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STANDARDIZATION OF ANTI-LETHAL TOXIN POTENCY TEST OF ANTIVENOMS PREPARED FROM TWO DIFFERENT *Agkistrodon halys* VENOMS

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ABSTRACT: In Korea, antivenoms for the treatment of patients bitten by venomous snakes have been imported from Japan or China. Although there is cross-reactivity between these antibodies and venoms from snakes indigenous to Korea (e.g. *Agkistrodon* genus), protection is not optimal. Antivenoms specifically prepared to neutralize Korean snake venoms could be more effective, with fewer side effects. To this end, we established an infrastructure to develop national standards and created a standardized method to evaluate the efficacy of two horse-derived antivenoms using mouse lethal toxin test. Additionally, we determined the antivenoms neutralizing activity against lethal doses (LD_{50}) of *Agkistrodon halys* (from Japan) and Jiangzhe *Agkistrodon halys* (from China) venoms. We also performed cross-neutralization tests using probit analysis on each pairing of venom and antivenom in order to check the possibility of using Jiangzhe *A. halys* venom as a substitute for *A. halys* venom, the current standard. Slope of *A. halys* venom with *A. halys* antivenom was 10.2 and that of *A. halys* venom with Jiangzhe *A. halys* antivenom was 9.6. However, Slope of Jiangzhe *A. halys* venom with *A. halys* antivenom was 4.7 while that of Jiangzhe *A. halys* venom with Jiangzhe *A. halys* antivenom was 11.5. Therefore, the significant difference in slope patterns suggests that Jiangzhe *A. halys* venom cannot be used as a substitute for the standard venom to test the anti-lethal toxin activity of antivenoms ($p<0.05$).

KEY WORDS: anti-lethal toxin potency, Jiangzhe *Angkistrodon halys*, lethal activity, probit analysis, slope

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INTRODUCTION

Three members of the *Agkistrodon* genus: *A. brevicaudus*, *A. saxatilis*, and *A. caliginosus*, have been vastly investigated (4). *Agkistrodon* snakes are widely distributed throughout Korea, Japan, Siberia, China, Central Asia, and Eastern Europe. Some scientists (8) insist that metalloproteinases and disintegrins (nonenzymatic platelet aggregation inhibitors) are important components of most viperine and crotalic venoms, but their immunogenicity and toxicity are still not well understood.

Hospital reports of about 410 snakebite accidents per year are most frequent from the last 10 days of April to the middle of October in Korea. These incidents often occur in farm villages and mountains but their exact reason is unknown. As *Agkistrodon saxatilis* and *Agkistrodon usuriensis* inhabit relatively high altitudes, the incidence of bites by these two species is small.

To ensure the efficacy of any antivenom, the interaction between venom and antivenom should always be evaluated (9). The first preparations of Japanese *A. halys* antivenom were commercially available in 1990; Chinese Jiangzhe *A. halys* antivenom came out to the market in 1997. Since then, their effectiveness in treating envenomation has been widely accepted.

According to bioassay principles (7), the potency of any antitoxin should be determined relative to a stable standard antitoxin, similarly to the titration of diphtheria or tetanus antitoxin. It has been reported that *A. halys* venom contains at least two lethal fractions and two hemorrhagic fractions, as demonstrated by DEAE-cellulose chromatography (12), and that there is a close association between the major part of its toxic activity and its main hemorrhagic fraction.

In the present paper, we indicate a new method of establishing an infrastructure to develop national standards and standardized a method to evaluate the anti-lethal toxin potency of two horse-derived antivenoms using an intravenous (mouse tail vein) test. This study was carried out during 1999 in our laboratory to confirm the hypothesis that Jiangzhe *A. halys* and *A. halys* venoms have similar lethal effects and to compare the efficacy of both *A. halys* and Jiangzhe *A. halys* antivenoms.

MATERIALS AND METHODS

Snake lethal venoms

The venoms used were *Mamushi* venom (Lethal toxin titer: 530 test doses/ampoule, Lot 3-2, 50 mg protein/ampoule), supplied by the National Institute of Infectious Diseases (NIID), Japan, and Jiangzhe *A. halys* venom, supplied by the Shanghai Institute of Biological Products (SIBP), China. Jiangzhe *A. halys* venom contains 10 mg protein/ampoule. For the lethal test, both stock venoms were diluted to a desired

potency immediately before use.

Antivenoms

Test antivenoms were: national standard equine *Mamushi* antivenom (Anti-lethal toxin titer: 14,200 U/ampoule, Lot C), produced by NIID, Japan, and equine Jiangzhe *Agkistrodon halys* antivenom, produced by SIBP, China.

Experimental animals

White inbred ICR mice of both sexes, aged 4-6 weeks (14-16 g), were used to determine the venoms lethal toxicity and the antivenoms anti-lethal toxin potency. The animals were fed on Purina Rodent Chow and received water *ad libitum* for 10 days prior to the study.

Venoms lethal toxicity

The venoms lethal toxicity was assayed by using intravenous (1) and intraperitoneal (3) injections into mice. Venom was subjected to 4-5 serial dilutions (1.4-fold increments) in 17 mM phosphate buffered sodium chloride solution (pH 7.0) containing 0.2% gelatin. Then, an aliquot of 0.1 ml of each dilution was either intravenously or intraperitoneally injected into 4-8 mice. The dilutions range was selected to cover the entire mortality range (from 0%-100%). Deaths up to 48 h after injections were ascribed to venom toxicity, although most deaths occurred within 24 h. The LD_{50} was calculated using both the Reed-Muench method (15) and probit analysis (2, 10, 16). The lethal toxicity of venoms was determined by the interpolation of their dose-response curves using Statistical Analysis Program (STA77), which was established by National Institute of Infectious Diseases (NIID), Japan, on March 2002.

Antivenoms effective dose 50% (ED_{50})

Aliquots of diluted *A*. *halys* and Jiangzhe *A. halys* venoms were mixed with the same volume of each serial dilution of antivenoms (4-5 serial dilutions, graded with 1.25-fold intervals from 5 U to 19.2 U). Each mixture was kept at room temperature for 1 h; then, 0.2 ml was intravenously or intraperitoneally injected into 8 mice. Animals were observed for 48 h, the number of dead animals was recorded and the antivenom ED_{50} was calculated using probit analysis (2). The antivenom protective potency was estimated according to the method of anti-lethal toxin potency of Jiangzhe *A. halys* antivenom described in the Results.

Bicinchoninic acid [BCA] protein assay (6)

Enzyme-linked immunosorbent assay (ELISA) was carried out to detect the amount

of proteins in *A. halys* antivenom and Jiangzhe *A. halys* antivenoms. We used bovine serum albumin diluted to various concentrations (25, 125, 250, 500, 750, 1000, 1500 and 2000 µg) to generate a standard concentration curve. Two different venom proteins were adsorbed to a solid surface. Total protein concentrations of antivenoms against *A. halys* and Jiangzhe *A. halys* venoms were determined by using an ELISA reader (Spectra-max 340 PC Molecular Device, U.S.A.). Absorbance of each dilution concentration was recorded at 562 nm. Graphical representation of the relationship between the standard dose of bovine serum albumin and the solution protein concentration was used to plot the results. Linear function was determined using Microsoft Excel 97 for Windows. The linear function of antivenom protein concentrations was computed.

Electrophoresis and immunoglobulin concentration

Electrophoresis was performed in order to analyze the mobility of immunoglobulin. It was carried out on cellulose acetate strip, pH 8.6, using veronal buffer. Protein was stained with Ponceau S solution and destained with a mixture of 7.5% acetic acid and 5% methanol. Protein purities were determined using a densitometer (Helena Lab. U.S.A.). Antivenoms nitrogen concentration was estimated according to the micro-Kjeldahl method, and equine whole IgG was evaluated using a densitometer. Standard protein markers (horse serum) were used.

Ouchterlony techniques (14)

Ouchterlony gel diffusion was carried out on 1-mm thin plates of 1.2% agar. First, venoms (*A*. *halys* venom lethal fraction, Jiangzhe *A*. *halys* venom, *A*. *halys* venom hemorrhagic fraction, *Bothrops atrox* venom, and *Agkistrodon rhodostoma* venom; 10 µg/ µl each) were placed in central wells. Antivenoms (2 U / 10 µl or 1 U / 10 µl) against their respective venoms were added to peripheral wells; plate was kept at room temperature for 24 h. The precipitin line was stained with Coomassie brilliant blue reagents.

RESULTS

Venoms lethal toxicity

According to the Reed-Muench method, the LD_{50} of *A. halys* and Jiangzhe *A. halys* venoms, when intravenously injected, was 28.7 µg / 16g and 39.04 µg / 16 g mouse, respectively. When intraperitoneally injected, the LD_{50} of *A. halys* and Jiangzhe *A. halys* venom was 44.89 µg/16 g and 29.2 µg/16 g mouse, respectively (Table 1).

Agkistrodon halys venom showed higher LD_{50} when intrapeitoneally injected than when intravenously injected. Jiangzhe *A. halys* venom had higher LD_{50} when

intravenously injected. There was no difference between Reed-Muench and probit method.

One lethal dose determination

To determine one lethal dose, 0.1 ml of venom solution was intravenously or intraperitoneally injected into mice. For the intraperitoneal injection tests, five serial dilutions containing 51; 71.4; 100; 140; 196 μg *A. halys* and 85.7; 120; 168; 235.2; 329.28 μg Jiangzhe *A. halys* venom were used.

Ten units (U) of Jiangzhe *A. halys* or *A. halys* antivenom were tested against 0.1 ml of either Jiangzhe *A. halys* or *A. halys* venom solution. The relative potency of the two parenteral administration methods was estimated by measuring the animals' response to various concentrations of each antivenom preparation. Control mice were given 0.2 ml PBS alone, without antivenom. When given intraperitoneally, the venoms lethal test doses were significantly higher than when administered intravenously (Table 2).

Cross-neutralization test

The neutralizing capacity of both antivenoms was designated as ED_{50}, which was defined as the unit of antivenom per test dose of venom (μg) capable of reducing the venom effects by 50%.

The ED_{50} of *A. halys* and Jiangzhe *A. halys* antivenoms intravenously administered against 115.4 μg *A. halys* venom [4 x LD_{50}] was 9.3 U and 7.4 U, respectively (p<0.05) The ED_{50} of *A. halys* and Jiangzhe *A. halys* antivenoms intravenously administered against 93.9 μg Jiangzhe *A. halys* venom [2.4 x LD_{50}] was 16.5 U and 8.4 U, respectively. There was a statistically significant difference between the neutralizing activity of *A. halys* and Jiangzhe *A. halys* antivenoms (p<0.05; Table 3).

When intraperitoneally injected into mice, the ED_{50} of *A. halys* and Jiangzhe *A. halys* antivenom against 89.4 μg [2.2 x LD_{50}] was 15.3 U and 7.3 U, respectively. In this case, the potency of the anti-lethal toxin effect of Jiangzhe *A. halys* antivenom was two-fold higher than that of *A. halys* antivenom (p>0.05; Table 3). The ED_{50} of Jiangzhe *A. halys* antivenom intraperitoneally injected against 107.3 μg Jiangzhe *A. halys* venom [3.7 x LD_{50}] was 9.7 U. For the *A. halys* antivenom intraperitoneal injection against 107.3 μg Jiangzhe *A. halys* venom [3.7 x LD_{50}], the regression response on the logarithmic scale was nonlinear (Table 3).

Antivenoms effective dose 50%

According to probit analysis of intravenous administration of antivenoms against 115.4 μg *A. halys* venom [4 x LD_{50}], the ED_{50} of *A. halys* antivenom was 10.12, and

that of Jiangzhe *A. halys* antivenom was 7.52. Jiangzhe *A. halys* antivenom potency was 1.34-fold higher than that of *A. halys* antivenom. When administered intravenously, *A. halys* and Jiangzhe *A. halys* antivenoms were capable of completely neutralizing one test dose of 115.4 µg *A. halys* venom [4 x LD_{50}] (Table 4.1 and Figure 1); 19.2 U Jiangzhe *A. halys* antivenom was sufficient to completely neutralize 93.9 µg Jiangzhe *A. halys* venom [2.4 x LD_{50}], but the same amount of *A. halys* antivenom did not completely neutralize it (Table 4.2 and Figure 2).

To test the efficacy of intraperitoneal administration of antivenoms versus the currently used method (mouse tail vein), we carried out cross-neutralization studies for each pairing of venom and antivenom. Both *A. halys* and Jiangzhe *A. halys* antivenoms were diluted to five different concentrations (6.4; 8.0; 10.0; 12.5; 16.0 U) in a total volume of 0.1 ml and tested against 89.4 µg *A. halys* venom [2.2 x LD_{50}].

Intraperitoneal administration of 10.0 U Jiangzhe *A. halys* antivenom completely neutralized 89.4 µg *A. halys* venom, but a similar response was not seen when *A. halys* antivenom was used, even up to the dose of 16.0 U (Table 5.1 and Figure 3).

The ED_{50} of *A. halys* and Jiangzhe *A. halys* antivenoms intraperitoneally administered against 107.3 µg Jiangzhe *A. halys* venom [3.7 x LD_{50}] was assessed (Table 5.2 and Figure 4). Although 16.0 U of *A. halys* antivenom was not capable of completely neutralizing 107.3 µg Jiangzhe *A. halys* venom, a dose as little as 12.5 U of Jiangzhe *A. halys* antivenom could lead to complete neutralization.

Slopes homogeneity

We compared the slopes of regression lines of the relationship between death rate and dose of both Chinese and Japanese antivenoms.

After substituting Jiangzhe *A. halys* venom for *A*. *halys* venom in the current method, we performed slope assays and comparisons between intravenous and intraperitoneal administration for each antivenom against each venom (Table 6).

Slope ratio can be used to test the relative potency of two preparations and is estimated by the relation between the two regression coefficients.

Agkistrodon halys and Jiangzhe *A. halys* antivenom intravenously injected against 115.4 µg *A. halys* venom [4 x LD_{50}] showed slopes of 10.2 and 9.6, respectively.

We then investigated the linearity and parallelism of log-dosage response curves for both antivenoms intravenously injected against 115.4 µg *A. halys* venom [4 x LD_{50}]. Results reinforced the above-stated conclusion that the log-dosage response curves were linear and parallel with each other. Properties of slopes ratio indicate relative potency of control (Japan antivenom) and tested materials (China antivenom). In terms of efficacy, this means that Jiangzhe *A*. *halys* antivenom is an adequate treatment for Japanese snakebite patients.

Slopes of *A. halys* and Jiangzhe *A. halys* antivenoms intravenously administered against 93.9 µg Jiangzhe *A. halys* venom [2.4 x LD_{50}] were 4.7 and 11.5, respectively. So, in this case, results were linear but non-parallel with each other, demonstrating that Jiangzhe *A*. *halys* venom cannot be used as a standard toxin to test the anti-lethal toxin potency of both antivenoms using the mouse tail vein injection method. This difference in the neutralizing capacity is probably related to antigenicity.

The intraperitoneal injection method showed that the slopes of *A. halys* and Jiangzhe *A. halys* antivenoms against 89.4 µg *A. halys* venom [2.2 x LD_{50}] were 2.5 and 15.5, respectively. Slopes of *A. halys* and Jiangzhe *A. halys* antivenoms against 107.3 µg Jiangzhe *A. halys* venom [3.7 x LD_{50}] were 1.4 and 8.8, respectively. Such results were linear but non-parallel with each other.

Anti-lethal toxin potency of Jiangzhe *A. halys* **antivenom**

One test dose of 10 L+ leveled *A. halys* toxin (the minimum amount of toxin which when combined with 10 I.U. of antitoxin is capable of killing a 16-g mice in two days) was determined by the multiple level beta-procedure. The anti-lethal toxin titer of *A. halys* antivenom (control) was 300 units potency, whereas that of Jiangzhe *A. halys* antivenom (tested material) was 323 units for the same mouse samples (Table 7), and the potency of *A. halys* and Jiangzhe *A. halys* antivenoms against *A. halys* venom was almost the same.

Therefore, the main lethal fraction of *A. halys* venom should be used as a test toxin. The ED_{50} of each test and standard antivenom was determined using one lethal dose of test toxin. The parallelism and linearity of the neutralization curves were assessed by statistical analysis.

Anti-lethal toxin potency of test antivenom was determined by comparing their relative potency with that of the standard antivenom using probit analysis.

Antivenom immunological analysis

The electrophoresis profile of each antivenom is shown in Figure 5. Protein composition of Jiangzhe *A. halys* antivenom (Figure 5, Lane 3) showed the mobility of a pure immunoglobulin preparation. In contrast, only 84.9% of *A. halys* antivenom demonstrated immunoglobulins mobility (Figure 5, Lane 4). So, Jiangzhe *A. halys* antivenom preparation is more purified than that of *A. halys* antivenom. Comparison of the antivenoms activity is shown in Table 8.

Total protein concentration of Jiangzhe *A. halys* antivenom is 12.60 mg/ml versus 13.44 mg/ml of *A. halys* antivenom, and the specific activity of *A. halys* antivenom is 1.1-fold higher than that of the Jiangzhe *A. halys* antivenom (Table 8).

Ouchterlony test demonstrated that *A. halys* and Jiangzhe *A. halys* antivenoms

cross-reacted with both venoms, which indicates that both *A. halys* and Jiangzhe *A. halys* venoms share identical antigenic structures. But, when exposed to either *B. atrox* or *A. rhodostoma* venoms, these antivenoms did not show a precipitated line using the precipitin test (Figure 6).

Table 1: Determination of the median lethal dose (LD_{50}) of venoms intravenously or intraperitoneally injected into mice.

Venom	Administration route	LD_{50} (µg venom/16 g mouse)	
		Reed-Muench Method (CL 95%)	Probit Method (CL 95%)
Agkistrodon halys	Intravenous	28.7 µg (17.7-46.86)	27.8 µg (18.02-47.88)
	intraperitoneal	44.89 µg (*)	38.2 µg (23.5-62.1)
Jiangzhe *Agkistrodon halys*	Intravenous	39.04 µg (22.7-67.1)	ND
	Intraperitoneal	29.2 µg (14.79-64.51)	29.8 µg (20.8-44.3)

CL 95%: 95% confidence limits

ND: Not determined

(*): Not calculated

Table 2: Determination of one lethal test dose of venoms intravenously or intraperitoneally injected into mice (1 Test Dose [µg] venom/16 g mouse).

Antivenom / Venom	Administration route	*Agkistrodon halys* (10 U)		Jiangzhe *Agkistrodon halys* (10 U)	
		Reed-Muench method (CL 95%)	Probit method (CL 95%)	Reed-Muench method (CL 95%)	Probit method (CL 95%)
Agkistrodon halys	Intravenous	115.4 µg [4xLD_{50}] (95.44-133.15)	115.3 µg [4.1xLD_{50}] (99.9-128.45)	144.4 µg [5xLD_{50}] (*)	ND
	Intraperitoneal	89.4 µg [2.2xLD_{50}] (70.78-112.86)	91.7 µg [2.4xLD_{50}] (75.83-110.81)	153.1 µg [3.8xLD_{50}] (*)	ND
Jiangzhe *Agkistrodon halys*	Intravenous	93.9 µg [2.4xLD_{50}] (*)	ND	132.5 µg [3.4xLD_{50}] (107.66-159.74)	134.6 µg (116.66-159.0)
	Intraperitoneal	<68µg [2.3xLD_{50}] (*)	ND	107.3 µg [3.7xLD_{50}] (84.94-135.44)	111.6 µg [3.7xLD_{50}] (88.39-140.89)

CL 95%: 95% Confidence limits

ND: Not determined

(*): Not calculated

Table 3: Cross-neutralization tests in mice (neutralizing unit of antivenom/1 Test Dose [µg] of snake venom).

Venom / Antivenom	*Agkistrodon halys*		Jiangzhe *Agkistrodon halys*	
	2.2xLD_{50} (89.4 µg) [ip]	4xLD_{50} (115.4 µg) [iv]	3.7xLD_{50} (107.3 µg) [ip]	2.4xLD_{50} (93.9 µg) [iv]
Agkistrodon halys	16.65 U (10.96-25.64)	9.3 U (7.9-10.9)	ND	16.5 U (12.6-19.99)
Jiangzhe *Agkistrodon halys*	7.45 U (6.87-8.27)	7.4 U (5.9-9.3)	9.7 U (8.21-10.79)	8.4 U (6.4-10.0)

Results indicate the minimum antivenom units for neutralization of 1 Test Dose of each venom.

ND: Not determined

ip : intraperitoneal injection method

iv : tail vein injection method (intravenous)

Table 4.1: Anti-lethal toxin effective dose (ED_{50}) of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms against 115.4 µg *Agkistrodon halys* venom (4 x LD_{50}) intravenously injected into mice.

Agkistrodon halys antivenom			Jiangzhe *Agkistrodon halys* antivenom		
Antivenom (U)	Number of animals	Number of deaths	Antivenom (U)	Number of animals	Number of deaths
6.4	8	8	6.4	8	7
8.0	8	8	8.0	8	1
10.0	8	3	10.0	8	2
12.6	8	0	12.6	8	0
16.0	8	1	16.0	8	0

Table 4.2: Anti-lethal toxin effective dose (ED_{50}) of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms against 93.9 µg Jiangzhe *Agkistrodon halys* venom (2.4 x LD_{50}) intravenously injected into mice.

Agkistrodon halys antivenom			Jiangzhe *Agkistrodon halys* antivenom		
Antivenom (U)	Number of animals	Number of deaths	Antivenom (U)	Number of animals	Number of deaths
5.0	8	8	5.0	8	8
7.0	8	8	7.0	8	8
9.8	8	6	9.8	8	6
13.72	8	6	13.72	8	1
19.2	8	3	19.2	8	0

Table 5.1: Anti-lethal toxin effective dose (ED_{50}) of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms against 89.4 µg *Agkistrodon halys* venom (2.2 x LD_{50}) intraperitoneally injected into mice.

Agkistrodon halys antivenom			Jiangzhe *Agkistrodon halys* antivenom		
Antivenom (U)	Number of animals	Number of deaths	Antivenom (U)	Number of animals	Number of deaths
6.3	8	8	6.3	8	8
8.0	8	6	8.0	8	2
10.0	8	7	10.0	8	0
12.5	8	7	12.5	8	0
16.0	8	5	16.0	8	0

Table 5.2: Anti-lethal toxin effective dose (ED_{50}) of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms against 107.3 µg Jiangzhe *Agkistrodon halys* venom (3.7 x LD_{50}) intraperitoneally injected into mice.

Agkistrodon halys antivenom			Jiangzhe *Agkistrodon halys* antivenom		
Antivenom (U)	Number of animals	Number of deaths	Antivenom (U)	Number of animals	Number of deaths
6.3	8	5	6.3	8	7
8.0	8	8	8.0	8	6
10.0	8	6	10.0	8	5
12.5	8	7	12.5	8	0
16.0	8	7	16.0	8	0

Table 6: Analysis of slopes of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms cross-neutralization curves using both intraperitoneal and intravenous injection into mice.

Venom / Antivenoms	Intraperitoneal route		Intravenous route	
	Slope with		Slope with	
	Agkistrodon halys venom (89.4 µg) [2.2xLD_{50}]	Jiangzhe *Agkistrodon halys* venom (107.3 µg) [3.7xLD_{50}]	*Agkistrodon halys* venom (115.4 µg) [4xLD_{50}]	Jiangzhe *Agkistrodon halys* venom (93.9 µg) [2.4xLD_{50}]
Agkistrodon halys	-2.5	+1.4	-10.2	-4.7
Jiangzhe *Agkistrodon halys*	-15.5	-8.8	-9.6	-11.5

Table 7: Anti-lethal toxin titer of Jiangzhe *Agkistrodon halys* antivenom against 10 L+ leveled *Agkistrodon halys* (Japan) venom

Antivenom	Antivenom (ml)	Buffer (ml)	Test Toxin (ml)	After 48 h injection		ED_{50} (ml)
				Dead	Alive	
Control - *Agkistrodon halys* (Japan)	0.32	0.68	1.0	4	0	0.56 ml
	0.40	0.60	1.0	4	0	
	0.50	0.50	1.0	2	2	
	0.63	0.37	1.0	0	4	
	0.80	0.20	1.0	1	3	
Jiangzhe *Agkistrodon halys* (China)	0.32	0.68	1.0	4	0	0.52 ml
	0.40	0.60	1.0	4	0	
	0.50	0.50	1.0	1	3	
	0.63	0.37	1.0	0	4	
	0.80	0.20	1.0	0	4	

Table 8: Comparison of *Agkistrodon halys* and Jiangzhe *Agkistrodon halys* antivenoms specific activities.

Test Items	*Agkistrodon halys* antivenom (Japan)	Jiangzhe *Agkistrodon halys* antivenom (China)
Total Protein (mg/ml)	13.44 mg/ml	12.6 mg/ml
Immunoglobulin content (mg/ml)	11.4 mg/ml	12.6 mg/ml
Specific activity (U/mg)	17.54 U/mg	15.87 U/mg

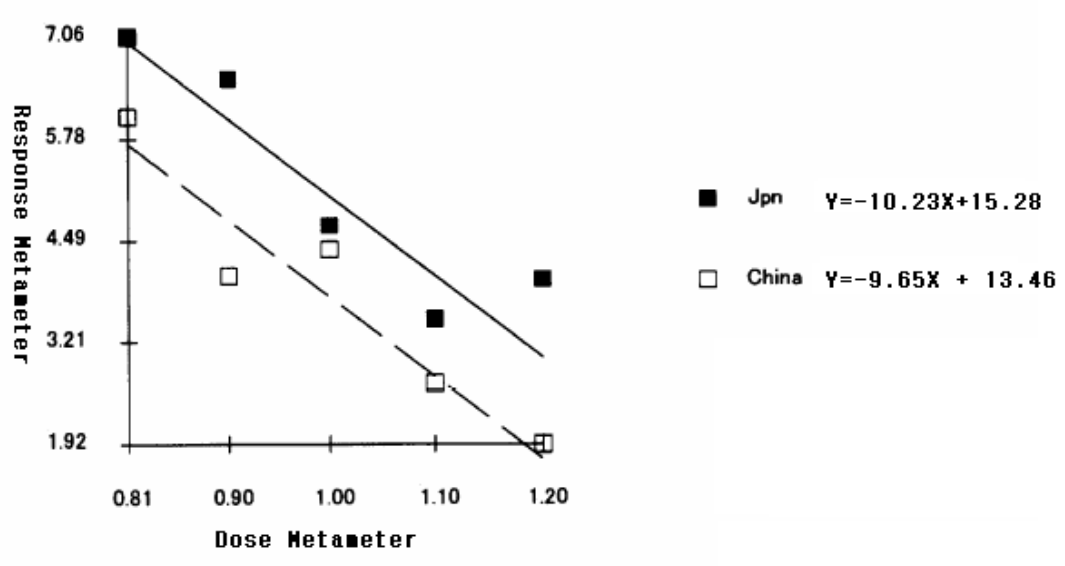

Figure 1: Median anti-lethal toxin effective dose (ED_{50}) of *Agkistrodon halys* (Jpn) and Jiangzhe *Agkistrodon halys* (China) antivenoms against 115.4 µg *Agkistrodon halys* venom (4 x LD_{50}) intravenously administered to mice.

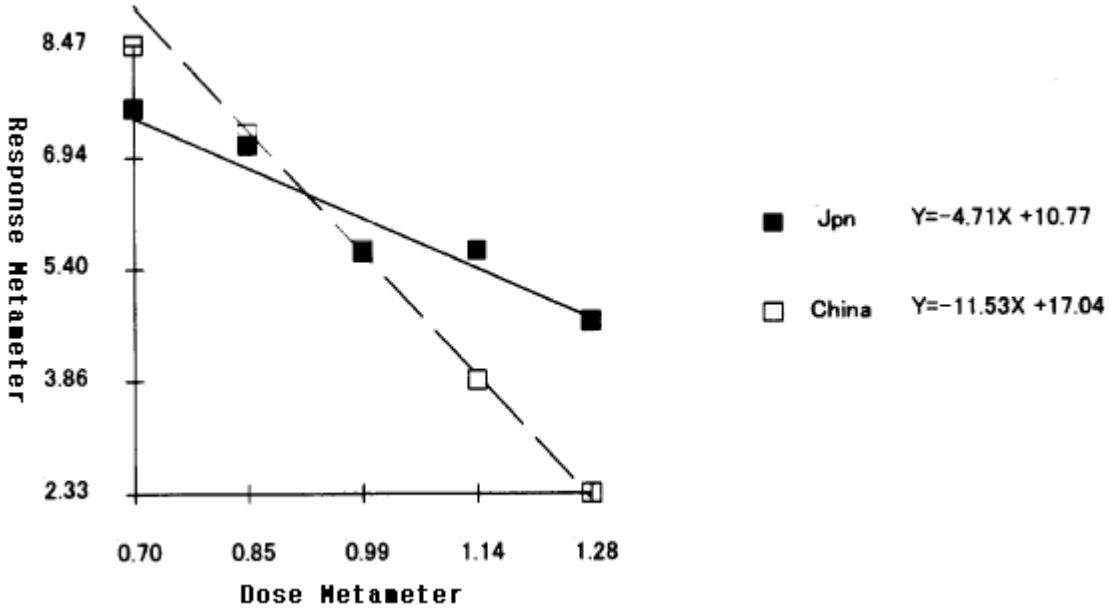

Figure 2: Median anti-lethal toxin effective dose (ED_{50}) of *Agkistrodon halys* (Jpn) and Jiangzhe *Agkistrodon halys* (China) antivenoms against 93.9 µg Jiangzhe *Agkistrodon halys* venom (2.4xLD_{50}) intravenously administered to mice.

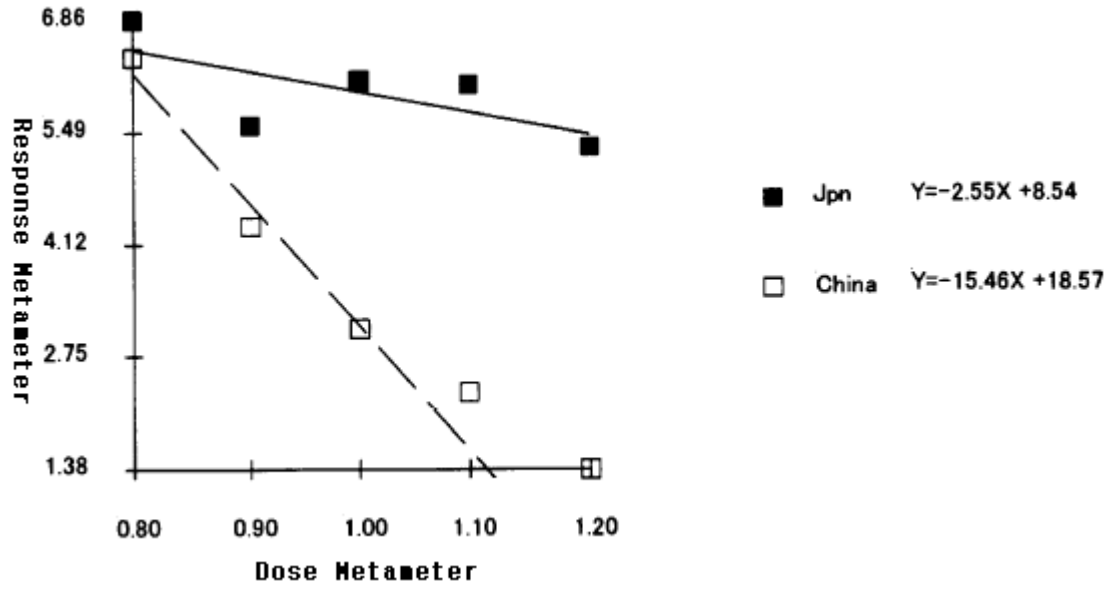

Figure 3: Median anti-lethal toxin effective dose (ED_{50}) of *Agkistrodon halys* (Jpn) and Jiangzhe *Agkistrodon halys* (China) antivenoms against 89.4 µg *Agkistrodon halys* venom (2.2 x LD_{50}) intraperitoneally administered to mice.

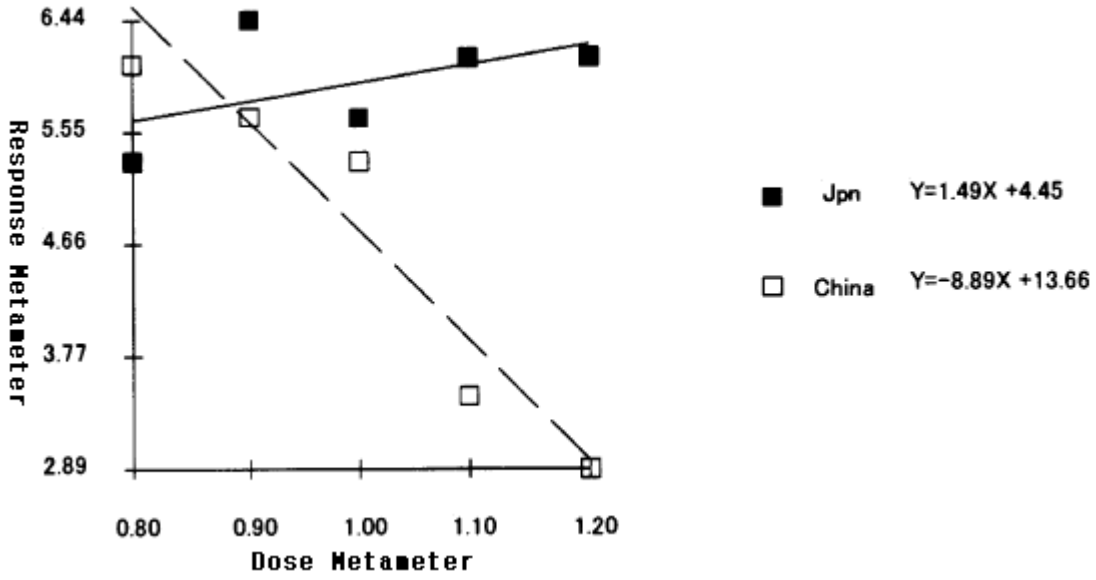

Figure 4: Median anti-lethal toxin effective dose (ED_{50}) of *Agkistrodon halys* (Jpn) and Jiangzhe *Agkistrodon halys* (China) antivenoms against 107.3 µg Jiangzhe *Agkistrodon halys* venom (3.7 x LD_{50}) intraperitoneally administered to mice.

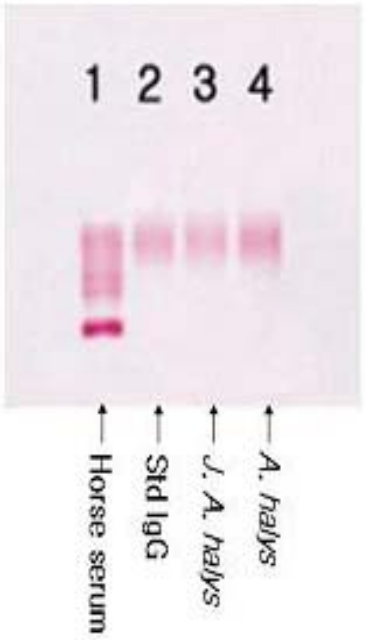

Figure 5: Analysis of electrophoresis. Lane 1: horse serum; Lane 2: standard immunoglobulin; Lane 3: Jiangzhe *Agkistrodon halys* antivenom against Jiangzhe *Agkistrodon halys* venom; Lane 4: *Agkistrodon halys* antivenom against *Agkistrodon halys* venom.

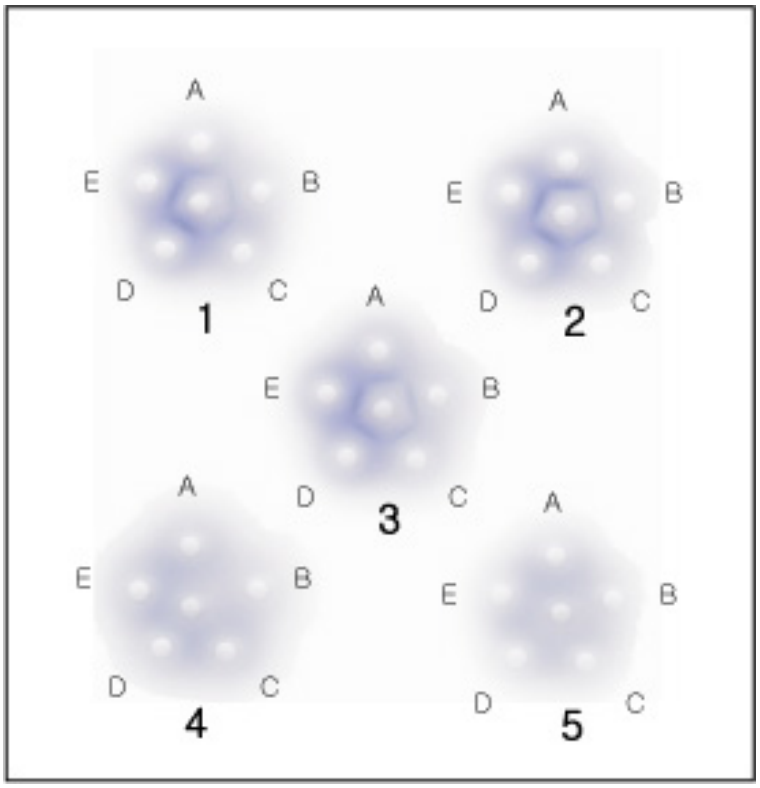

Figure 6: Analysis of Ouchterlony reactions. Venoms in central wells – 1: *Agkistrodon halys* venom lethal fraction; 2: Jiangzhe *Agkistrodon halys* venom; 3: *Agkistrodon halys* venom hemorrhagic fraction; 4: *Bothrops atrox* venom; 5: *Agkistrodon rhodostoma* venom. Antivenoms in peripheral wells – A: *Agkistrodon halys* antivenom (2U); B and C: Jiangzhe *Agkistrodon halys* antivenom (2U); D: Jiangzhe *Agkistrodon halys* antivenom (1U); E: *Agkistrodon halys* antivenom (1U).

DISCUSSION

There are approximately 16 snake species in Korea. Among them, only three belong to the *Agkistrodon* genus. Snake venom biological specificity has been determined through intra and inter-species analysis. To obtain a precise classification of snakes, it is important to know their geographical distribution, age, the season when accidents occurred, and whether their venoms are homologous or heterologous. Effective treatment for snake envenomation is difficult. It is recommended that, in each country, antivenoms be produced from venoms obtained from its indigenous snakes (13). However, if an antivenom has significant immunological cross-reactivity with a venom from a different species of a different region or country, it can be used as an effective treatment for snakebites (9). In order to treat envenomation by snakes of the *Agkistrodon* genus, which is indigenous to Korea, the commercial antivenoms Jiangzhe *A. halys* (from China) and *A. halys* (from Japan) have been imported, and

the quality control test for these products have been based on *A. halys* antivenom and venom standards.

Grasset (5) proposed that the method for antivenom potency determination should be unified using a multiple-level or single-level procedure (choosing the suitable level), a highly active and stable standard venom, and a standard antivenom. He also suggested that expression of the antivenom potency should be standardized to a suitable international unit. This proposal will only hold true if the neutralization curves of both standard and test antivenoms against the standard venom are all linear and parallel with each other. So, to comply with this suggestion, our first step was to select both standard antivenom and venom. Although *A. halys* venom has been used as standard, the present study used Jiangzhe *A. halys* venom as its substitute and demonstrated anti-lethal toxin potency methods for Jiangzhe *A. halys* and *A. halys* antivenoms.

Jiangzhe *A. halys* snakes (18) were captured from Chiangsu and Chechiang, provinces of China. *Agkistrodon halys* snakes were captured from the mainland of Japan. Japanese partially-purified test toxins (11) were prepared using zone electrophoresis of crude venom in a column packed with starch. Each antivenom was prepared by obtaining venom-specific neutralizing globulins present in the serum of healthy horses hyperimmunized against crude *Agkistrodon* venoms. To standardize anti-lethal toxin potency assays (17) for the characterization of Jiangzhe *Agkistrodon halys* venoms, we verified the relationship between injection route and lethality (LD_{50}) using two groups of mice: the first group was injected with *A. halys* venom and the second received Jiangzhe *A. halys* venom. The test dose, intraperitoneally administered, was significantly higher than that intravenously injected.

The capacity of two horse-derived antivenoms to neutralize lethal toxin activities induced by *A. halys* and Jiangzhe *A. halys* venoms was tested.

To substitute the standard method of intraperitioneal injection by the method of mouse tail vein injection, we used cross-neutralization test of *A. halys* and Jiangzhe *A. halys* venoms and antivenoms. In the intraperitioneal test, *A. halys* antivenom against *A. halys* venom showed linearity ($p>0.05$). So, the intraperitoneal test can be used as the anti-lethal toxin activity test.

To substitute Jiangzhe *A. halys* venom for *A. halys* venom in the anti-lethal activity test, we also performed cross-neutralization test. Slope of *A. halys* venom with *A. halys* antivenom was 10.2 and that of *A. halys* venom with Jiangzhe *A. halys* antivenom was 9.6. However, slope of Jiangzhe *A. halys* venom with *A. halys* antivenom was 4.7 and that of Jiangzhe *A. halys* venom with Jiangzhe *A. halys* antivenom was 11.5. Therefore, it is considered that Jiangzhe *A. halys* venom cannot be used as a standard to test anti-lethal activity ($p<0.05$).

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