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RESEARCH ARTICLE

Evaluation of the stability of Yamakagashi (*Rhabdophis tigrinus*) Equine Antivenom after 20 years storage

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ABSTRACT

In 2000, an equine Yamakagashi (*Rhabdophis tigrinus*) antivenom (Lot 0001) was test-manufactured as an unapproved drug for treatment of Yamakagashi bites. It was stocked on the premise of super-legal use from the viewpoint of emergency health crisis management. The antivenom showed a strong neutralizing ability against the hemorrhagic and coagulation activity of the Yamakagashi venom in its potency test. One vial of the antivenom can effectively neutralize at least about 4 mg of Yamakagashi venom. Its efficacy has also been confirmed in patients with severe cases of *R. tigrinus* bite that has been used in emergency. In 2020, this antivenom (Lot 0001) has reached 20 years after its production. To evaluate the integrity and potency of the antivenom, quality control, safety and potency tests had been conducted almost every year since 2012. Physical and chemical tests (property test, moisture content test, insoluble foreign matter test, osmotic pressure ratio test, pH test, protein content test, endotoxin test, sterility test) of the antivenom, showed no significant changes throughout the years, when compared to the results immediately after its production in 2000. All the parameters measured were also within the standard values. In animal safety tests (test for absence of toxicity and pyrogen), there was no change in the test results during the storage period and no abnormalities were observed. The potency test (anti-coagulant activity) after 20 years of the product, showed the same potency as those recorded immediately after production. Therefore, in all of the stability monitoring tests conducted so far, the product did not show any significant change compared to the results immediately after production. This confirms the stability of the product during the stockpiling period to the present, that is, 20 years after production.

Keywords: Yamakagashi; *Rhabdophis tigrinus*; antivenom; snake venom; long term storage.

INTRODUCTION

Rhabdophis tigrinus, also known as the tiger keelback or Yamakagashi in Japanese, is a venomous snake of the family Colubridae. It is widely distributed in the mountainous regions of China and Taiwan, along the southern coasts of South Korea and Russia, and in East Asia, including most parts of Japan (Sengoku, 1983). In Japan, this snake inhabits almost all areas except Hokkaido (Figure 1). Yamakagashi does not have a tubular fang like Mamushi (*Gloydius blomhoffii*), and the tube that connects to the secretory gland opens at the base of the fang, so a momentary bite does not inject much venom (Figure 2). It was once thought to be a non-venomous snake, as it causes almost no pain or swelling when bitten. However, in recent years, there have been reports of fatal cases due to dysfunction of the blood coagulation system after being bitten by Yamakagashi (Mori *et al*., 1983; Ogawa & Sawai, 1986). Yamamagashi venom has a prothrombin-activating effect and a weak thrombin-like action that also acts directly on fibrinogen, and exhibits strong coagulation activity against plasma, causing hypo-fibrinogenemia in bite patients (Sakai *et al*., 1983, 1990).

The most common venomous snakebites in Japan are caused by Mamushi (*Gloydius blomhoffii*) and Habu (*Protobothrops flavoviridis*). Antivenom drugs against these two snakebites has been approved as therapeutic agents in

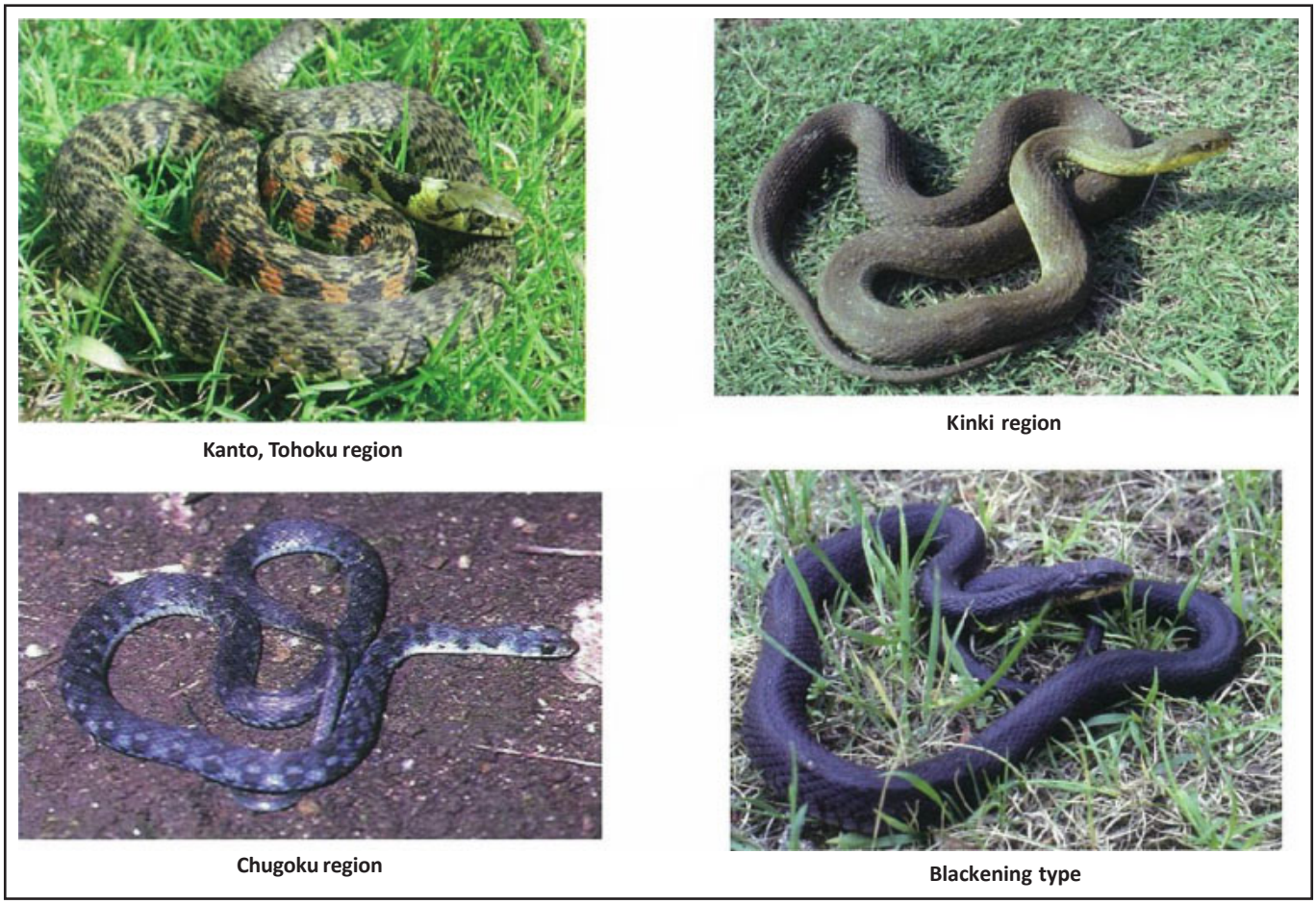
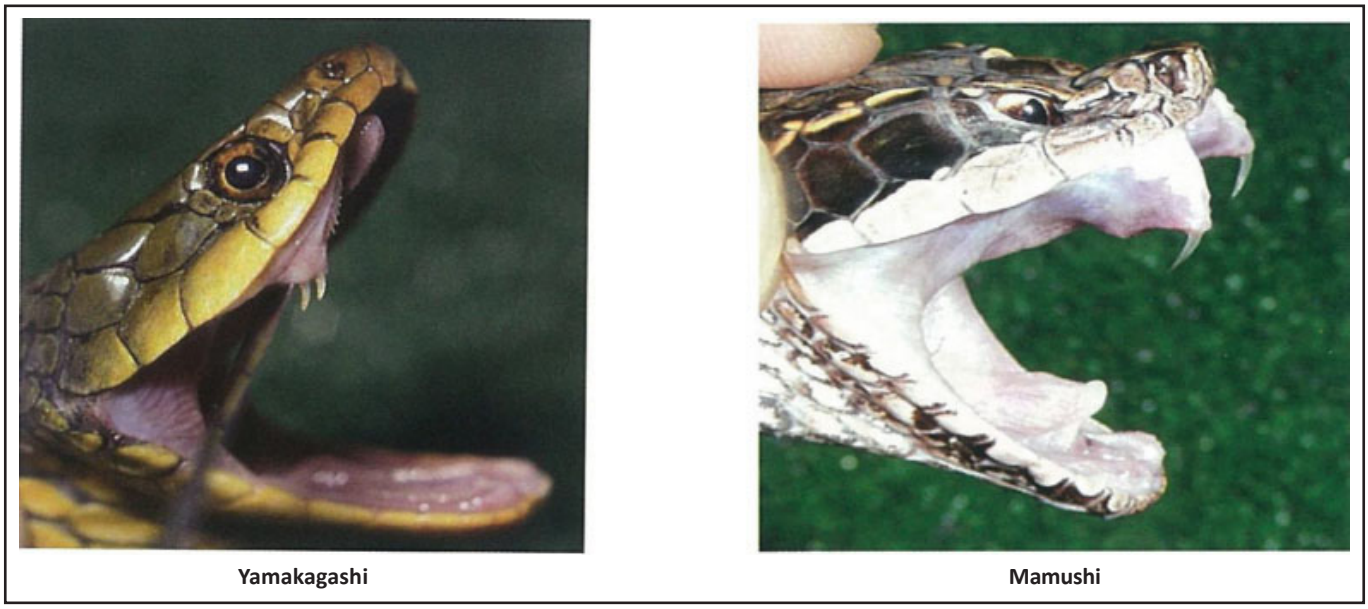


Figure 1. Appearance (snake color variation) of Yamakagashi (*Rhabdophis tigrinus*).
(Based on the pamphlet "Snake Identification and Diagnosis of Viper Snake Bite" created by the Health and Labor Sciences Research Project "Regulatory Science Research Project for Pharmaceuticals and Medical Devices" on 2011).



The Yamakagashi's fangs are not tubular like Mamushi's fangs, but are located slightly behind the mouth. The length of the fangs is short, about 2 mm. There is an opening of the Duvernoy's glands at the base of the fangs. The Mamushi's fangs are very fine, and if bitten, the wound becomes two stabs at approximately 1 cm intervals.

Figure 2. Difference between the fangs of Yamakagashi (*Rhabdophis tigrinus*) and Mamushi (*Gloydius blomhoffii*).
(Based on the pamphlet "Snake Identification and Diagnosis of Viper Snake Bite" created by the Health and Labor Sciences Research Project "Regulatory Science Research Project for Pharmaceuticals and Medical Devices" on 2011).

Japan. "Freeze-dried Mamushi antivenom" and "Freeze-dried Habu antivenom" are commercially available and their supply chain system are presently in place (Nakai *et al*., 2003; Ministry of Health, Labor and Welfare, Japan, 2004a, 2004b). However, there are still no commercially approved antivenom drug for Yamakagashi snakebite in Japan. This is because of the extremely small number of cases of Yamakagashi bite in Japan and it was considered as commercially non-viable to develop the antivenom. Although there are not many cases of Yamakagashi bites in Japan, its antivenom is an indispensable therapeutic drug for its lifesaving properties.

A Yamakagashi antivenom (Lot 0001) was first test-manufactured in 2000 (Kurata, 1998; Kurata, 1999). Although this antivenom is an unapproved drug in Japan, it is extremely valuable for Yamakagashi bite patients. The use of Yamakagashi antivenom in clinical research today is being carried out, even in 2021 due to its social necessity. Thus, within this framework, the Yamakagashi antivenom can be used clinically, with the consent of the patient and the doctor who is treating the patient. For the clinical use of the Yamakagashi antivenom, its quality needs to be confirmed and guaranteed.

For other domestically approved equine antitoxin products in Japan, namely, Habu (*Protobothrops flavoviridis*) antivenom, Mamushi (*Gloydius blomhoffii*) antivenom, Botulinum antitoxin, Gas gangrene antitoxin and Diphtheria antitoxin, all have an expiration date of 10 years after manufacture (Association of Biologicals Manufacturers of Japan, 2006). On the other hand, this unapproved drug, Yamakagashi antivenom (Lot 0001), was test-manufactured in 2000, and reached 20 years after production in 2020. The unapproved Yamakagashi antivenom may continue to be administered to humans in the event of severe cases of Yamakagashi bites in Japan. However, the Research Project under the Ministry of Health, Labor and Welfare, Japan, in 2011, required that even if the Yamakagashi antivenom is used for emergency, its quality evaluation from the aspect related to the stability of the preparation is indispensable. It was then decided that the safety and effectiveness of the antivenom should be confirmed from time to time. From 2012 onwards, almost every year, monitoring tests related to stability and safety were carried out.

Quality tests of the Yamakagashi equine antivenom (Lot 0001) were conducted in 2012 (12th year after production), in 2013 (13th year), and in 2015 (15th year) to 2020 (20th year), after its production in 2000.

Based on the above background, the stability of the Yamakagashi antivenom during the stockpiling period was evaluated and confirmed over time in this study, and the results are presented in this paper.

MATERIALS AND METHODS

In 2000, we used horses as the animal to experimentally produce a new Yamakagashi antivenom. From the viewpoint of emergency health crisis management, we stockpiled the antivenom on the premise of using it under special cases requiring its usage. Venom was collected from the Duvernoy's gland of about 500 Yamakagashi and injected into two horses. After monitoring the increase of the antivenom titer, sera were collected from the horse. The immuno-globulin fraction was purified with a method conforming to a commercially available horse antivenom product (Sakuma, 2002), and then freeze-dried. A total of 1,369 vials of Yamakagashi antivenom products (Lot 0001) was produced (Figure 3) (Morokuma *et al*., 2011).

The items and test methods of the stability test over time of the Yamakagashi antivenom that were conducted are as follows. The following tests 1) to 10) were conducted at KM Biologics Co., Ltd., and the test 11) was conducted at the Japan Snake Institute and National Institute of Infectious Diseases.

1) Property test

The test was conducted by applying General rules of "Minimum Requirements for Biological Products" (MRBP). At that time, the dissolution time of the product was also confirmed. The property test was conducted every year after 2012, except for 2014. The dissolution time carried out at the same time as the property test, was not confirmed only in FY2015.

2) Test for moisture content

The test was conducted by applying the general test method "Moisture content measurement method" of MRBP. These tests were conducted every year after 2012, except in 2014.

3) Insoluble foreign matter test (2nd method)

The test was conducted by applying the general test method "Injectable foreign matter inspection method (second method) for injections" of "The Japanese Pharmacopoeia" (JP). This test was conducted only in 2013.

4) Osmotic pressure ratio test

The test was conducted by applying the general test method "Osmotic pressure measurement method" of JP. This test was also conducted only in 2013.

5) Test for pH

The test was conducted by applying the general test method "pH measurement method" of MRBP. These tests were conducted in 2012 and 2013.

6) Test for protein content

The test was conducted by applying the general test method "Protein nitrogen quantification method" of MRBP. These tests were also conducted in 2012 and 2013.

7) Endotoxin test

The JP general test method "Endotoxin test method" was applied mutatis mutandis. These tests were also conducted in 2012 and 2013.

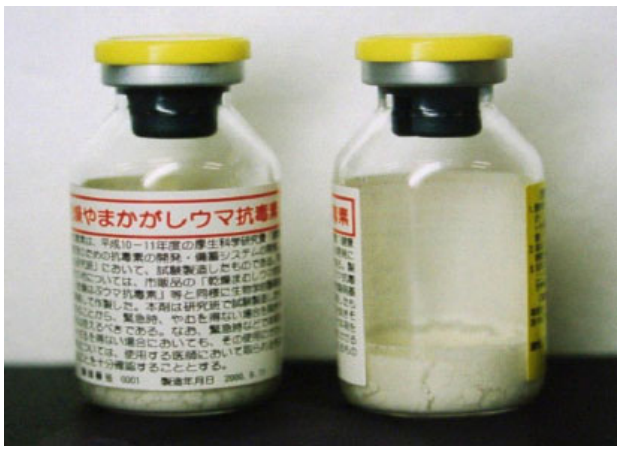


Figure 3. Original Freeze-dried anti-Yamakagashi, Equine Antivenom (Lot 0001).

8) Sterility test (MF method of JP)
The test was conducted by applying the general test method "Sterility test method" of JP mutatis mutandis. These tests were also conducted in 2012 and 2013.

9) Test for freedom from abnormal toxicity (guinea pig)
The test was carried out by applying the general test method "Test method for freedom from abnormal toxicity" of MRBP mutatis mutandis. These tests were conducted every year after 2012, except in 2014.

10) Pyrogen test
The test was conducted by applying the general test method "Pyrogen test method" of MRBP mutatis mutandis. These tests were conducted in 2012 and 2013.

11) Potency test (anticoagulant activity)
0.05 mL of a diluted solution of antivenom and 0.05 mL of Yamakagashi venom (800 μg/mL) with varying concentrations were mixed, and heated at 37°C for 30 minutes. Next, 0.1 mL of $CaCl_2$ solution was mixed and 0.1 mL of normal rat plasma was added. As a test control, a variable amount of Yamakagashi venom and a $CaCl_2$ solution were mixed, and normal rat plasma was similarly added.

A dose-response curve was obtained for the coagulation time and the concentration of the venom in both cases, and the amount of venom that coagulated in 20 seconds was calculated as the neutralization value (Sakai & Sawai, 1984; Kawamura *et al*., 1989; Sakai *et al*., 1990). This potency test (anticoagulant activity) was conducted in 2013 and 2017.

RESULTS

Table 1 shows the quality test results immediately after the production of Yamakagashi equine antivenom (Lot 0001) in 2000. From this result, one vial of the products was shown to neutralizes about 13 mg of Yamakagashi venom for the hemorrhagic activity and about 4 mg of this venom for coagulation activity. The general property test met all the standards as specified for other commercially available equine antivenom products and has the same quality (Association of Biologicals Manufacturers of Japan, 2006).

The results of the stability monitoring tests of this antivenom are shown in the following 11 items, which is summarized in Table 2.

1) Property test
The results of the tests conducted almost every year from 2012 to 2020 are all "a pale yellow dry preparation, a pale yellow slightly cloudy liquid when the solvent is added", and these results were the same as those immediately after production in 2000. The dissolution time was 79 seconds immediately after production, while the test in 2013 was relatively longer at 185 seconds, but it fluctuates between 81 and 146 seconds in the test from 2012 to the latest in 2020. Therefore, there was no tendency for the dissolution time to increase as the storage period of the product increased (Table 3).

2) Test for moisture content
Moisture content was 0.26% immediately after manufacturing, but it was 0.35 to 0.73% in the 2012-2019 test. Although there was a slightly upward trend, but all were of low values at 3.0% or less than the standard, and thus passed the test (Table 3).

3) Insoluble foreign matter test (2nd method)
In the test conducted in 2013, no insoluble foreign matter was found in the dissolved formulation, and the test was successful (Table 2).

4) Osmotic pressure ratio test
The osmotic pressure ratio was 1.25 immediately after production in 2000, and 1.19 in 2013 when the test was conducted. No change was observed in the product even during the storage period of 13 years (Table 2).

5) Test for pH
The pH was 7.18 in 2012 and 7.12 in 2013, both conforming to the standards of 6.8 to 7.4 (Table 2).

6) Test for protein content
The protein content was 29.8 mg/mL immediately after production in 2000, while it was 31.7 mg/mL and 30.4 mg/mL in 2012 and 2013, respectively, and no particular change was observed (Table 2).

7) Endotoxin test
In the tests conducted in 2012 and 2013, the endotoxin content remained negative at 0.020 EU / mL or less, and there were no particular abnormalities (Table 2).

8) Sterility test (Japanese Pharmacopoeia MF method)
In the sterility tests conducted in 2012 and 2013, no bacterial growth was observed in both tests, and the test standards were met (Table 2).

9) Test for absence of abnormal toxicity (guinea pig)
In the tests conducted almost every year from 2012 to 2020, the test results showed "no abnormalities" in any of the animals, and all conformed to the standard values (Table 4).

10) Pyrogen test
In the tests conducted in 2012 and 2013, the total increase in body temperature of the three rabbits was 0.11! and 0.12!, both of which were negative and passed the test (Table 2).

11) Potency test (anticoagulant activity)
As a result of measuring the anticoagulant activity of the 13th year (in 2013) and 17th year (in 2017) of the product in vitro using normal rat plasma, it was confirmed that one vial product neutralizes 16.9 mg and 15.6 mg of Yamakagashi

Table 1. Property of Freeze-dried anti-Yamakagashi, Equine Antivenom (Lot 0001)
(The results of the tests conducted at the time of manufacture in 2000 are shown).

Item	Results[a]
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 [two rabbits])
Potency test	
• Anti-coagulant activity	Each vial neutralized 4 mg of venom.
• Anti-hemorrhagic activity	Each vial neutralized 13 mg of venom.

a, Performed at the National Institute of Infectious Diseases (NIID).

Table 2. Stability test[a] of Yamakagashi antivenom (Lot 0001) in 2020

Test year / Item (Years since manufacture)	2,000 (0)	2,012 (12th)	2,013 (13th)	2,015 (15th)	2,016 (16th)	2,017 (17th)	2,018 (18th)	2,019 (19th)	2,020 (20th)
Property	Pale yellow dry Formulation, Slightly yellowish liquid after dissolution	same as 2000	same as 2000	same as 2000	same as 2000	same as 2000	same as 2000	same as 2000	same as 2000
Moisture content (%)	0.26	0.35	0.48	0.46	0.47	0.57	0.53	0.67	0.73
Insoluble foreign matter	Not admitted (pass)	–	pass	–	–	–	–	–	–
Dissolution time (Second)	79	81	185	–	107	114	146	133	91
Osmotic pressure ratio	1.25	–	1.19	–	–	–	–	–	–
pH	7.13	7.18	7.12	–	–	–	–	–	–
Protein content (mg/mL)	29.8	31.7	30.4	–	–	–	–	–	–
Endotoxin content (EU/mL)	<0.004	<0.020	0.020	–	–	–	–	–	–
Sterility	No organisms (pass)	pass	pass	–	–	–	–	–	–
Test for freedom from abnormal toxicity	Normal (pass)	pass	pass	pass	pass	pass	pass	pass	pass
Pyrogen test (°C [three rabbits])	0.17	0.11	0.12	–	–	–	–	–	–
Potency test • Anti-coagulant activity[#1]	Neutralized 4mg of venom	–	Neutralized 16.9mg of venom	–	–	Neutralized 15.6mg of venom	–	–	–
• Anti-hemorrhagic activity[#2]	Neutralized 13mg of venom	–	–	–	–	–	–	–	

a, Performed at the KM Biologics Co. Ltd.
#1, Performed at the Japan Snake Institute (JSI).
#2, Performed at the National Institute of Infectious Diseases (NIID).

Table 3. Stability test[a] of Yamakagashi antivenom (Lot 0001) in 2020
– Property, Moisture content and Dissolution time –

Test year / Item (Years since manufacture)	2,000 (0)	2,012 (12th)	2,013 (13th)	2,015 (15th)	2,016 (16th)	2,017 (17th)	2,018 (18th)	2,019 (19th)	2,020 (20th)
Property	Pale yellow dry Formulation, Slightly yellowish liquid after dissolution	same as 2000	same as 2000	same as 2000	same as 2000	same as 2000	same as 2000	same as 2000	same as 2000
Moisture content (%)	0.26	0.35	0.48	0.46	0.47	0.57	0.53	0.67	0.73
Dissolution time (Second)	79	81	185	–	107	114	146	133	91

a, Performed at the KM Biologics Co. Ltd.

Table 4. Stability test of Yamakagashi antivenom (Lot 0001) in 2020
– Test for freedom from abnormal toxicity –

Test year / Item (Years since manufacture)	2,000 (0)	2,012 (12th)	2,013 (13th)	2,015 (15th)	2,016 (16th)	2,017 (17th)	2,018 (18th)	2,019 (19th)	2,020 (20th)
Test for freedom from abnormal toxicity#1	Normal (pass)	pass	pass	pass	pass	pass	pass	pass	pass

#1, Performed at the KM Biologics Co. Ltd.

Table 5. Stability test of Yamakagashi antivenom (Lot 0001) in 2020
– Potency test –

Test year / Item (Years since manufacture)	2,000 (0)	2,012 (12th)	2,013 (13th)	2,015 (15th)	2,016 (16th)	2,017 (17th)
Potency test • Anti-coagulant activity#1	Neutralized 4mg of venom	–	Neutralized 16.9mg of venom	–	–	Neutralized 15.6mg of venom
• Anti-hemorrhagic activity#2	Neutralized 13mg of venom	–	–	–	–	–

#1, Performed at the Japan Snake Institute (JSI).
#2, Performed at the National Institute of Infectious Diseases (NIID).

venom, respectively. Considering that the potency titer (anticoagulant activity) immediately after production in 2000 neutralized 4 mg of Yamakagashi venom with one vial, and even considering the variation of the test method, no decrease in potency titer was observed during the storage period of the product (Table 5).

DISCUSSION

In the past, Yamakagashi antivenom has been experimentally produced using rabbits and goats as experimental animals (Kawamura *et al*., 1989). There was no report of the follow up of these experimentally produced antivenom. In our study in 2020, the Yamakagashi equine antivenom (Lot 0001) test-produced in 2000 will be 20 years since its production. Therefore, it was necessary to confirm the quality of the product at the present time, and tests were conducted on parameters that are particularly susceptible to the passage of time. From the result of the physical and chemical tests, that include the property test (including dissolution time), insoluble foreign matter test, osmotic pressure ratio test, pH test, protein content test, endotoxin test and sterility test, no particular change as compared with the results immediately after production was observed. The moisture content tended to increase after production (0.26% to 0.73%), but it was of a sufficiently low value of 3.0% or less of the standard, and thus conformed to the test. In the animal tests for the drug safety, no abnormal toxicity nor pyrogen effects were detected indicating no change in the test results during the storage period and also no abnormality was observed. In the potency test (anticoagulant activity) from the aspect of the efficacy of the product, the titer was sufficiently retained as these immediately after production, even at the present time 20 years after production. It was thus confirmed that this Yamakagashi equine antivenom is still an effective and viable product.

During the storage period, this Yamakagashi equine antivenom (Lot 0001) was used in July of the year following its manufacture (2001), on a 5-year-old boy who was bitten by Yamakagashi in Saitama, Japan. With the consent of doctor and patient, the antivenom showed significant positive effects after administration (Kato *et al*., 2003). In this case, Disseminated Intravascular Coagulation (DIC) occurred immediately after the left second finger was bitten by a Yamakagashi. Bleeding from the bite site, swelling of the second finger, and nose bleeding appeared the following day. Blood test showed abnormalities in the coagulation system, so plasmapheresis and Yamakagashi equine anti-venom were administered. Six hours after administration of the antivenom, all blood test values returned to normal and blood coagulation improved. This showed that the antivenom was significantly effective.

The second case in which this Yamakagashi antivenom was used occurred in Saga in August 2005, that is five years after the preparation of the product (Satake, Personal communication). A 14-year-old boy was bitten at his right middle finger while trying to catch and play with a snake near his home. Approximately 12 hours after the bite, the child showed a tendency for headache, persistent bleeding from the bite site, and developed DIC due to significant coagulation abnormalities. Based on clinical symptoms, the patient was diagnosed with a Yamakagashi bite, and 24 hours later, Yamakagashi antivenom was administered. Continuous bleeding stopped 30 minutes after the administration. Three hours later, all blood test values return to normal except for fibrinogen. The fibrinogen level improved the next morning. No adverse reactions were observed in the above two cases in which the Yamakagashi antivenom (Lot 0001) was used for the first time. After that, although the frequency of occurrence of Yamakagashi bite was low, patients who were similarly treated with Yamakagashi antivenom improved greatly (Hifumi *et al*., 2014). More recently, two severe cases of Yamakagashi bites occurred in succession in 2017. The first case occurred in Fukuoka on July 25 of the same year (Aso Iizuka Hospital, Personal communication). A 10-year-old boy was bitten by Yamakagashi for about 5 minutes, and a blood test immediately afterwards revealed a serious case with FDP (fibrin/fibrinogen degradation product) of 70 µg/mL and fibrinogen of about 100 mg/dL with DIC. Approximately 9 hours after the bite, the Yamakagashi antivenom was administered, and after that, blood parameters improvement was observed leading to stable condition. The patient was discharged from the hospital safely on the July 29th. The second case occurred in Hyogo prefecture on July 29 of the same year (Hyogo Prefectural Amagasaki General Medical Center, Personal communication). A 10-year-old boy was bitten by a snake on his right wrist in the evening and immediately returned home without going to the hospital. He continued to bleed from his wounds after returning and was taken to the hospital again at night with a headache. Subsequent blood tests showed an FDP of 800 µg/mL and fibrinogen below the measurement sensitivity. Therefore, Yamakagashi bite was suspected, and Yamakagashi anti-venom was administered the next morning, about 15 hours after the bite. The patient had temporarily been placed in a coma, but regained consciousness by administration of Yamakagashi antivenom. He was discharged safely on August 2 in 2017. Successful treatment of both cases of Yamakagashi bites that occurred in 2017 were attributed to the use of Yamakagashi antivenom (Lot 0001), which has been 17 years since its manufacture.

From the above clinical observation, it was proved that the Yamakagashi antivenom (Lot 0001) was stable as a preparation even 20 years after its test-production, and still retain its safety and efficacy. Considering that all domestically approved horse antitoxin products in Japan such as Habu antivenom and Mamushi antivenom have a uniform expiration date of 10 years after production, our observation that Yamakagashi antivenom being an unapproved drug, showed stability ever 20 years after production, is a new finding.

Since there is no approved drug for the treatment of Yamakagashi bites in Japan, in the future, in the event of a serious case of Yamakagashi bite, it is still necessary to use Yamakagashi antivenom (Lot 0001) as an emergency therapeutic drug, with the consent of the attending physician and the patient. However, even if it is not urgent in the future, there is a concern on the quality deterioration of the product, and its efficacy is not guaranteed forever. In order to avoid long-term use of the Yamakagashi antivenom (Lot 0001) that are more than 20 years old, we are working on test-production of new product lots through the activities of the Japan Agency for Medical Research and Development (AMED) research group. For this purpose, it is first necessary to collect the Yamakagashi venom, which is an antigen for immunizing horses. However, the number of *Rhabdophis tigrinus* is decreasing in Japan today, and its capture is not proceeding as planned. As a result, immunity to horses has not yet been reached, and the production of new Yamakagashi antivenom lots has been delayed considerably. At the same time, we will also seek ways to convert this Yamakagashi equine antivenom into an approved drug.

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Conflict of interest

The author declares that they have no conflict of interests.

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