（Tamura, 1941）Yamaguti, 1971

Dicrocoelium megareceptaculum Tamura, 1941

Paradistomum megareceptacum Uchida, Uchida et Itagaki, 1977 (*sic*)

Paradistomum habui Kagei, 1972

Paradistomum sp. Hasegawa et Iwatsuki, 1984

宿主：シマヘビ，アオダイショウ，ヤマカガシ，ニホンマムシ，ハブ，トカラハブ
サキシママダラ

寄生部位：胆嚢・胆管・腸

分布：日本（本州，九州，宝島，奄美大島，西表島），台湾

文献：Tamura, 1941; Kagei, 1972; Kifune et al., 1977; 内田他, 1977c; 長谷川・岩附, 1984; 養命酒中央研究所, 1999

注：*Paradistomum* 属は国内では２種，本種と *P. habui* が知られている．これらの区別点について，Kifune et al. (1977)は，後者では腹吸盤が口吸盤よりも大きい事，卵黄腺の広がる範囲が前者では全長の３分の１，後者では短く５分の１と述べている．しかし，Kagei (1972)は後者の原記載において，腹吸盤の長さの口吸盤に対する比率を，0.98-1.54 としており，変異の幅は非常に大きい．内田他（1977）の前者の記述を見ても，ほぼ同大とはいえ腹吸盤がわずかに大きく，卵黄腺の長さは，本文の記述では３分の１となっているものの，貼付されている写真で見れば４分の１以下である．養命酒中央研究所（1999）は，ニホンマムシに寄生した本属の吸虫を *P. habui* として記載しているが，*D. megareceptaculum* の原記載の中で，ニホンマムシも宿主に含めている Tamura (1941)の論文を見落としており，細かい測定値は載っていないものの，付図を見ると腹吸盤は口吸盤よりも小さく，卵黄腺の長さは右が４分の１程度，左は５分の１よりも短く，上記の基準では同定できない．長谷川・岩附（1984）もサキシママダラで見つかった本属の吸虫の同定に迷っている．これまでの測定値を表１にまとめておいたが，変異の大きさを考えると，これらを同種と見るのが妥当と思われる．台湾においても，本種はナミヘビ科およびクサリヘビ科の多数の種に寄生している (Fischthal and Kuntz, 1975)．

Mesocoeliidae Dollfus, 1929

Mesocoelium brevicaecum Ochi in Goto et Ozaki, 1929

Mesocoelium elongatum Goto et Ozaki, 1929

Mesocoelium lanceatum Goto et Ozaki, 1929

Mesocoelium ovatum Goto et Ozaki, 1930

Mesocoelium pearsei Goto et Ozaki, 1930

Mesocoelium minutum Park, 1939

Mesocoelium dubium Yuen, 1965

宿主：シマヘビ

寄生部位：腸

分布：日本，ベトナム，マレーシア

文献：越智, 1930; 福井, 1963

注：本種は多くの両生類と一部の爬虫類に寄生している．国内における *Mesocoelium* 属について，Freitas (1963)は *M. geoemydae* 以外の７種を１種にまとめ，本属の２種が日本に分布するとした．その後 Odening (1968)は，Freitas の見解を受け入れたが，*M. japonicum* Goto and Ozaki, 1930 をシノニムに置くのを保留し，マレーシアとベトナムから見つかった個体を本種に置いた．ここでは Odening に従っている．なお内田他（1977a）も，*M. japonicum* を本種とは別種としている．しかし長谷川（1984）は，これらの著者がすべてを１種においているように記述しており，不適切であろう．また，Hasegawa (1990)は，本種がハブから記録されたと述べているが，引用の誤りである．

Mesocoelium geoemydae Ozaki, 1936

Mesocoelium geomydae Zerecero, 1950; Yamaguti, 1971 (*sic*)

宿主：ヒメハブ

寄生部位：小腸

分布：日本（奄美大島，沖縄島）

文献：内田他(1977c)

注：最初リュウキュウヤマガメで見つかったが，その後ヒメハブから記録された．

Opisthorchiidae Looss, 1899

Oesophagicola laticaudae Yamaguti, 1933

宿主：ヒロオウミヘビ

寄生部位：食道

分布：日本（石垣島）

文献：Yamaguti, 1933; 福井，1963

Hemiuridae Looss, 1899

Pulmovermis cyanovitellosus Coil et Kuntz, 1960

Laticaudatrema amamiensis Telford, 1967

宿主：エラブウミヘビ

寄生部位：気管・肺・気嚢

分布：日本（奄美諸島），台湾（蘭嶼）

文献：Coil and Kuntz, 1960; Telford, 1967

注：上記２種は独立して記載されたが，Yamaguti（1971）はこれらをシノニムに置いた．

Liolopidae Odhner, 1912

Harmotrema laticaudae Yamaguti, 1933

宿主：ヒロオウミヘビ，エラブウミヘビ

寄生部位：小腸

分布：日本（琉球列島）

文献：Yamaguti, 1933; Telford, 1967

科の所属不明

Ophiotreminoides orientalis Coil et Kuntz, 1960

宿主：アオマダラウミヘビ

寄生部位：小腸

分布：台湾（蘭嶼）

文献：Coil and Kuntz, 1960

Cryptogonimidae Ward, 1917

Acanthostomum marinum (Coil et Kuntz, 1960)

Ateuchocephala marinus Coil et Kuntz, 1960

宿主：エラブウミヘビ

寄生部位：小腸

分布：台湾（蘭嶼）

文献：Coil and Kuntz, 1960

Proterodiplostomidae Dubois, 1936

Proalarioides serpentis Yamaguti, 1933

宿主：シマヘビ，ヤマカガシ，ニホンマムシ

寄生部位：腸

分布：日本

文献：Yamaguti, 1933; 福井，1963; 養命酒中央研究所，1999

注：Yamaguti (1936)は実験的にアオダイショウに感染させた．

Diplostomatidae Poirier, 1886

Pharyngostomum cordatum (Diesing, 1850) 壺型吸虫幼虫（メタセルカリア）

Pharyngostomum sp. Hasegawa et Iwatsuki, 1984; Hasegawa, 1985

宿主：シマヘビ，ヤマカガシ，サキシママダラ，ガラスヒバア，ヒメハブ

寄生部位：腸，筋肉，腸間膜，（小腸，肺）

分布：アジア，ヨーロッパ，アフリカ

文献：内田他，1977b; 長谷川・岩附，1984; 長谷川，1985; Fischthal and Kuntz, 1975

注：沖縄県での記録はいずれも，*Pharyngostomum* sp.として記載されており，推定で本種と述べられている．一方，香川県や台湾では，本種と同定されているので，ここでは沖縄のものも同種に置いた．上記の寄生部位で，かっこに入っているのは，台湾での記録である．終宿主はネコである．

Didymozoidae Monticelli,1888

type Torticaecum Yamaguti, 1942　幼虫

宿主：セグロウミヘビ

寄生部位：小腸

分布：日本，台湾，東南アジア

文献：Fischthal and Kuntz, 1975

注：もともと日本のさまざまな海水魚から記録されたもので，ヘビで稀に見つかる．セグロウミヘビの記録は，台湾でのものである．なお，この名称は幼虫のいくつかある型の１つであり，特定の成虫とのつながりは不明であって，属や種は決定できない．しばしば *Torticaecum nipponicum* として言及されているが，これを通常の意味での学名と考えるべきではない（Bray et al., 2008）

２．宿主別のリスト

シマヘビ

Allopharynx japonica Tamura, 1941

Encyclometra japonica Yoshida et Ozaki, 1929

Paradistomum megareceptaculum（Tamura, 1941）

Mesocoelium brevicaecum Ochi in Goto et Ozaki, 1929

Proalarioides serpentis Yamaguti, 1933

Pharyngostomum cordatum (Diesing, 1850) 幼虫

Ochetosoma sp.

アオダイショウ

Paradistomum megareceptaculum（Tamura, 1941）

ヤマカガシ

Encyclometra japonica Yoshida et Ozaki, 1929

Paradistomum megareceptaculum（Tamura, 1941）

Proalarioides serpentis Yamaguti, 1933

Pharyngostomum cordatum (Diesing, 1850) 幼虫

ニホンマムシ

Encyclometra japonica Yoshida et Ozaki, 1929

Paradistomum megareceptaculum（Tamura, 1941）

Proalarioides serpentis Yamaguti, 1933

ハブ

Paradistomum megareceptaculum（Tamura, 1941）

トカラハブ

Paradistomum megareceptaculum（Tamura, 1941）

ヒメハブ

Mesocoelium geoemydae Ozaki, 1936

Pharyngostomum cordatum (Diesing, 1850) 幼虫

ガラスヒバア

Pharyngostomum cordatum (Diesing, 1850) 幼虫

サキシママダラ

Paradistomum megareceptaculum（Tamura, 1941）

Pharyngostomum cordatum (Diesing, 1850) 幼虫

エラブウミヘビ

Pulmovermis cyanovitellosus Coil et Kuntz, 1960

Harmotrema laticaudae Yamaguti, 1933

Acanthostomum marinum (Coil et Kuntz, 1960)

ヒロオウミヘビ

Oesophagicola laticaudae Yamaguti, 1933

Harmotrema laticaudae Yamaguti, 1933

アオマダラウミヘビ

Ophiotreminoides orientalis Coil et Kuntz, 1960

セグロウミヘビ

type Torticaecum Yamaguti, 1942 幼虫

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表1. *Paradistomum megareceptaculum* の測定値の比較
Table 1. Comparisons of measurements (mm) of *Paradistomum megareceptaculum*

著者	Tamura	Kifune et al.	内田ほか	Kagei	養命酒	長谷川・岩附
宿主	シマヘビ, ほか	アオダイショウ	ヤマカガシ	ハブ, トカラハブ	ニホンマムシ	サキシママダラ
寄生場所	胆嚢、胆管	小腸	胆管	胆嚢	胆嚢、胆管	胆嚢
産地	広島・山口	福岡	東京	奄美大島、宝島	鹿児島	西表島
体長	6.25～9.4	8.3～9.5	3.5～4.2	4.64～6.56	3.9～6.3	4.6
体幅	2.00～2.80	2.8～3.8	1.75～2.3	1.88～3.16	1.0～2.3	1.8
口吸盤	0.4～0,8	0.73～0.82	0.6～0.7	0.52～0.76		0.54
	0.6～0.82	0.78～0.85	0.5～0.55	0.54～0.76		0.51
腹吸盤	0.6～0.73	0.57～0.64	0.75～0.82	0.60～0.89		0.40
	0.55～0.82	0.67～0.74	0.55～0.68	0.56～0.89		0.46
咽頭	0.15～0.24	0.26～0.30	0.15～0.17	0.18～0.26		0.15
	0.18～0.24	0.29～0.32		0.18～0.26		0.13
食道	0.13～0.17	0.04～0.07	0.043～0.051	0.04～0.18		0.19
陰茎嚢	0.55～0.8	0.51～0.67		0.24～0.40		0.35
	0.25～0.4	0.25～0.33		0.16～0.24		0.24
精巣	0.45～1.08	0.91～1.22	0.45～0.47	0.37～0.78		0.22～0.32
	0.5～1.0	0.70～1.02	0.28～0.37	0.38～0.84		0.11～0.13
卵巣	0.25～0.46	0.36～0.43	0.22	0.22～0.32		0.32
	0.33～0.51	0.41～0.58		0.26～0.42		0.19
受精嚢	0.6～0.85	1.16～1.63	0.34	0.70～0.96		
	0.55～1.0	1.02～1.56	0.23	0.62～1.14		
卵	0.044～0.048	0.046～0.055	0.048～0.053	0.053～0.058		0.055～0.056
	0.03～0.032	0.027～0.030	0.030～0.035	0.029～0.032		0.028～0.029

注:数値が2段になっているのは, 上段が長さ, 下段が幅

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Original Article

Surveillance of the Clinical Use of Mamushi (*Gloydius blomhoffii*) Antivenom in Tertiary Care Centers in Japan

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SUMMARY: We report the results of the first large-scale questionnaire surveillance on the clinical use of pit viper antivenom in tertiary care centers in Japan. The questionnaire surveillance was conducted over a period of 3 years (April 2006 to March 2009). Completed questionnaires were received from the tertiary care centers of 108 (49.3%) medical institutions. In that period, 574 cases of pit viper bites, including 2 severe cases, were reported. Antivenom was administered in 44% of the cases of pit viper bites, and of these cases, 2.4% had adverse reactions but no severe symptoms. Approximately half of the clinicians indicated that antivenom was effective. Antivenom was recognized to be safe; however, the remarkable finding was that although the severity of treated cases was unclear, some clinicians reported using cepharanthine as the first choice of treatment for pit viper bites.

INTRODUCTION

Japanese mamushi, *Gloydius blomhoffii*, a species of pit viper distributed throughout Japan excluding Ryukyu Islands, is sighted from spring to autumn. It is important that many people are bitten by this pit viper in the mountains and fields of rural Japan. The annual number of pit viper bites remains unclear because there is currently no system to report pit viper bites to the public health department in Japan. Some reports estimate the number of pit viper bites to be 1,000 with 10 deaths annually (1).

Fatalities due to pit viper bites are generally low, but severe cases involving cardiac, pulmonary, and/or renal dysfunction can be lethal (2–4). These symptoms are caused by the snake's venom, which has lethal and hemorrhagic activities (5). Passive immunization against the venom is crucial for the clinical treatment of bites. Antivenom can neutralize both the hemorrhagic and lethal activities of venom. However, since they are derived from horse serum, these exogenous serum products frequently cause shock and anaphylaxis (6). A satisfactory treatment strategy has been proposed on the basis of the progress of symptoms following pit viper bites (1). An essential and rationalized therapy for severe cases of pit viper bites is rapid intravenous administration of antivenom. Therefore, antivenom should be administered to the snakebite victim safely and quickly. The annual antivenom production in Japan is 3,000 doses, which is gradually decreasing because of the limited opportunity for use (7).

Any snakebite victim should immediately visit a hospital's emergency department, unless the snake has been positively identified as nonvenomous, because of the potential lethal effects of snake venom. We conducted this survey to elucidate the number of snakebite cases and related therapy in tertiary care centers in Japan. This is the first large-scale questionnaire surveillance on the clinical use of pit viper antivenom in tertiary care centers in Japan.

We received reports of 574 cases of pit viper bites, including 2 severe cases, from tertiary care centers of 67 medical institutions. Antivenom was administered in 44% of pit viper bite cases, and adverse reactions to the serum were reported in only 2.4% of cases, with no severe symptoms.

METHODS

We used a newly designed questionnaire to survey the clinical use of pit viper antivenom at all 219 tertiary care centers in Japan. The questionnaire was sent in October 2009. The completed questionnaires were collected within 3 months. The content of the questionnaire is provided in Table 1. The questionnaire surveillance was conducted for a period of 3 years (April 2006 to March 2009).

RESULTS

Completed questionnaires were received from the tertiary care centers of 108 (49.3%) medical institutions that reported 574 cases of pit viper bites, including 3 cases of complications. Among the centers that responded, 67 reported having treated cases of pit viper

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Table 1. Questionnaire on the clinical use of pit viper antivenom

1. How many pit viper bites have you treated in the last 3 years?
2. Please select two or three grounds listed below for the diagnosis of a pit viper bite.
   (1) identification of the pit viper, (2) appearance of the bite, (3) clinical judgment, (4) declaration of the patient, (5) local signs, (6) systemic signs, and (7) hematological parameters.
3. How many doses of antivenom were used for treating pit viper bite(s)?
4. What was the outcome of the pit viper bite(s)?
5. Did you encounter serum sickness and/or adverse reaction to antivenom in any patient? Could you tell us the outcome of the same?
6. Did you use drugs other than antivenom for treating the pit viper bite(s)? (1) Cepharanthine, (2) Others.
7. What do you think of the effectiveness of antivenom?
   (1) Effective for both severe and mild cases
   (2) Effective for only severe cases
   (3) Ineffective
   (4) Other
8. What do you think of antivenom utility?
   (1) Essential
   (2) Can be used only for severe cases
   (3) Is an alternate drug
   (4) Other

bites, and 52 (78%) of these centers reported administering antivenom in 253 cases.

Respondents were asked to rationalize their diagnosis of pit viper bites based on the following 7 criteria: (1) identification of the pit viper, (2) appearance of the bite, (3) clinical judgment, (4) declaration of the patient, (5) local signs, (6) systemic signs, and (7) hematological parameters. The responses are summarized in Fig. 1. Among the 7 criteria, criterion 4 was most commonly used for establishing a diagnosis; the other commonly used criteria included 2, 1, 5, 6, 7, and 3, in decreasing order of frequency. Criteria 4, 2, 1, and 5 accounted for 90% of diagnoses.

The distribution of cases of pit viper bites in each tertiary care center is illustrated in Fig. 2. The geometric mean number of cases in these centers was calculated to be 5.3 cases. The care centers were classified into 3 groups based on the number of cases reported: (i) no case, (ii) less than 10 cases, and (iii) more than 10 cases (Fig. 2). Among the 108 centers that responded, 41 had no cases, 50 had less than 10 cases, and 17 had over 10 cases.

Following antivenom treatment, 6 cases including 2 severe cases from K. Red Cross Hospital were reported as having adverse effects. We reconfirmed the details of the 2 severe cases, which experienced mild anaphylaxis with rapid recovery. No cases of severe adverse reaction were observed.

Other than the pit viper antivenom, cepharanthine (CEP) was administered in 52 centers, which coincided with the number of institutions where antivenom was administered. While the severity of cases in which antivenom was administered remains unclear, CEP without antivenom was administered in 17 cases. That is, CEP was the first choice for treatment of pit viper bites in these 17 cases, even though the details of these cases are still unclear.

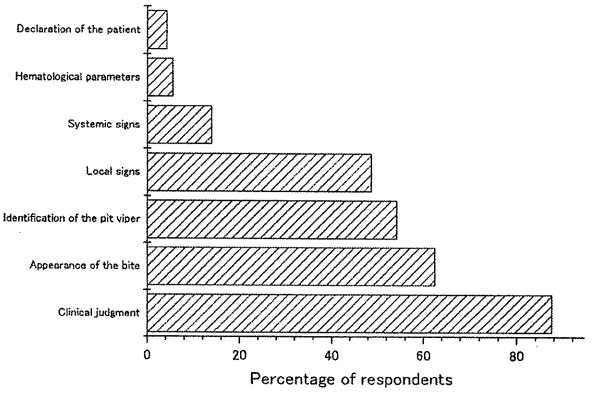

Fig. 1. Criteria for the diagnosis of pit viper bites. Respondents were asked to rationalize their diagnosis of pit viper bites based on 7 criteria. The percentage of respondents for each criterion is presented.

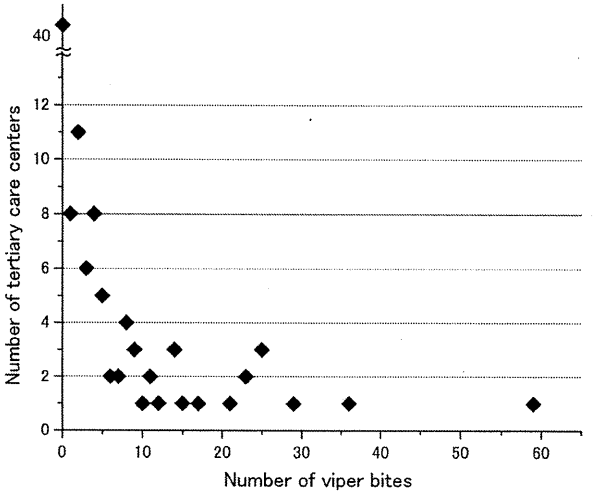

Fig. 2. Distribution of pit viper bite cases at each tertiary care center. The number of cases of pit viper bites at each tertiary care center is presented.

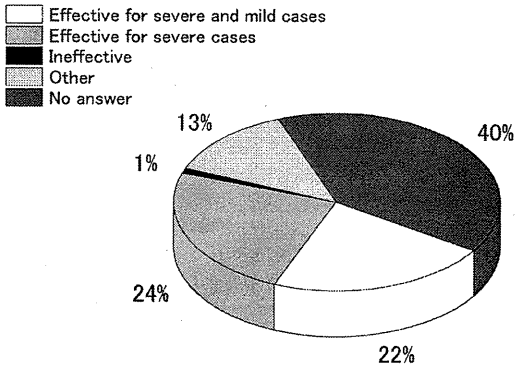

Fig. 3. Effectiveness of antivenom treatment. The responses evaluating the effectiveness of antivenom for pit viper bites were placed in the following 5 categories of answers: (i) effective for severe and mild cases, (ii) effective for severe cases, (iii) ineffective, (iv) other; and (v) no answer.

Antivenom was effective in 46% of cases of pit viper bites, but was not considered entirely effective in 13% of cases, and further study is required for evaluation of

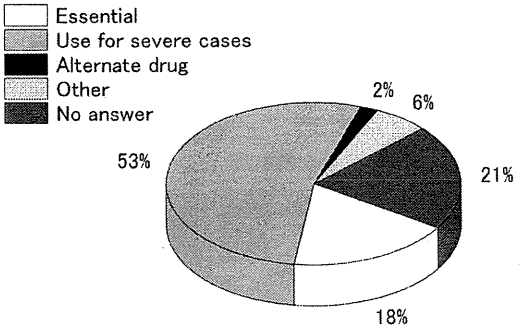

Fig. 4. Necessity of antivenom treatment. The clinicians' evaluation of the necessity of antivenom use for pit viper bites were assigned to the following 5 categories: (i) essential, (ii) use for severe cases, (iii) alternate drug, (iv) other; and (v) no answer.

its efficacy (Fig. 3).

Over 70% of the clinicians answered that they considered it necessary to use the antivemon for treating pit viper bites, whereas 6% considered the evidence inconclusive and could not evaluate its necessity because of the lack of experience in the use of antivenom during the past 10 years (Fig. 4).

DISCUSSION

Snakebites are not systematically reported in most countries. Moreover, very few countries possess a reliable epidemiological reporting system capable of providing precise data on snakebites (8). This survey is the first large-scale questionnaire surveillance on the clinical use of pit viper antivenom in tertiary care centers in Japan. It was difficult to estimate the total annual number of pit viper bite cases from our surveillance because of the lack of surveillance power as not all pit viper bite cases are transferred to tertiary care centers in Japan. However, questionnaires were received from approximately half of the tertiary care centers, and 574 pit viper bite cases during the 3 years of the study were reported. Therefore, approximately 400 cases of pit viper bites would be expected annually in all tertiary care centers in Japan.

The remarkable finding is that although the degree of severity of cases was unclear, some clinicians answered that CEP was their first choice for treatment of pit viper bites. Other centers reported adopting CEP as their first-line therapy because previous studies had suggested adverse reactions in patients receiving antivenom and because the efficacy of the antivenom had not been proven. CEP is a biscoclaurine (bisbenzylisoquinoline) amphipathic alkaloid isolated from *Stephania cepharantha* Hayata. CEP or extracts from this plant are widely used, primarily in Japan, to treat a variety of acute and chronic diseases. Conditions treated with CEP include alopecia areata (9), radiotherapy-induced leucopenia (10), malaria (11), and septic shock (12). Other pharmacological activities mediated by CEP include inhibition of plasma membrane lipid peroxidation that leads to membrane stabilization (13), inhibition of histamine release (14), immunomodulation (15), anti-allergic effects (16), anti-inflammatory effects (17), anti-HIV effects (18), inhibition of platelet aggregation (19), and antitumor activity (20).

CEP has not been reported to neutralize circulating venom. Discussions on this subject took place almost 15 years ago, and it must be confirmed that CEP does not neutralize pit viper venom (21,22). No reports outside Japan recommend CEP for the treatment of pit viper bites (23). The legal ramifications for a doctor failing to administer antivenom to a pit viper bite victim also require consideration (21).

Venom sometimes causes human death, and the only antidote that can neutralize circulating venom is antivenom, which consists of concentrated immunoglobulins from the plasma of domestic animals such as the horse that has been repeatedly immunized with one or more different snake venoms. These immunoglobulins specifically target venom toxins. After intravenous injection of the venom into the snakebite patient, the antibodies bind and neutralize venom toxins, thereby preventing and, in some cases, reversing the dangerous effects of envenomization. However, antivenoms themselves can cause complications, including potentially fatal anaphylactic shock. Incorrect risk-benefit assessment can lead to the unnecessary use of antivenom in patients with mild symptoms. According to our survey, mild complications occurred in only 2.4% of cases, and hence, antivenom was considered clinically safe. This incidence rate is one-fifth of that reported in clinical trial data (3) and in a previous study (24).

Approximately half of the clinicians mentioned that antivenom was effective and useful in patients with pit viper bites. Furthermore, there is the case report of a pit viper bite victim who initially presented with mild symptoms in the emergency department, but developed multiple organ failure a few days later, because antivenom had not been administered. However, as some clinicians mentioned, there are no prospective studies evaluating the clinical effect of antivenom. This large-scale questionnaire surveillance documented the relative safety of using antivenom. Future prospective, observational, multi-center studies should take into account patient characteristics such as age, gender, clinical severity, antivenom administration, intensive care unit stay, hospital stay, outcome, and complications.

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Conflict of interest None of declare.

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Original Article

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

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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.

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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

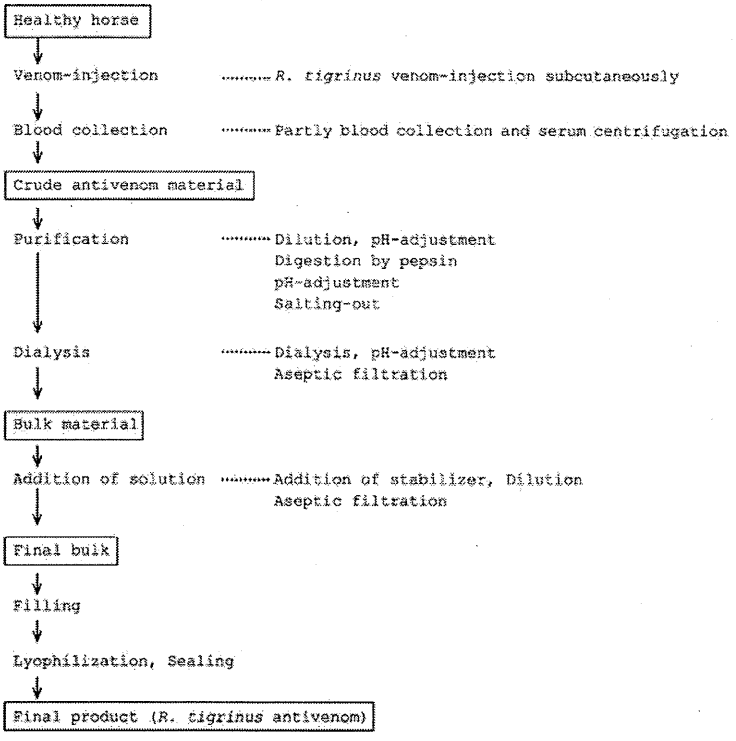

Fig. 1. Procedure of purification of *Rhabdophis tigrinus* antivenom.

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,

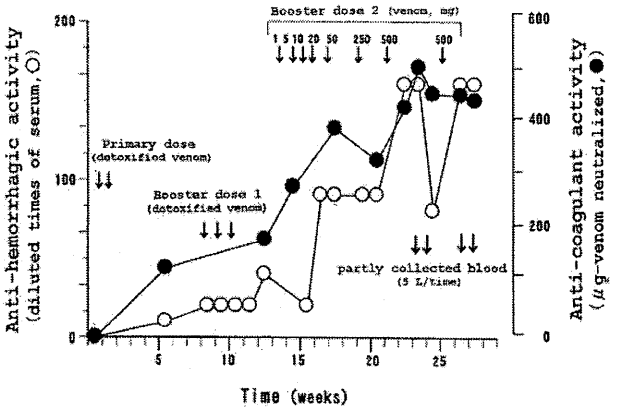

Fig. 2. Change of *Rhabdophis tigrinus* antivenom-titer in horse immunized by conventional method (Animal 1313).

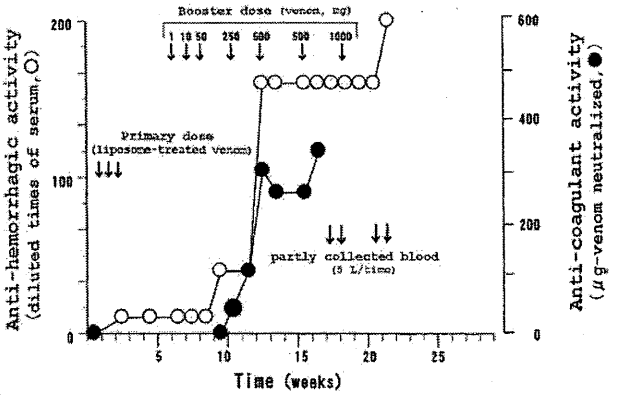

Fig. 3. Change of *Rhabdophis tigrinus* antivenom-titer in horse immunized by liposome-treated venom (Animal 1319).

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-

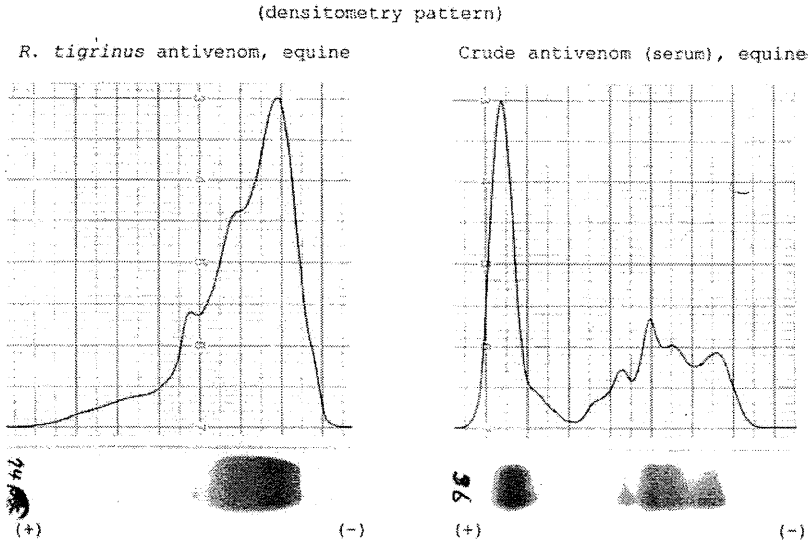

Fig. 4. Cellulose acetate membrane electrophoresis of *Rhabdophis tigrinus* antivenom. The purity analysis of the crude serum revealed the peaks of albumin and globulin, while the purified antivenom only showed immunoglobulins peaks (γ-globulin and T-globulin) without the evidence of albumin.

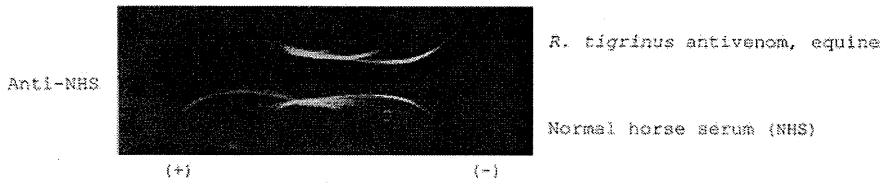

Fig. 5. Immunoelectrophoresis of *Rhabdophis tigrinus* antivenom.

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 non-venomous, 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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ヘビの判別と毒蛇咬症の診断

ヘビの判別

南西諸島を除いて九州から北海道にかけて９種類の陸棲ヘビが生息している。そのうち毒蛇はニホンマムシ、ツシママムシとヤマカガシだけであるが、ほとんどのヘビで色彩変異が大きく、さらに幼蛇と成蛇で模様が異なるヘビも多いことなどが、ヘビの判別を難しくしている。

ニホンマムシは褐色の地に体の左右に丸い模様のある個体が多いが、模様の黒い個体や時には模様のない個体も見られる。また、ヘビではしばしば真っ黒の個体（黒化型）が出現する。ヤマカガシやシマヘビでは比較的多く見られるが、マムシでは珍しい。

長崎県の対馬に住むマムシは、近年ニホンマムシとは別種（**ツシママムシ**）に分類された。ニホンマムシより少し小型で、体色も少し薄い。

ヤマカガシは、北海道には生息していないが、九州、四国、本州の水田などではよく見られるヘビで、他のヘビに比べて色彩の地域差が大きい。関東、東北地方では赤と黒の斑紋が特徴的で、中部地方西部や近畿地方ではくすんだ緑色一色の個体やそこに赤い斑紋が混ざった個体が見られる。また、中国地方では全体が青みがかった赤い斑紋のない個体が見られる。九州地方では赤と黒の斑紋が特徴であるが、関東産とは斑紋の形が異なる。

アオダイショウは大型で、人家付近などでも見られるヘビである。しかし、親子で模様が異なるヘビの代表で、その幼蛇がマムシと間違われることの多いヘビである。多くのヘビが頭を三角にして威嚇するが、特にアオダイショウの幼蛇は地域によってはキマムシ（木に登るマムシ）、シロマムシ、イワマムシ、カママムシなどと呼ばれていて、マムシの１種と誤解されていることが多い。

シマヘビも水田ではよく見られるヘビで、その幼蛇も成蛇とは全く模様が異なる。また、縞のないシマヘビもしばしば見られる。**ジムグリ**は山やその近辺に生息し、あまり珍しいヘビではないが、臆病なヘビで隠れていることが多い。**ヒバカリ**は水田などに生息する小型のヘビで、見かけることは少ない。**シロマダラ**も小型で、しかも夜行性でもあるため、見かけることの少ないヘビであるが、模様などから逃げ出した外国産のペットのヘビと間違われることがある。**タカチホヘビ**は、湿地の落ち葉の下などにいるヘビで、ほとんど見かけることはない。

日本のヘビ（九州以北）

毒ヘビ	（全長ｃｍ）	無毒ヘビ	（全長ｃｍ）
ニホンマムシ	(40 ～ 70)	アオダイショウ	(150 ～ 200)
ツシママムシ	(40 ～ 60)	シマヘビ	(80 ～ 150)
ヤマカガシ	(70 ～ 150)	ジムグリ	(70 ～ 100)
		ヒバカリ	(40 ～ 60)
		シロマダラ	(30 ～ 70)
		タカチホヘビ	(30 ～ 60)