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Original Article

Standardization of Regional Reference for Mamushi (*Gloydius blomhoffii*) Antivenom in Japan, Korea, and China

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SUMMARY: The mamushi (*Gloydius blomhoffii*) snakes that inhabit Japan, Korea, and China produce venoms with similar serological characters to each other. Individual domestic standard mamushi antivenoms have been used for national quality control (potency testing) of mamushi antivenom products in these countries, because of the lack of an international standard material authorized by the World Health Organization. This precludes comparison of the results of product potency testing among countries. We established a regional reference antivenom for these three Asian countries. This collaborative study indicated that the regional reference mamushi antivenom has an anti-lethal titer of 33,000 U/vial and anti-hemorrhagic titer of 36,000 U/vial. This reference can be used routinely for quality control, including national control of mamushi antivenom products.

INTRODUCTION

Snakebites are a threat to human life in areas inhabited by poisonous snakes. Various antivenom products have been used for the treatment of snakebites (1). Venomous snakes belonging to "mamushi" species inhabit countries in the Far East Asia, including China, Korea, and Japan (2). The mamushi species, *Gloydius blomhoffii*, is widely distributed throughout Japan, and its variants also inhabit China and Korea (*Gloydius blomhoffii brevicaudus*). These snakes were newly proposed to be regrouped into *Gloydius* spp. from *Agkistrodon* spp. in 1997 (2), and their venoms were shown to have very similar immunological characteristics. Although fatality rates of mamushi bites are generally low (3), severe cases can be lethal with cardiac, pulmonary, and/or renal dysfunction (3,4). These symptoms are caused by the snake's venom, which has lethal and hemorrhagic activities (5). Passive immunization against the venom is crucial in the clinical treatment of bites. Antivenom products can neutralize both lethal and hemorrhagic activities of the venom. In Japan and China, these products are manufactured domestically, while in Korea they are imported from Japan and China after confirmation of their potency against venom prepared from mamushi captured locally. The quality of the products has been controlled according to the minimum requirements prescribed in each of these three countries (6-8). However, the lack of international standards from the World Health Organization (WHO) precludes comparison of potency among these three Asian countries. Thus, a common reference antivenom was prepared in these countries and confirmed to be suitable as a regional reference antivenom.

In the present study, the potency of a candidate regional reference mamushi antivenom produced by Shanghai Institute of Biological Products (SIBP) (Shanghai, China) was calibrated against Japanese national standard mamushi antivenom using the quality control test methods described in the Japanese minimum requirements at the National Institute of Infectious Diseases (NIID) (Tokyo, Japan) and Chemo-Sero-Therapeutic Research Institute (Kaketsuken) (Kumamoto, Japan) and in the Korea minimum requirements at the Korea Food and Drug Administration (KFDA) (Seoul, Korea). The reference antivenom will be used in routine quality control tests in these countries.

MATERIALS AND METHODS

Production of a candidate regional reference mamushi antivenom: The candidate regional reference mamushi antivenom (Lot 011201, 3,000 vials) was produced at SIBP according to the procedure for Chinese commercial antivenom products. The candidate was made from pooled horse serum containing a sufficient antibody titer against mamushi venom. The venom and toxoid (venom detoxified with formaldehyde) prepared from Chinese mamushi were used as antigen to immunize the horse. To ensure stability of quality under storage for long periods, the candidate was freeze-dried similarly to Japanese national standard mamushi antivenom.

Animals: Mice (more than 3 mice/group; body weight, approximately 16 g) were used for determination of anti-lethal titer. The mouse strains were Slc:ddY at NIID and Kaketsuken, ICR at KFDA, and Kunmin at SIBP. For determination of anti-hemorrhagic titer, two rabbits (Japanese white strain) weighing approximately 2.5 kg were used in three countries.

Determination of anti-lethal titer: The antibody titer of the candidate regional reference material against the lethal activity of mamushi venom was determined at SIBP, NIID,

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Table 1. Composition of assay mixtures for mamushi antivenom titer determination

	preparation	(1)	(2)	(3)	(4)	(5)
antivenom	200 U/ml (anti-lethal test) or 20 U/ml (anti-hemorrhagic test)	0.720	0.600	0.500	0.417	0.347
PBS-G[1)]		0.280	0.400	0.500	0.583	0.653
venom	10 test dose/ml	1.000	1.000	1.000	1.000	1.000

[1)]: 0.017 mol/l phosphate buffered sodium chloride solution containing 0.2 w/v% gelatin (pH 7.0). (ml)

Kaketsuken, and KFDA, using Japanese national standard mamushi antivenom (Lot C-48, anti-lethal titer: 2,100 U/vial, NIID) with the test methods prescribed in China (8), Japan (NIID and Kaketsuken) (6,9,10), and Korea (7).

The Japanese standard antivenom was dissolved in 0.017 M phosphate buffered sodium chloride solution containing 0.2 w/v% gelatin (pH 7.0) (PBS-G) to make a solution of 200 U/ml and serially diluted with PBS-G such that 1 ml of each dilution contained 160, 125, 100, 80, or 64 U of antivenom (Table 1). The candidate (Lot 011201) was dissolved and serially diluted in a manner similar to the Japanese standard antivenom. Japanese mamushi test venom (Lot 3-2, lethal titer: 530 test dose/ampoule, or Lot 4, lethal titer: 450 test dose/vial) was reconstituted and diluted in PBS-G to a concentration of 10 test doses/ml. Aliquots of 1 ml of appropriately diluted Japanese standard antivenom or the candidate (Table 1) were mixed with 1 ml of venom and kept at room temperature for 1 h. Mice were injected intravenously with 0.2 ml of each mixture at NIID, KFDA, and Kaketsuken, and intraperitoneally at SIBP. The number of deaths was recorded for 2 days at NIID, KFDA, and Kaketsuken, or for 3 days at SIBP. The 50% effective doses (ED_{50}) of the standard antivenom and the candidate were calculated by the probit method from the number of dead mice associated with each dilution. The potency of the candidate was determined relative to that of the standard antivenom.

Determination of anti-hemorrhagic titer: The potency of the candidate regional reference mamushi antivenom was examined against the Japanese national standard mamushi antivenom (Lot C-48, anti-hemorrhagic titer: 3,300 U/vial, NIID) as standard at SIBP, NIID, and Kaketsuken using the Japanese method (6,9,11) and at KFDA using the Korean method (7).

The methods described in Japanese (6) and Korean (7) minimum requirements are essentially identical: the standard antivenom was dissolved in PBS-G to a concentration of 20 U/ml and serially diluted with PBS-G such that 1 ml of the dilution contained a total of 16, 12.5, 10, 8, or 6.4 U (Table 1). The candidate (Lot 011201) was dissolved and serially diluted in the same way as the standard antivenom. Japanese mamushi test venom (Lot 3-2, hemorrhagic titer: 1,200 test dose/ampoule, or Lot 4, hemorrhagic titer: 1,200 test dose/vial) was reconstituted and diluted into PBS-G to a final concentration of 10 test doses/ml. Aliquots of 1 ml of appropriately diluted standard antivenom or candidate were mixed with 1 ml of venom and kept at room temperature for 1 h (Table 1). Thus, aliquots of 0.2 ml of these mixtures were injected intradermally into the shaved backs of two rabbits at two sites for each rabbit per mixture. Twenty-four hours after injection, the rabbits were killed by pentobarbital anesthesia and the skin was stripped off. The cross-diameters of the hemorrhagic spots were measured from the inner side of the skin.

ED_{50} was expressed in terms of number of hemorrhagic spots measuring 10 mm in average cross-diameter. The potencies were determined relative to the standard antivenom by the parallel line method (12,13).

Estimation of stability: The stability test was performed at KFDA (7). The stability of the candidate was determined by accelerated thermal degradation test. In standard routine quality control testing, the accelerated thermal degradation test is performed by keeping the antitoxin vials at 20°C, 37°C, and 45°C for 3, 6, and 9 months in triplicate. In the present study, for simplicity, only one vial was subjected to the assay at each temperature and time. The stability of the candidate was determined by comparing the antivenom potencies of vials stored at 20°C, 37°C, and 45°C against that of vials kept at 4°C.

Estimation of safety: At SIBP, the candidate was subjected to pH testing, sterility testing, and pyrogen testing for suitability as a Chinese commercial mamushi antivenom. In the pH test, the hydrogen ion concentration of the candidate was measured with a pH meter using a glass electrode. In the sterility tests, the candidate was determined for freedom from microorganisms. In the pyrogen test, the pyrogenic activity of the candidate was determined based on febrile response of the rabbit to intravenous injection of the candidate. These methods were conducted according to the procedures prescribed in the Chinese minimum requirements (8).

RESULTS

Determination of anti-lethal titer: At SIBP, the anti-lethal titer was examined three times using 10 vials of the candidate to confirm the homogeneity of the candidate preparation. The titer of the candidate was determined to be 29,450 ± 1,650 U/vial (Table 2). No significant difference in anti-lethal potency was observed among the three examinations ($P = 0.05$). Then, the titers were determined in collaboration of NIID, Kaketsuken, and KFDA. As shown in Table 3, the titers were 31,437 (95% confidence interval: 29,111 - 33,949 U/vial at NIID, 31,572 (27,066 - 36,827) U/vial at Kaketsuken, and 36,391 (32,832 - 40,335) U/vial at KFDA. The general common potency of the anti-lethal titer determined from the results of the nine tests performed at these three facilities was 32,909 (31,080 - 34,846) U/vial, which was rounded off to 33,000 U/vial.

Determination of anti-hemorrhagic titer: At SIBP, the anti-hemorrhagic titer of the candidate was determined three times using 10 vials of the candidate to confirm the homogeneity of the candidate preparation. Potency testing indicated that the anti-hemorrhagic titer was 31,000 ± 2,550 U/vial (Table 2). No significant difference in anti-hemorrhagic potency was observed among three tests ($P = 0.05$). Then, the anti-hemorrhagic titer was measured in collaboration of three facilities. The results were 34,454 (95% confidence interval: 33,112 - 35,850) U/vial at NIID (triplicate assays), 37,543 U/vial at Kaketsuken (single assay), and 36,063 (34,411-37,793) U/vial at KFDA and NIID (triplicate assays) (Table 4). From the results of seven tests performed at these

Table 2. Homogeneity for the candidate mamushi antivenom by potency tests at SIBP

vial No.	anti-lethal titer			anti-hemorrhagic titer		
	1	2	3	1	2	3
1	30,000	28,680	28,680	30,000	30,000	30,000
2	28,680	30,070	27,360	30,000	30,000	30,000
3	31,440	28,680	24,960	30,000	30,000	30,000
4	28,680	27,360	31,440	30,000	30,000	30,000
5	31,440	30,070	31,440	30,000	30,000	30,000
6	30,000	27,360	31,440	30,000	30,000	30,000
7	28,680	30,070	31,440	30,000	37,500	30,000
8	28,680	30,070	28,620	30,000	37,500	30,000
9	28,680	30,070	28,620	30,000	30,000	37,500
10	31,440	27,360	31,440	30,000	30,000	37,500
geometric mean	29,770 ± 1,200	28,980 ± 1,180	29,560 ± 2,140	30,000	31,500 ± 3,000	31,500 ± 3,000
general geometric mean		29,450 ± 1,650			31,000 ± 2,550	

Potency tests were performed using 10 vials in triplicate. (U/vial)

Table 3. Collaborative study for anti-lethal titer determination

facility	test	potency	95% confidence interval
Japan NIID[1)]	1	30,594	
	2	31,999	
	3	31,328	
	common potency	31,437	29,111 - 33,949
Japan Kaketsuken[2)]	1	36,310	
	2	29,036	
	3	27,342	
	common potency	31,572	27,066 - 36,827
Korea KFDA[3)]	1	31,256	
	2	46,114	
	3	37,638	
	common potency	36,391	32,832 - 40,335
general common potency		32,909	31,080 - 34,846

(U/vial)

[1)]: National Institute of Infectious Diseases.
[2)]: The Chemo-Sero-Therapeutic Research Institute.
[3)]: Korea Food and Drug Administration.

Table 4. Collaborative study for anti-hemorrhagic titer determination

facility	test	potency	95% confidence interval
Japan NIID	1	37,847	
	2	33,033	
	3	29,561	
	common potency	34,454	33,112 - 35,850
Japan Kaketsuken		37,543	
Korea KFDA	1	42,607	
	2	35,402	
	3	42,607	
	common potency	36,063	34,411 - 37,793
general common potency		36,226	35,440 - 37,030

Abbreviations are in Table 3. (U/vial)

three facilities, the general common potency anti-hemorrhagic titer was 36,226 (35,440 - 37,030) U/vial, which was rounded off to 36,000 U/vial.

Estimation of stability: The results of the accelerated thermal degradation test performed at KFDA are shown in Table 5. The titers of the candidate stored at 20°C, 37°C, and 45°C for 9 months were 92.9, 83.1, and 83.1% of that stored at 4°C, respectively. There were no statistically significant differences between potencies and regression coefficients of the candidates stored at these temperatures for these periods ($P = 0.05$). These results indicated that there was no significant loss of anti-lethal activity of the candidate with increasing storage temperature or storage time.

Estimation of safety: The pH 6.9 determined by pH test was within the range (6.8 - 7.4) described in the Chinese minimum requirements (8). In the sterility test, there was no evidence of microbial growth. Thus, the antivenom candidate met the requirements of the test for sterility (8). The results of the pyrogen test fulfilled the requirements (8) regarding pyrogenicity.

DISCUSSION

For quality control of biological products, such as antivenoms against the bites of poisonous snakes with restricted geographical distributions or those with wider distributions but with significant geographical variation in venom activity, the WHO recommends the establishment of standard materials by individual country or by region (eastern Asia, etc.), because of the difficulty in coverage of such a wide variety of snake venoms by WHO (14). Standard mamushi antivenom is one such case. Thus, we established a regional reference mamushi antivenom for common use in China, Korea, and Japan. The antivenom raised against the venom of the snakes from China is capable of completely neutralizing those of the same species from Korea and Japan (15). Thus, we chose Chinese mamushi venom as an immunogen and the regional reference antivenom was produced in SIBP in China. From the viewpoint of improving the accuracy of the quality control test, a standard material was required to have characteristics similar to a commercial antivenom. This candidate was prepared at SIBP using the same manufacturing procedure as used for the commercial antivenom. The safety tests performed at SIBP indicated that the candidate had characteristics similar to the commercial products. The results of the stability tests performed at KFDA indicated that the candidate was sufficiently stable on long-term storage. The candidate showed an anti-lethal activity titer of 33,000 U/vial and an anti-hemorrhagic activity titer of 36,000 U/vial. The three participating countries evenly shared 3,000 vials of products (1,000 vials/country) and they will be used in

Table 5. Stability for the candidate mamushi antivenom by accelerated thermal degradation test at KFDA

	stored at (°C)	stored for 3	6	9	common (months)
Potency[1)] 95% confidnce interval	20	0.825 (0.635 - 1.011)	0.830 (0.974 - 0.974)	0.929 (0.799 - 1.078)	0.870 (0.792 - 0.954)
	37	0.710 (0.544 - 0.866)	0.860 (0.743 - 0.997)	0.831 (0.715 - 0.964)	0.818 (0.747 - 0.964)
	45	0.872 (0.663 - 1.098)	0.837 (0.717 - 0.971)	0.831 (0.715 - 0.964)	0.840 (0.766 - 0.921)
	common	0.796 (0.703 - 0.902)	0.843 (0.775 - 0.916)	0.862 (0.794 - 0.936)	0.842 (0.799 - 0.888)
Regression coefficient[2)]	20	10.716	14.246	12.834	12.281
	37	11.003	17.363	12.722	12.090
	45	8.959	16.411	12.722	11.681
	common	10.213	15.954	12.760	12.386

[1)]: Anti-lethal titers relative to the candidate stored at 4°C for the same period.
[2)]: Regression coefficient of potency.

routine quality control tests in the region.

The anti-lethal titers of the commercial products are determined in all of Japan, Korea, and China as lot-release tests. The major difference in the methods used for anti-lethal titer determination among these three countries is the route of administration of neutralized venom into mice: intravenous administration is employed in Korea (7) and Japan (6), while intraperitoneal administration is performed routinely in China (8). The route of administration was not the subject of calibration in the present collaborative study, with each country following its own method. This study design was designed based on practical considerations concerning the routine use of the product, and we found no significant differences in the results related to route of administration. However, the interchangeability of route of administration should be confirmed in future studies. Titer determination against hemorrhagic activities of venom is performed routinely for quality control testing in Japan and Korea. However, anti-hemorrhagic titer determination is not recommended by the WHO from the viewpoint of animal welfare (14). Thus, whether anti-hemorrhagic titer determination is necessary should be discussed further.

This study is the first to establish a regional reference antivenom as recommended by the WHO (14). Venomous snakes, spiders, and cnidarians inhabit restricted regions or areas, and various antivenom products have been used on a regional basis for treatment of their bites and stings. Regional reference antivenoms are required for quality control of such antivenom products. Thus, this international collaborative study will provide important information and experience for the establishment of regional reference standards.

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