

Potency of horse-derived antivenoms against *Bitis parviocula* and *Echis pyramidium* venoms

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Abstract

Background: Envenomation in Ethiopia especially rural area is the most serious health hazards. The greater risk of snakebite and unavailability and cost forces us to find out solution.

Objective: To assess the efficacy of two horse-derived antivenoms against venoms of *Bitis parviocula* and *Echis pyramidium* snake species.

Methods: Venom specimens (*B. parviocula* and *E. pyramidium*) were captured in Southwestern (Dima) and Eastern (Yandui Diressa) parts of Ethiopia.

Results: The study conferred 16.2U of VECSEARA antivenom did not completely neutralize 107.3 µg *B. parviocula* venom, compared to 8.4 U of FAB-AFRIQUE antivenom which completely neutralized the venom. FAB-AFRIQUE antivenom of 7.3U completely neutralized 84.4 µg *E. pyramidium* venom, but a similar response was not seen when VACSEARA antivenom was used, even at the dose of 15.3U. The potency of the anti-lethal toxin effect of FAB-AFRIQUE antivenom was two-fold higher than that of VACSEARA antivenom. There was a statistically significant difference between the cross-neutralizing activity of FAB-AFRIQUE and VECSEARA antivenoms ($p < 0.05$).

Conclusion: The two horse-derived antivenom protections on lethality mice were effective. The potency of polyvalent antivenoms is increasing from VACSEARA to FAB-AFRIQUE.

Keywords: Antivenom, *Bitis parviocula*, *Echis pyramidium*, FAB-AFRIQUE, VACSEARA

Introduction

Venomous snakes are found throughout the world except in few islands, forests and areas of high altitudes. In Ethiopia, there are 19 venomous snake specieses (Largen and Spawls 2010). Globally, there are 5.5 million snakebites and as high as 125,000 snakebite related deaths (Kasturiratn et al. 2008). Snakebites are major public health problems particularly in the rural tropics. Populations in these regions experience high morbidity and mortality because of a scarcity of antivenom, which is the only specific treatment. A large number of victims survive with permanent physical sequelae due to local tissue necrosis (Hansdak et al. 1998). Although the burden of snake bite in Ethiopia is poorly documented, certain epidemiological surveys has indicated as it is a serious public health problem due to low health seeking behavior of the victims and lack of antivenoms for treatment (Aga et al. 2014). Venoms are a complex mixture of components which have a diverse array of actions both on prey and human victims. The venoms may contain a mixture of the following activities: neurotoxic (pre/post synaptic), cardiotoxic, myolytic, coagulant, anticoagulant, haemostatic, haemorrhagic and nephro- or hepatotoxic actions (Chippaux et al. 1991). Both interspecific and intraspecific variation in snake venom composition can affect the neutralisation capacity of antivenoms snakebite syndromes management (Fry et al. 2003). Anti-venom is made by immunizing mammals such as horse, goat, rabbit with particular snake venom and

the specific immunoglobins are isolated from the blood. The subject animal will undergo an immune response to the venom, producing antibodies against the venom's active molecule which can then be harvested from the animal's blood and used to treat envenomation. Antivenom is classified into two types; monovalent antivenom when they are effective against a given species venom; polyvalent when they are effective against a range of species (Lake 2004). Among the member of those indigenous species, *B. parviocula* and *Echis pyramidum* are medically important viperine venomous snakes, but the immunogenicity and toxicity of their venoms are still not yet understood (Largen and Spawls 2010). *Bitis* and *Echis* are indigenous venomous snake species relatively widely distributed in Ethiopia. *B. parviocula*, known as Death Adder, is restricted to deciduous forest and montane grasslands of the Ethiopian highlands (David 2016) and it is one of the medically important snake of the *Bitis* species. The venom of *Bitis* species is cytotoxic with strong haematotoxic and some cardiotoxic effects and large volumes are produced. This species is responsible for more fatalities than any other African snake. In Ethiopia, the saw-scaled viper, *E. pyramidum*, one of the venomous snakes belongs to the family viperidae is widely distributed. The rural population and the army are more exposed to the bites of this venomous snakes. The venom largely consists of enzymes and toxins affecting hemocoagulation. Victims of bites suffer from, pain, nausea, vomiting, swelling, low blood pressure and bleeding (Al-Shammari 2007).

Potency of horse-derived antivenoms against Ethiopian *Bitis parviocula* and *Echis pyramidum* Venoms

The only available treatment against snake bite is the usage of anti-venom. Ethiopia imports antivenoms in limited quantities and this discourages the victims to seek medical assistance (Aga et al. 2014). To ensure the efficacy of any antivenom the interaction between venom and antivenom should be evaluated (Kawamura 1974). The study of cross-neutralization of venoms by antivenoms is classically performed, at the preclinical level, by assessing the ability of a particular antivenom to neutralize the most important and clinically relevant toxicological activities of snake venoms using standard laboratory tests in experimental animals (Gutiérrez et al. 1990).

The immune-reactivity of antivenom to the venoms of snakes is based on the ability of antivenom to immunodeplete homologous or heterologous venom proteins. This helps to support that antivenoms prepared from the venom of some snakes may be partially or fully effective against snakes in the same genera but from different species (Calvete et al. 2010). There are some observations that some antivenom proved effective against the venom of interspecies although no venom of the genus was used to immunize the horses during preparation of the antivenom (Dzikouk et al. 2002). In the present study, we determined cross-neutralization and efficacy of two commercial antivenoms (FAV-AFRIQUE and VACSERA) against venoms of *B. parviocula* and *E. pyramidum*.

Materials and methods

Collection of snakes: The Ethiopian mountain adder (*Bitis parviocula*) was collected from Dima (Kaffa) and *Echis pyramidum* was collected from Yangudi Rassa National Park. The collected snakes were immediately transferred into large plastic jars and then transported in a ventilated pickup car to the ware house in Addis Ababa for acclimatization.

Venom collection: During extraction venom glands were massaged to ensure maximal quantities of venom. Fresh venom samples were examined for determination of its variability including color and turbidity (Tu 1996). The venom samples were centrifuged at 13,000 rpm for 10 min at 4°C. Venoms from individual snakes were kept separately. Venoms were then freeze-dried for 3-4 hours and stored at 4°C until further analysis.

Experiment animals: Six weeks aged Swiss albino mice of both sexes from the EPHI Experimental Animals Breeding Center were used to determine the venoms of median lethal dose (LD₅₀) and the antivenom anti-lethal toxin potency. During the 10 days prior to the experiment the animals were fed and received water *ad libitum*.

Commercial antivenoms: FAV AFRICA is manufactured by Sanofi Pasteur SA, Exp. Date

October 21st, 2015, Ref. 69007, Lyon, France. FAV AFRICA is a polyvalent equine F(ab')₂ antivenom (Fragments F(ab')₂D'immunoglobuline E'equines Antivenimeuses) for Sub-Saharan African snakes. Test antivenoms were 250 U/ampoule. VACSERA is polyvalent equine immune plasma antivenom (Snake Venom Antiserum Polyvalent equine) Liquid form manufactured by Holding Company for Biological Products and Vaccines, Ref. 51, Wezaret El Zeras St., Agouza, Giza, Egypt. Test antivenom was 200 U/ampoule.

Venom lethal toxicity: The purpose of this study was to determine lethal dose in mice of two viper venoms determined by Russell method (1985). In brief; five groups of six mice each were housed in individual cages. Stock snake venoms were prepared by dissolving 1 ml of 0.85% physiological buffer saline solution at the highest concentration of venom that would be used for injection. Serial dilutions of 2-fold, using saline solution, were made to obtain four additional concentrations. The dilution range were selected to cover the entire mortality range (from 0% - 100%). All venom solutions were stored at 0°C and warmed to 37°C just before injecting the mice. The lethal toxicity was determined by intravenous and intraperitoneal administration of 0.2 ml of venom according to Gingruch and Hohenadel (1956). Each group of mice received a dose range of 2.10-8.3 µg/mice intravenously and 3.48-21.13 µg/mice intraperitoneally. This equivalent to 1.95-6.5 µg/mice intravenously for *B. parviocula* and 2.90-16.4 µg/mice intraperitoneally for *E. pyramidum*. The endpoint of lethality of mice was determined after 48hrs. The lethal toxicity of venoms was determined by calculating probit analysis using SPSS.

Lethal dose 50%: Lethal dose 50% is the amount of venom that causes the death of 50 percent of injected mouse. One lethal dose, 0.1 ml of venom solution was intravenously or intraperitoneally injected into mice (WHO 2008). For the intravenous or intraperitoneal injection tests, five serial dilutions containing 51, 71.4, 100, 140, 196 µg *E. similar to pyramidum* venom and 85.7, 120, 168, 235.2, 329.28 µg for *B. parviocula* venom were used. Ten units (U) of FAV-AFRIQUE and VACSERA antivenoms were tested against 0.1 ml of the two venom solutions to determine the 50% cross neutralization potentials of heterologous venoms.

Antivenoms effective 50% (ED₅₀): One lethal toxin dose (112 µg and 103.3 µg) of diluted *B. parviocula* and *E. pyramidum* venoms were mixed with the same volume of each serial dilution of antivenoms (5 serial dilutions, graded with 1.25-fold intervals from 5U to 19.2U (5.0, 7.0, 9.8, 13.72, 19.2U). Each mixture was kept at room temperature for 1h; then, 0.2 ml was intravenously injected into 8 mice similar

to the neutralization test. Animals were observed for 48hrs, the numbers of dead animals were recorded and the antivenom ED₅₀ was calculated using probit analysis (Meier and Theakston 1986).

Cross-neutralization potential of antivenom test: One lethal toxin (107.3µg and 84.4µg) of *B. parviocula* and *E. pyramidium* venoms was mixed with the same volume of each serial dilution of antivenoms (4-5 serial dilution, graded with 1.5 fold intervals from 6U to 16U). Five different concentrations of antivenoms (6.4; 8.0; 10.0; 12.5; and 16.0 U) were tested against one lethal toxin of different LD₅₀ of *B. parviocula* and *E. pyramidium* venoms. Each mixture was kept at room temperature for 1h; then 0.2ml was injected intravenously and intraperitoneally into 8 mice.

Statistical analysis: Data were analyzed statistically by one way analysis of variance (ANOVA). When the values were significantly different (P<0.05), the differences between pairs of means were analyzed by the Tukey test.

Results

Venoms lethality toxicity: For lethality, results are reported as mean and 95% confidence limits (given in parentheses). The LD₅₀ of *B. parviocula* and *Echis pyramidium* venoms when injected intravenously were 28µg/18g and 32µg/18g mouse respectively. The intraperitoneally injected LD₅₀ of *B.parviocula* and *Echis pyramidium* venoms were 31.1µg/18g and 34.5 µg/18g mouse respectively.

One lethal dose determination: The result of one lethal dose determination in relation to potency of the administration routes is shown in Table 1.

Antivenom effective dose 50%: The one lethal intravenous administration dose of FAV-AFRIQUE and VACSERA antivenoms against *B.parviocula* venom were 112 µg and 141.5 µg, respectively (Table 1). In addition, FAV-AFRIQUE antivenom had one lethal dose of 104.3 µg against *E. pyramidium* venom. On the otherhand, the one lethal test dose varied significantly when both antivenoms are administered by intraperitoneal route (Table 1).

Table 1: Determination of one lethal test dose of venoms intravenously injected into mice (one test dose [µg])

Route of Administration	<i>B. parviocula</i> (10U) (CI 95%)	<i>E. pyramidium</i> (10U) (CI 95%)
Intravenous	112 µg [2.4LD ₅₀] (97.18-124.45)	141.5 µg [3.5LD ₅₀] (*)
Intraperitoneal	ND	124 µg [3.2LD ₅₀] (102.61-153.39)
Intravenous	104.3µg [2.7LD ₅₀] (86.33-125.41)	ND
Intraperitoneal	91 µg [2.5LD ₅₀] (75.13-110.28)	89.1 µg [2.2LD ₅₀] (70.25-112.35)

CI 95%: 95% Confidence limits, ND: Not determined, (*): Not calculated

In the neutralization of lethality, results are also reported as µg or mg venom neutralized per ml antivenom as indicated in Tables 2 and 3. At 6.0U concentration of FAV-AFRIQUE antivenom, 87.5% of the animals were died whereas all animal were died at the same concentration of VECSERA antivenom

(Table 2). At 10U concentration of FAV-AFRIQUE antivenom, no animals died whereas 37.5% of the animals died at the same concentration of VECSERA antivenom. The efficacy of the two antivenoms differ significantly (p<0.05) when applied to *B. parviocula* venom.

Table 2: Anti-lethal toxin effective dose (ED₅₀) of FAV-AFRIQUE and VECSERA antivenom against 112µg *B. parviocula* venom intravenously injected into mice

FAV-AFRIQUE antivenom			VECSERA antivenom		
Antivenom (U)	No. of animals	No. of death	Antivenom (U)	No. of animals	No. of death
6.0	8	7	6.3	8	8
8.0	8	3	8.0	8	5
10.0	8	0	10.0	8	3
12.5	8	0	12.5	8	1
16.0	8	0	16.0	8	0

Table 3: Anti-lethal toxin effective dose (ED₅₀) of FAV-AFRIQUE and VECSERA antivenom against 104.3µg *E. pyramidium* venom intravenously injected into mice

FAV-AFRIQUE antivenom			VECSERA antivenom		
Antivenom (U)	No. of animals	No. of death	Antivenom (U)	No. of animals	No. of death
5.0	8	7	5.0	8	8
7.0	8	2	7.0	8	5
9.8	8	1	9.8	8	3
13.72	8	0	13.72	8	2
19.2	8	0	19.2	8	2

Cross-neutralization potential of antivenom test: Neutralization is reported as effective dose 50% for lethal toxic effects. Table 3 showed the different neutralizing potencies of the two antivenoms against *B. parviocula* and *E. pyramidium* venoms. The result to

the effective potential of intravenous and intraperitoneal administration of the two antivenoms was effective. The cross-neutralization results indicated that the ED₅₀ of VACSERA and FAB-AFRIQUE antivenoms when administered

Potency of horse-derived antivenoms against Ethiopian *Bitis parviocula* and *Echis pyramidium* Venoms

intraperitoneally against 104.3µg *B. parviocula* and *E. pyramidium* venom were 2.7 and 2.2 respectively. These differences were statistically significant ($p<0.05$). These values were obtained by 16.2 U and 15.3 U of VACSERA antivenom. In contrast, the 2.7 and 2.2 ED₅₀ values for FAV-AFRIQUE antivenom

were 8.4U and 7.3U, respectively (Table 4). In the intravenous administration route, more VACSERA antivenom was utilized as compared to FAV-AFRIQUE antivenom to neutralize *B. parviocula* and *E. pyramidium* venoms.

Table 4: Cross-neutralization tests in mice (neutralizing unit of antivenom/1 Test Dose [µg] of snake venom)

Antivenom	<i>B. parviocula</i> venom		<i>Echis pyramidium</i> venom	
	2.4LD ₅₀ 112.2 µg [iv]	2.7LD ₅₀ 104.3 µg [ip]	4LD ₅₀ 115.4µg [iv]	2.2LD ₅₀ 84.4µg [ip]
VACSERA antivenom	9.3 U (7.34-12.1)	16.2U (12.1-21.1)	9.7 U (7.8-10.87)	15.3 U (10.61-26.2)
FAV-AFRIQUE antivenom	7.4 U (5.6-9.4)	8.4 U (6.1-10.5)	6.5U ND	7.3 U (6.8-9.12)

Results indicate the minimum antivenom unit for neutralization of 1 test Dose of each venom, ND: Not determined

Discussion

Parental or intraperitoneal administration of sheep or horse-derived antivenoms constitute the cornerstone in the treatment of snakebite envenomation. Since venoms present conspicuous biochemical and pharmacological variations between and within species, it is recommended that antivenoms should be produced in each country with venoms obtained from snakes collected in that particular country (Chippaux et al. 1991).

The results of this study indicated that the two antivenoms were effective in neutralizing the toxic effects of *B. parviocula* and *E. pyramidium* venoms. This suggests that there is a significant immunological cross-reactivity between *B. parviocula* and *E. pyramidium* venoms. Cross-neutralization of several antivenoms produced in different countries was demonstrated when they were tested against the venoms of several snakes from Africa (David et al. 2016). On the other hand, when the effective doses 50% of these antivenoms were compared, the FAV-AFRIQUE antivenom showed the highest neutralizing ability against produced with *B. parviocula* and *E. pyramidium* venoms. Local tissue damage is one of the most typical and dangerous consequences of *B. parviocula* and *E. pyramidium* envenomation. These effects result in severe lesions and in a number of cases in sequelae. The rapid development of these local effects makes difficult for efficient neutralization by antivenoms (Goswami et al. 2014). Thus, it is necessary to have antivenoms with high neutralizing capacity against the toxins responsible for these effects in order to reduce the local tissue damage. Our results indicate that the two antivenoms were similarly efficient in neutralizing the two snake antivenoms. However, FAV-AFRIQUE antivenom was more potent than VACSERA antivenom when used against *B. parviocula* and *E. pyramidium* venoms.

Conclusions

The tested FAV-AFRIQUE and VACSERA antivenoms are efficient in neutralizing the toxic

effects induced by *B. parviocula* and *E. pyramidium* venoms from Ethiopia.

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