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STANDARDIZATION OF AN ANTIHEMORRHAGIC POTENCY TEST OF ANTIVENOMS PREPARED FROM TWO DIFFERENT *Agkistrodon halys* VENOMS

LEE K. H. (1), HUR S. J. (1), KIM S. N. (1), YOO S. H. (1), SHIN I. S. (1), WON H. J. (1), SHIN K. H. (1), HONG S. H. (1), LEE S. H. (2), MIN H. K. (1), PARK S. N. (1,2)

(1) Department of Biologics Evaluation, Korea Food and Drug Administration, Seoul, Korea; (2) National Institute of Toxicological Research, Korea Food and Drug Administration, Seoul, Korea.

ABSTRACT: To establish Korea National Standards for venoms and antivenoms, it is necessary to have standardized assay methods. In this study, we standardized a method to evaluate the antihemorrhagic potency of two horse-derived antivenoms using rabbit intracutaneous injection. We expressed the capability of these antivenoms to neutralize the hemorrhagic activities triggered by the venoms of *Agkistrodon halys* from Japan and Jiangzhe *Agkistrodon halys* from China as Minimum Hemorrhagic Dose (MHD). We also performed cross-neutralization tests employing the parallel line assay on different pairings of venoms and antivenoms to check the possibility of using Jiangzhe *Agkistrodon halys* venom as a substitute for the standard *Agkistrodon halys* venom in measurements of the antihemorrhagic activity, since *A. halys* venom is not easily available. Slope function ratio (S.R.) was 0.957 for *Agkistrodon halys* venom either with *Agkistrodon halys* antivenom or with Jiangzhe *Agkistrodon halys* antivenom ($p>0.05$). Similarly, S.R. was 0.348 for Jiangzhe *Agkistrodon halys* venom either with *Agkistrodon halys* antivenom or with Jiangzhe *Agkistrodon halys* antivenom ($p>0.05$). Thus, in this study we established antihemorrhagic potency test methods for both *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms and we could also show it is possible to use Jiangzhe *Agkistrodon halys* venom as a standard.

KEY WORDS: antihemorrhagic potency, minimum hemorrhagic dose, Jiangzhe *Agkistrodon halys*, parallel line assay.

CORRESPONDENCE TO:
SUE NIE PARK, Blood Products Division, Department of Biologics Evaluation, Korea Food and Drug Administration, 231, Jinheoungno, Eunpyeong-Gu, Seoul 122-704, Korea. Phone: 82 2 380 1711. Fax: 82 2 383 8322 or 82 2 380 1349. Email: suenie@kfda.go.kr.

INTRODUCTION

Hemorrhagic proteinases, or hemorrhagic factors [HRs] (7), are largely present in the venoms of snakes from the genus *Agkistrodon* (*Crotalidae* family) and those from the *Viperidae* family. They have been purified from several snake venoms and some of their properties have been investigated (4). It has been reported that HRs are metalloproteinases but their enzymatic properties are apparently different from one another.

Hemorrhagic activities seem to play a leading role in the lethality of *Agkistrodon halys* venom (8). Some pharmacists insist that purified hemorrhagic components of *A*. *halys* venom are immunologically related (2) but their mechanism of hemorrhagic activity is still unclear.

The antihemorrhagic potency of *A. halys* antivenom can be determined by multiple level beta-procedure in which the amount of antivenom varies with a fixed dose of venom (6). The hemorrhagic fractions of *A. halys* venom should be used as test toxins. The effective dose (ED) of both the test and the standard antivenoms should be determined with no less than three doses of the test toxin according to the method described by Kondo *et al.* (5). Parallelism and linearity of the neutralization curves should be ascertained by statistical analyses. Antihemorrhagic potency of the test antivenom should be determined by measuring the relationship between its potency and the standard antivenom potency according to the parallel line assay (3) using neutralization curves.

In our laboratory, we established an antihemorrhagic potency test using *A. halys* antivenoms against *A. halys* venom. We confirmed that the antivenoms are effective in neutralizing the most relevant hemorrhagic effects of *A. halys* venom.

In this study, we first tested and compared *A. halys* and Jiangzhe *A. halys* antivenoms against *A. halys* venoms. We also attempted to determine whether cross-neutralization of hemorrhage occurs experimentally and whether antivenoms produced from Jiangzhe *A. halys* and *A. halys* venoms are capable of neutralizing not only their own specific venom but also venoms from other species by using rabbit intracutaneous injection. This way we tried to check the possibility of using Jiangzhe *A*. *halys* venom as a substitute for the standard *A*. *halys* venom in antihemorrhagic activities, since *A*. *halys* venom is not readily available in Korea.

MATERIALS AND METHODS

Antivenom

Test antivenoms were: national standard equine *Mamushi* antivenom (Lot C, antihemorrhagic titer: 23,430 U/ampoule), produced by the National Institute of Infectious Diseases (NIID) in Japan and equine Jiangzhe *Agkistrodon halys*

antivenom, produced by the Shanghai Institute of Biological Products (SIBP) in China and commercially available in Korea.

Snake hemorrhagic venom

The venoms used were *Mamushi* venom (Hemorrhagic – hemorrhagic titer: 1,100 test doses/ampoules, Lot 3-2, 50 mg protein/ampoule) supplied by the National Institute of Infectious Diseases (NIID) in Japan and Jiangzhe *A. halys* venom by the Shanghai Institute of Biological Products (SIBP), in China.

Immediately before use, venoms were dissolved in 17 mM phosphate buffered sodium chloride solution (PBS, pH 7.0) containing 0.2 w/v % gelatin to a desired concentration.

Experimental animals

Male and female adult white rabbits of approximately 2.5 kg were utilized to determine the hemorrhagic activity of venoms and the antihemorrhagic activity of antivenoms.

Hemorrhagic activity

The hemorrhagic activity of venoms was assayed by the method developed by Kondo *et al.* (5). From four to five different doses of each venom diluted in 0.1 ml PBS at increasing concentrations as needed were intracutaneously injected into the depilated skin on the back of 3 rabbits. Rabbits were sacrificed by chloroform inhalation 24 h after injection and the tested skin areas were immediately removed and placed on a glass plate so as not to distort their original shape. The cross-diameter of each hemorrhagic spot was measured from the visceral side of the skin through the glass plate, mean value was calculated and the results were statistically analyzed. The Minimum Hemorrhagic Dose (MHD) of each venom was defined as the lowest dose of venom causing hemorrhagic reaction over an area of approximately 9 mm in diameter 24 h after the intracutaneous injection. The MHD of a test sample is determined by interpolation of its dose-response curve using the Statistical Analysis Program STA77.

Antivenom effective dose by the skin test

An effective dose (ED) is defined as the quantity of antivenom that, when mixed with a given dose of a test toxin, causes a hemorrhagic spot of approximately 9 mm in diameter. We prepared several dilutions of a test toxin containing 47 MHD of *A. halys* venom in a 0.1-ml volume. To determine dose-response curves and measure cross-reactivity, venoms were mixed with equal volumes of antivenom diluted in solutions

of 1.25-fold increments in concentration. All mixtures were incubated for 1 h at room temperature. Then, 0.2 ml of each mixture was injected into the depilated skin on the back of three rabbits and hemorrhagic response after 24 h was observed from the visceral side of the removed skin using the method developed by Kondo *et al.* (5).

Unit of antihemorrhagic activity

One unit of antihemorrhagic activity was defined as the amount of specific antibody that neutralized 47 MHD of *A. halys* venom and 43 MHD of Jiangzhe *A. halys* venom. The antihemorrhagic potency is expressed as units per ml of antivenom. The effective dose (ED_{10mm}) of an antivenom with a test toxin at 47 MHD of *A. halys* venom resulting in a 10mm-diameter hemorrhagic spot corresponds to one unity of antihemorrhagic activity.

RESULTS

Hemorrhagic activities of venoms

Each venom (4-5 different doses) was intracutaneously injected into the depilated skin on the back of 3 rabbits. *Agkistrodon halys* venom was diluted to six concentrations: 0.1 µg, 0.3 µg, 0.9 µg, 2.7 µg, 8.1 µg, and 24.3 µg in 0.1-ml solutions; Jiangzhe *A. halys* venom was also diluted to: 0.1 µg, 0.3 µg, 0.9 µg, 2.7 µg, 8.1 µg, and 24.3 µg in 0.1-ml solutions (Table 1).

Expression of the hemorrhagic activities of *A. halys* toxins usually does not directly correlate with their weight in µg. It is necessary to express their weight and biological activities (MHD, etc) to represent their characteristics because the purities of toxins from *A*. *halys* and Jiangzhe *A*. *halys* are different.

The MHD of each venom was determined by the emergence of a hemorrhagic spot of approximately 9 mm in diameter on the visceral side of the removed skin of rabbits (Figure 1). The MHD of *A. halys* venom was 1.78 µg / 0.1 ml and that of Jiangzhe *A. halys* venom was 0.42 µg / 0.1 ml (Table 2).

When the hemorrhagic activity of *A*. *halys* venom was considered as 1 unit of virulence, the relative virulence of Jiangzhe *A. halys* venom was approximately 4.2 (Table 2).

Response to the MHD showed a linear correlation between *A. halys* venom and Jiangzhe *A. halys* venom (data not presented). The MHDs of *A. halys* and Jiangzhe *A*. *halys* venoms were estimated by the Reed and Muench method (9). When the mean diameter of the red spot exceeded 9 mm, it was considered as a positive reaction.

Determination of test doses of venoms

The hemorrhagic activities of *A. halys* and Jiangzhe *A. halys* venoms were determined using fixed amounts of *A. halys* and Jiangzhe *A. halys* antivenoms. Results of the tests carried out to determine one test dose of *A. halys* venom against 1 unit of antihemorrhagic activity of *A. halys* and Jiangzhe *A. halys* antivenoms are shown in Table 3 and Figures 2 and 3.

The weight range of *A. halys* venom used was between 22.72 µg and 136.36 µg.

When the antihemorrhagic potency of *A. halys* antivenom was considered as 1 unit/ml that of Jiangzhe *A. halys* antivenom was 0.837 unit/ml. *Agkistrodon halys* antivenom appears to be slightly more effective than Jiangzhe *A. halys* antivenom when their weights are compared.

We also determined one test dose of Jiangzhe *A. halys* venom against 1 unit of antihemorrhagic activity of either *A. halys* or Jiangzhe *A. halys* antivenom (Table 4 and Figure 4). The weight range of Jiangzhe *A. halys* venom used was between 6.0 µg and 486 µg.

In this case, when the antihemorrhagic potency of *A. halys* antivenom was considered as 1 unit/ml that of Jiangzhe *A. halys* antivenom was 1.287 units/ml.

Determination of hemorrhagic test doses of the toxins

The hemorrhagic test doses of *A. halys* and Jiangzhe *A. halys* venoms were calculated and are shown in Table 5.

Neutralization reactions of *A. halys* venom correlated well with the antivenoms used and demonstrated a first-order relationship (Figure 4). The test dose of *A. halys* venom against 1 unit of *A. halys* antivenom was 60.78 µg, and for 1 unit of Jiangzhe *A. halys* antivenom it was about 76.43 µg (Table 5). The test dose of Jiangzhe *A. halys* venom against either 1 unit of *A. halys* or Jiangzhe *A. halys* antivenom was approximately 10.4 µg.

Antihemorrhagic Potency of the antivenoms

Three different amounts of *A. halys* antivenom and Jiangzhe *A. halys* antivenom against *A. halys* venom were used to determine antihemorrhagic potency test doses (Table 6). The test dose of *A. halys* venom used was 83.0 µg (47 MHD). Antihemorrhagic potency of an antivenom was estimated by analyzing the relationship between its potency and the standard antivenom potency in the parallel line assay. Relative potency of the two antivenom preparations against *A. halys* venom was estimated by the ratio between their regression coefficients. Slope ratios of both *A. halys* and Jiangzhe *A. halys* antivenoms against *A. halys* venom were 0.957 (Figures 5 and 6). Results of the log-dosage response curves were linear and

parallel (p>0.05). When the antihemorrhagic potency of *A. halys* antivenom was considered as 1 unit/ml that of Jiangzhe *A. halys* antivenom was 0.991 unit/ml (Table 6).

Five different amounts of *A. halys* antivenom and Jiangzhe *A. halys* antivenom against Jiangzhe *A. halys* venom were used to determine antihemorrhagic potency test doses (Table 7). The test dose of Jiangzhe *A. halys* venom was 18 µg (43 MHD). Relative potency of the two antivenom preparations against Jiangzhe *A. halys* venom was estimated by the ratio between their regression coefficients. Slope ratios of both *A. halys* and Jiangzhe *A. halys* antivenoms against Jiangzhe *A. halys* venom were 0.348 (Figure 7). Results of the log-dosage response curves were linear and parallel (p>0.05). When the antihemorrhagic potency of *A. halys* antivenom was considered as 1 unit/ml that of Jiangzhe *A. halys* antivenom was 1.156 units/ml (Table 7).

Antihemorrhagic potency of Jiangzhe *A. halys* **antivenoms**

The test method established in this study was utilized in order to check the possibility of adopting it in the determination of antihemorrhagic potency of an unknown antivenom sample in the lot release test of *Jiangzhe A. halys* antivenom. The current lot release test in Korea requires potency higher than 300 units/ml. The antihemorrhagic activity of the unknown sample applied for lot release was tested using 20 units of *A. halys* antivenom (330 U/ml) as control. Detailed experiments are shown in Table 8. We used 5 different rabbits for testing 5 different dilutions of antivenoms. For each dilution, five spots were injected. $ED_{10\ mm}$ for the control and the unknown sample was 0.4308 ml and 0.4204 ml, respectively. Antivenom potency of the unknown sample was found to be 300 units/ml not only by the current lot release test method in Korea (data not shown) but also by the newly established method in this study.

Table 1: Diameters of response spots triggered by the intracutaneously injected venoms.

Agkistrodon halys							Jiangzhe *Agkistrodon halys*						
Venom Dose (µg)	Spot1 (mm)	Spot2 (mm)	Spot3 (mm)	Spot4 (mm)	Spot5 (mm)	Mean (mm)	Venom Dose (µg)	Spot1 (mm)	Spot2 (mm)	Spot3 (mm)	Spot4 (mm)	Spot5 (mm)	Mean (mm)
0.1	-	-	-	-	-	-	0.1	-	-	-	-	-	-
0.3	-	-	-	-	-	-	0.3	7.6	7.4	9.1	-	9.0	8.2
0.9	-	7.6	7.1	-	7.7	7.5	0.9	8.7	10.9	9.7	9.8	9.9	9.8
2.7	8.9	10.7	11.5	9.7	10.4	10.2	2.7	11.8	11.8	12.4	11.6	15.0	12.5
8.1	9.6	11.3	12.0	12.3	11.9	11.4	8.1	13.6	14.4	15.9	17.0	13.3	14.8
24.3	11.9	13.1	15.0	14.5	13.8	13.7	24.3	16.4	17.3	19.5	17.7	19.3	18.0

Table 2: Minimum Hemorrhagic Doses (MHD) of venoms and their relative virulence.

Venoms	MHD (µg)	Relative virulence
Agkistrodon halys venom	1.78 (0.92~3.46)*	1
Jiangzhe *Agkistrodon halys* venom	0.42 (0.18~0.99)*	4.2 (3.089~6.085)*

* 95% Confidence Interval

Table 3: Measurements of the neutralization of *Agkistrodon halys* venom by one unit of antihemorrhagic activity (1U) of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms.

Antivenoms (1 U)	*Agkistrodon halys* Antivenom				Jiangzhe *Agkistrodon halys* Antivenom			
A. halys venom (µg)	136.36	90.90	45.45	22.72	136.36	90.90	45.45	22.72
Rabbit 1 (mm)	8.9	9.1	4.6	4.9	12.2	8.3	4.9	4.8
Rabbit 2 (mm)	16.3	13.9	6.3	9.1	19.7	12.5	4.9	3.3
Rabbit 3 (mm)	12.6	11.5	9.1	6.5	-	10.3	5.6	4.2
Mean (cm)	1.087	1.054	0.807	0.821	1.190	1.010	0.710	0.608
Variance	0.017	0.008	0.022	0.018	0.022	0.008	0.001	0.007

- No response

Factor	Sum of Squares	Degree of Freedom	Mean Squares	F test (Variance ratio)
Preparations	0.042	1	0.0416	3.00
Regression	2.466	1	2.4665	177.79*
Parallel	0.051	1	0.0510	3.68
Linearity	0.111	4	0.0277	2.00
Between Doses	2.670	7	0.3814	27.49*
Error	0.888	64	0.0139	
Total	3.558	71		

Relative potency of Jiangzhe *Agkistrodon halys* antivenom = 0.837 U/ml

95% Confidence Interval = 0.677~1.028

A. halys: Y = 0.624X – 0.191

Jiangzhe *A. halys*: Y = 0.624X – 0.239

* 1% Significance Level

Table 4: Measurements of the neutralization of Jiangzhe *Agkistrodon halys* venom by one unit of antihemorrhagic activity (1U) of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms.

Antivenoms (1U)	*Agkistrodon halys* antivenom					Jiangzhe *Agkistrodon halys* antivenom			
Jiangzhe *A. halys* venom (µg)	6.0	18.0	54.0	162.0	486.0	6.0	18.0	54.0	162.0
Rabbit 1 (mm)	-	12	16	18.5	22	-	13	16.5	19.5
Rabbit 2 (mm)	-	13	20.5	21.4	20	-	13.5	18	17
Rabbit 3 (mm)	-	14.5	14	18.5	23	-	14	20	18.5
Mean (mm)	-	13.167	16.833	19.467	21.667	-	13.500	18.167	18.333
Variance	-	1.583	11.083	2.803	2.333	-	0.250	3.083	1.583

- No response

Factor	Sum of Squares	Degree of Freedom	Mean Squares	F test (Variance ratio)
Preparations	0.254	1	0.2541	0.09
Regression	282.572	1	282.5720	97.51*
Parallel	3.808	1	3.8080	1.31
Linearity	17.396	3	5.7987	2.00
Between Doses	304.030	6	50.6717	17.49*
Error	92.733	32	2.6979	
Total	396.764	38		

Relative potency of Jiangzhe *Agkistrodon halys* antivenom = 1.287

95% Confidence Interval = 0.830~1.984

A. halys: Y = 6.017X + 5.251

Jiangzhe *A. halys*: Y = 6.017X + 5.910

*1% Significance Level

Table 5: Determination of the Hemorrhagic Test Dose of the venoms tested in rabbits.

Antivenoms / Venom (µg)	*Agkistrodon halys* (1 U)	Jiangzhe *Agkistrodon halys* (1 U)
Agkistrodon halys	60.78 µg (42.44~87.05)*	76.43 µg (54.16~107.86)*
Jiangzhe *Agkistrodon halys*	10.4 µg (6.0~18.0)*	10.4 µg (6.0~18.0)*

The test dose of a venom was expressed as g / 2.5 kg rabbit.

* 95% Confidence Interval

Table 6: Results of the antihemorrhagic potency (AHP) test of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms against *Agkistrodon halys* venom containing 83 µg (47 MHD) of the test toxin.

Type of Antivenom		*Agkistrodon halys* antivenom (U)			Jiangzhe *Agkistrodon halys* antivenom (U)		
Amount of antivenom		0.64	0.8	1	0.64	0.8	1
Diameters of hemorrhagic spots in millimeter (AHP)	Rabbit 1	12.5	9.1	9.9	15.8	13.7	10.2
	Rabbit 2	15.8	6.7	7.6	11.8	8.3	6.5
	Rabbit 3	8.8	9.3	8.2	13.2	8.4	7.5
Mean (cm)		1.080	0.918	0.930	1.130	0.993	0.899
Variance		0.016	0.006	0.003	0.004	0.015	0.010

Factor	Sum of Squares	Degree of Freedom	Mean Squares	F test (Variance ratio)
Preparations	0.000	1	0.0001	0.01
Regression	0.172	1	0.1721	12.20*
Parallel	0.002	1	0.0024	0.17
Linearity	0.009	2	0.0044	0.32
Between Doses	0.183	5	0.0367	2.60
Error	0.339	24	0.0141	
Total	0.522	29		

Relative potency of Jiangzhe *Agkistrodon* halys antivenom = 0.991

95% Confidence Interval = 1.288~0.755

A. halys: Y = 0.957X + 0.874

Jiangzhe *A. halys*: Y = 0.957X + 0.878

* 1% Significance Level

Table 7: Results of the antihemorrhagic potency (AHP) test of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms against Jiangzhe *Agkistrodon halys* venom containing 18 µg (43 MHD) of the test toxin.

Type of Antivenoms		*Agkistrodon halys* antivenom (U)					Jiangzhe *Agkistrodon halys* antivenom (U)				
Amount of antivenom		0.64	0.8	1.0	1.3	1.6	0.64	0.8	1.0	1.3	1.6
Diameters of hemorrhagic spots in millimeter (AHP)	Rabbit 1	12.4	10.8	10.8	11.4	11.5	11.1	11.5	11.4	9.2	9.8
	Rabbit 2	11.8	12.8	11.9	10.7	9.1	14.5	13.8	14	9.9	8
	Rabbit 3	14.9	13.0	12.2	12.4	10.9	10.9	10.4	10	11	7.5
Mean (cm)		1.113	1.085	1.065	1.060	1.019	1.081	1.073	1.068	1.000	0.923
Variance		0.003	0.002	0.001	0.001	0.003	0.005	0.004	0.005	0.002	0.004

Factor	Sum of Squares	Degree of Freedom	Mean Squares	F test (Variance ratio)
Preparations	0.006	1	0.0060	1.04
Regression	0.123	1	0.1228	21.24*
Parallel	0.002	1	0.0016	0.28
Linearity	0.012	6	0.0021	0.36
Between Doses	0.143	9	0.0159	2.75**
Error	0.231	40	0.0058	
Total	0.374	49		

Relative potency of Jiangzhe *Agkistrodon halys* antivenom = 1.156

95% Confidence Interval = 1.665~0.861

A. halys: Y = 0.348X + 1.027

Jiangzhe *A. halys*: Y = 0.348X +1.005

* 1% Significance Level

** 5% Significance Level

Table 8: Antihemorrhagic toxin titers of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms against 83 µg-leveled *Agkistrodon halys* venom.

Antivenoms		Rabbit 1	Rabbit 2	Rabbit 3	Rabbit 4	Rabbit 5	$ED_{10\ mm}$
	Buffer (ml)	0.68	0.6	0.5	0.37	0.2	
	A.V. (ml)	0.32	0.4	0.5	0.63	0.8	
Agkistrodon halys (Japan) as control	(mm)	13.0x12.0	9.82x8.38	10.1x9.74	6.85x8.32	6.9x5.2	0.4308 ml
		19.6x11.84	7.0x6.46	8.3x6.9	-	-	
		8.42x8.7	9.84x8.74	8.14x8.26	-	-	
		12.0x8.0	7.0x9.7	11.46x9.66	-	-	
		11.84x11.88	8.7x11.68	4.74x4.84	-	-	
Jiangzhe *Agkistrodon halys* (China)	(mm)	16.74x15.14	15.24x12.22	11.80x8.6	6.6x3.6	4.76x3.1	0.4203 ml
		13.4x10.25	8.2x8.46	6.7x6.32	-	-	
		14.62x11.86	8.76x8.1	8.08x7.0	5.14x6.4	-	
		13.1x9.8	13.3x8.2	11.7x9.73	7.78x4.9	2.0x2.0	
		9.8x8.56	6.8x5.3	4.0x5.0	-	-	

- No response

A.V.: Antivenoms

$ED_{10\ mm}$: Effective dose of antivenom in neutralizing 20 U/ml venom resulting in 10mm-diameter hemorrhagic spots.

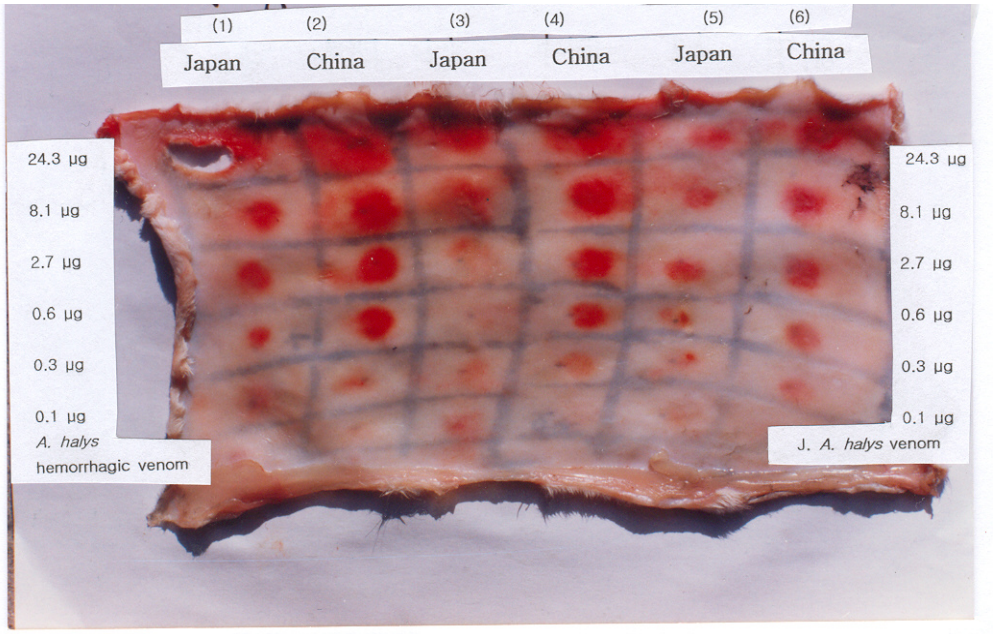

Figure 1: Minimum hemorrhagic doses and appearance of rabbit skin intracutaneously injected with either *Agkistrodon halys* [*A. halys*] venom from Japan (1, 3, 5) or Jiangzhe *Agkistrodon halys* [J. *A. halys*] venom from China (2, 4, 6).
A, B, C: Experiments carried out by three different experimenters.

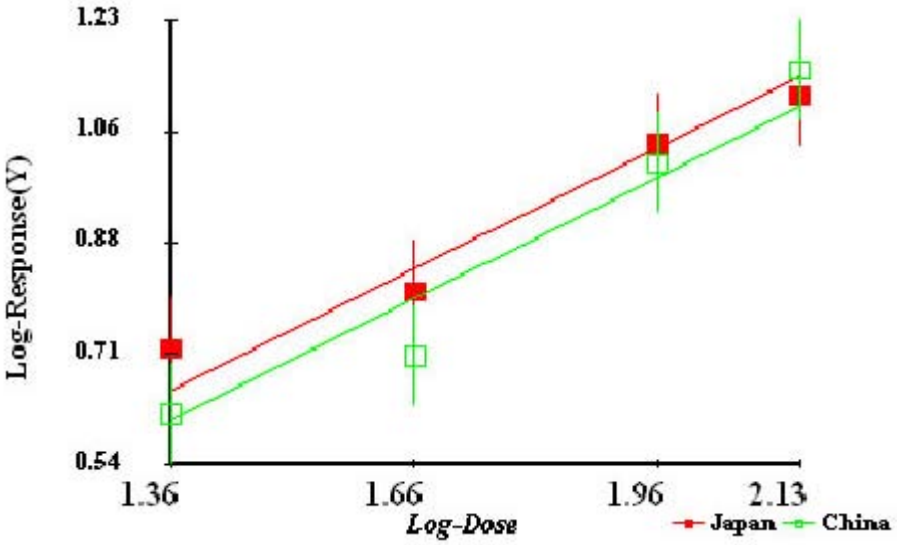

Figure 2: Graphical analysis of the neutralization of *Agkistrodon halys* venom by one unit of hemorrhagic activity (1U) of *Agkistrodon halys* (Japan) and Jiangzhe *Agkistrodon halys* (China) antivenoms.

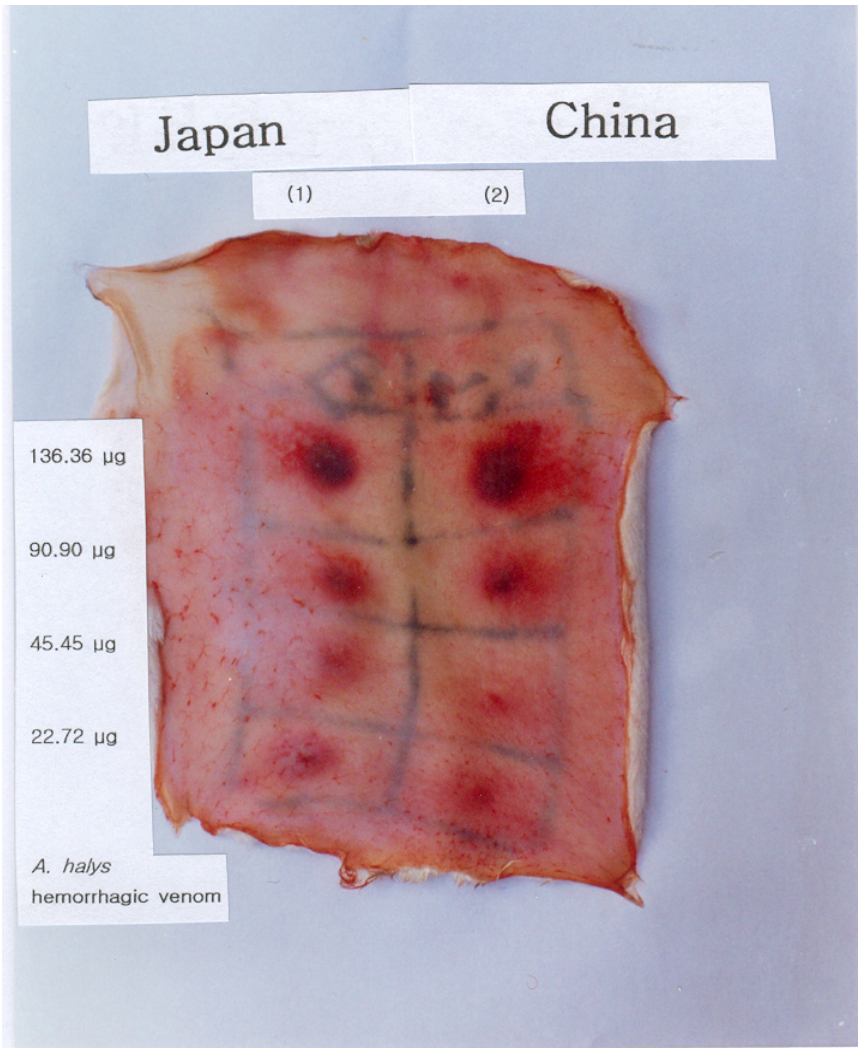

Figure 3: Hemorrhagic spots on the skin of rabbits intracutaneously injected with one unit of antihemorrhagic activity of *Agkistrodon halys* antivenom from Japan (1) and Jiangzhe *Agkistrodon halys* antivenom from China (2) against the test dose of *Agkistrodon halys* venom for determination of their antihemorrhagic titers.

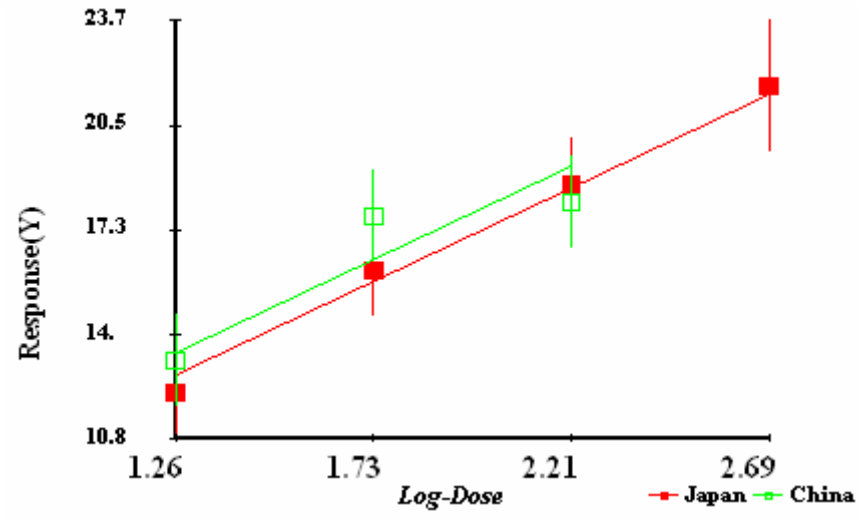

Figure 4: Graphical analysis of the neutralization of Jiangzhe *Agkistrodon halys* venom by one unit of antihemorrhagic activity (1U) of *Agkistrodon halys* (Japan) and Jiangzhe *Agkistrodon halys* (China) antivenoms.

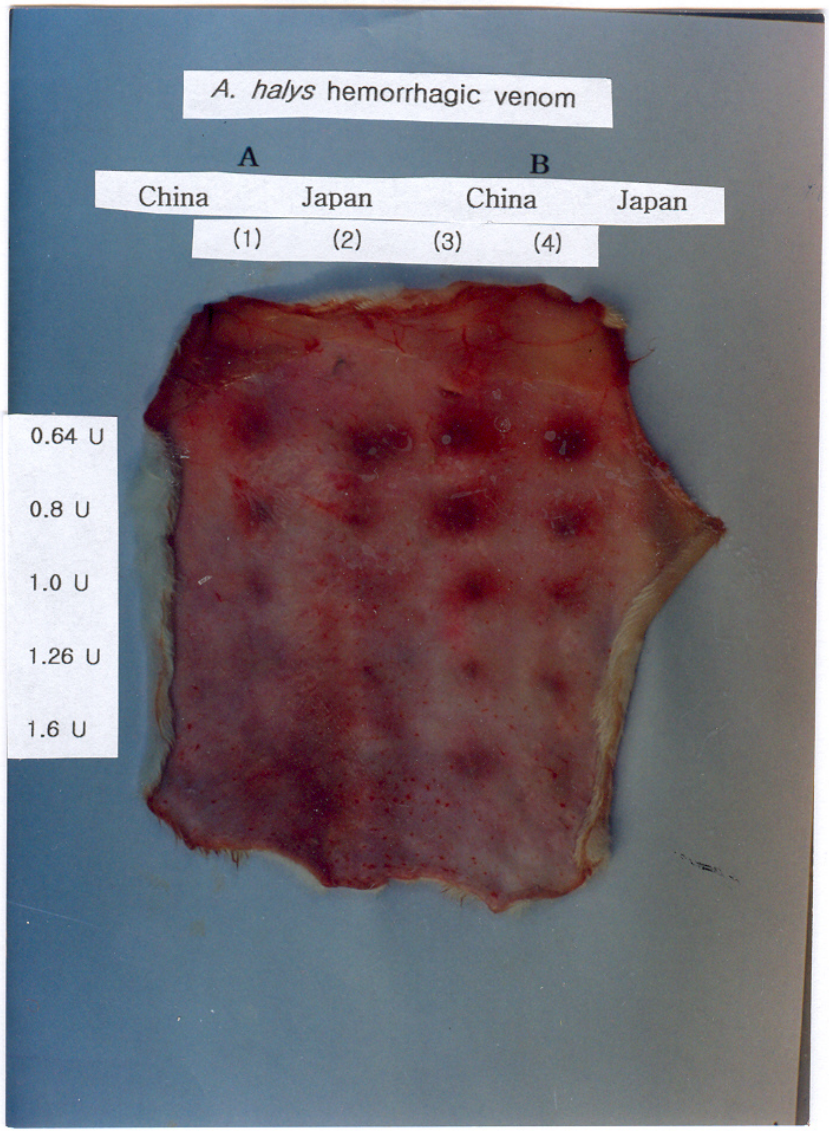

Figure 5: Neutralization of *Agkistrodon halys* venom by *Agkistrodon halys* antivenom from Japan (2, 4) and Jiangzhe *Agkistrodon halys* antivenom from China (1, 3). Equal volumes of each diluted antivenom (0.64 U/ml-1.0 U/ml) were mixed with 83 µg of *Agkistrodon halys* venom

A, B: Duplicate tests.

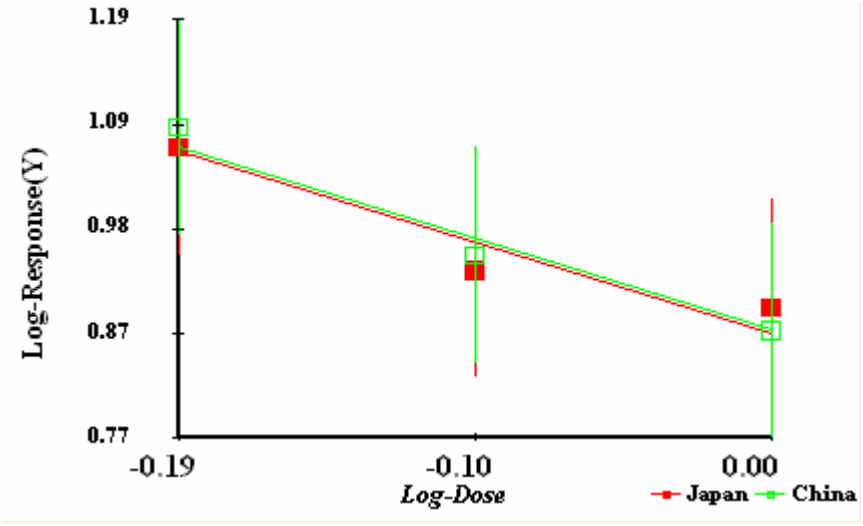

Figure 6: Slope function and parallelism of the hemorrhagic neutralization of *Agkistrodon halys* venom by *Agkistrodon halys* (Japan) and Jiangzhe *Agkistrodon halys* (China) antivenoms.

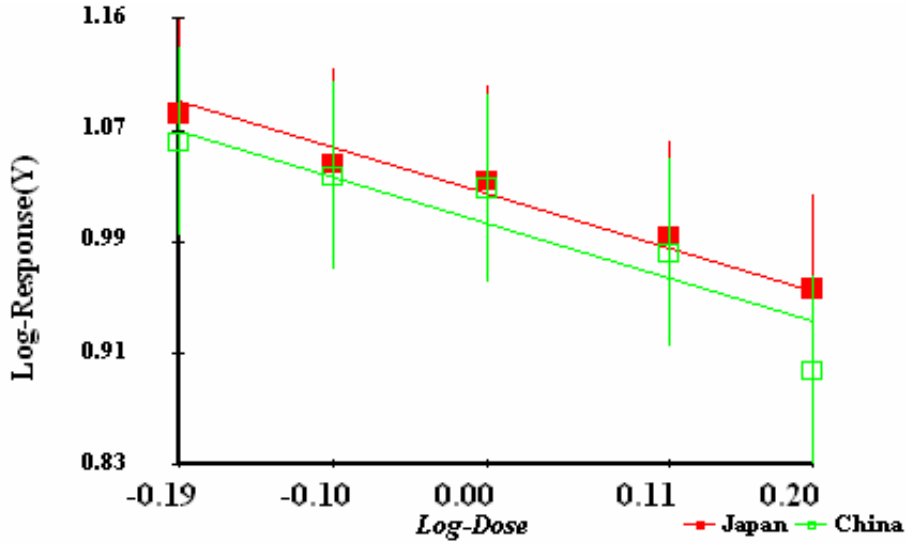

Figure 7: Slope function and parallelism of the hemorrhagic neutralization of Jiangzhe *Agkistrodon halys* venom by *Agkistrodon halys* (Japan) and Jiangzhe *Agkistrodon halys* (China) antivenoms.

DISCUSSION

It has been demonstrated that the hemorrhagic factors present in *A. halys* and Jiangzhe *A. halys* venoms have distinct immunological specificities. Furthermore the antihemorrhagic activities of their antivenoms can be determined only when individual factors are used separately as test toxins instead of the crude venom.

The most widely used type of simultaneous trial is that in which a simple response metameter has a linear regression on its log dose (3).

For such an assay, it is required that the lines for the standard (*A. halys* antivenom) and the test preparation (Jiangzhe *A. halys* antivenom) be parallel. In view of the important role played by the hemorrhagic as well as the lethal activity of *A. halys* venom in snake envenoming, the potency of *A. halys* antivenom should be determined according to its antihemorrhagic and anti-lethal potencies. Hemorrhagic activity of venoms (7) was only suppressed by treatment with chelating agents, such as EDTA-2Na (Disodium Ethylene Diamine Tetraacetate Dihydrate), EGTA (ethyleneglycol-bis [beta-aminoethylether] N,N'-tetraacetic acid), and o-phenanthroline, suggesting that they are metalloproteinases.

During the course of our study, we found out that two hemorrhagic principles (*A. halys* HR and Jiangzhe *A. halys* HR) showed indistinguishable hemorrhagic action on rabbit skin. We also noticed that there is an extensive cross-reactivity between the hemorrhagic principles from the two venoms studied, evidenced by the capability of their antivenoms to neutralize the hemorrhagic activity of heterologous venoms. Thus, hemorrhagic metalloproteinases from the two *Agkistrodon halys* venoms tested may share relevant neutralizing epitopes (1).

Both antivenoms tested were effective in neutralizing the hemorrhagic activities of the two snake venoms used. So, in emergencies, Jiangzhe *A. halys* antivenom may be useful as an antitoxin for Japanese snakebite cases.

The MHD of *A*. *halys* venom for hemorrhagic response was 1.78 µg / 0.1 ml and that of Jiangzhe *A. halys* venom was 0.42 µg / 0.1 ml. We found out that 1 unit/ml of *A*. *halys* antivenom neutralized 50% of the hemorrhagic activity induced by 83.0 µg (47 MHD) of *A*. *halys* venom (as a challenge dose). When the test dose of *A*. *halys* venom was 83 µg (47 MHD), the antihemorrhagic potency of *A*. *halys* antivenom was 1 unit/ml in the rabbit samples used, whereas that of Jiangzhe *A. halys* was 0.991 unit/ml in the same samples. Although *A*. *halys* antivenom appears to be slightly more effective than that of Jiangzhe A. halys, they may be considered comparable. So, we can use these two antivenoms in the quality control of *A. halys* venoms. *Agkistrodon halys* and Jiangzhe *A*. *halys* antivenoms showed almost the same efficacy against Jiangzhe *A*. *halys* venom: 1 and 1.156 U/ml, respectively ($p>0.05$). So, Jiangzhe *A*. *halys* venom may be used as a substitute for *A*. *halys* venom in tests for antihemorrhagic potency of Jiangzhe *A*. *halys* antivenom.

In the slope-ratio assay, both the standard and the test venom showed straight-line and parallel dose-response curves. Slopes measure the change rate of the dependent variable as the independent variable changes, and the greater the slope the steeper the line. Jiangzhe *A*. *halys* and *A*. *halys* antivenoms showed equal

potency against *A*. *halys* venom. Their slope ratios were 0.957, which indicates positive relationship. Their log-dosage response curves were linear and parallel (p>0.05) and had common slopes. The efficacy of *A*. *halys* and J. *A*. *halys* antivenoms against Jiangzhe *A*. *halys* venom was almost equal and their slope ratios were 0.348, indicating a positive relationship (p>0.05). Their log-dosage response curves were linear and parallel (p>0.05). We concluded it is possible and useful to utilize Jiangzhe *A*. *halys* venom as a standard for testing antihemorrhagic activity since the availability of *A. halys* venom is limited in Korea.

In addition, antivenom potency of the unknown sample was found to be 300 units/ml by both the current lot release test method in Korea (data not shown) and the newly established method in this study. Thus, we may consider the unknown antivenom tested passes the current specification for antivenom lot release test in Korea using the test methods established in this study, which suggests the possibility of adopting it in the antivenom lot release system in Korea.

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