

Toxicity evaluation of viper snake venoms and *in vivo* neutralization efficacy test of FAV-AFRIQUE and VECSERA antivenoms

Gashaw Gebrewold^{1*}, Timothy J.Colston³, Brihanu Hurisa¹, Abebe Mengesha, Sintayehu Abate, Kelbessa Urga and Kifle Dagne²

¹Ethiopian Public Health Institute, P.O.BOX 1242, Addis Ababa, Ethiopia

²Addis Ababa University, Department of Microbial, Cellular and Molecular Biology, P.O. BOX 1176, Ethiopia

³University of Mississippi, Biology Department, University, MSC, USA 38677

*Corresponding author: gwold71@gmail.com

Abstract

Introduction: Snake bite is a public health hazard that leads to high morbidity and mortality in the rural areas of Ethiopia. Thousands of injuries associated with bites of venomous snake occur every year in the country. The most common types of snake venoms which cause cytotoxic and hemotoxic clinical effects are viper species.

Objective: To determine the lethal values of reference snake venoms in Swiss mice and to assess efficacy of FAV-AFRIQUE and VECSERA antivenoms drugs.

Method: A cross-sectional study was conducted to evaluate venom toxicity, comparison between routes of administration mice (n=8) as treatment and control, and neutralization test of two polyvalent antivenoms. The categorical bivalent variables were assessed using non-regression analysis as 95% confidence level and Graph Pad Prism 5 software and using statistical program (SPSS) of linear modeling.

Result: The lethality values obtained from four venomous snakes indicated that *Bitis arietans somalica* and *Echis pyramidum* are found to be more toxic than *B. parviocula*. *Causus rhombeatus* is less toxic than other species examined. Comparing the two injection routes revealed that viper venoms were 2.5 times less toxic when injected in intraperitoneal compared to intravenous. The point of interception in the four plots indicated that the LD₅₀ of *B.a.somalica* venom was the most toxic venom followed by *E. pyramidum*, *B. parviocula* and *C. rhombeatus* venoms. In neutralization tests *B. parviocula*, *Echis pyramidum* and *B.a.somalica* venoms were achieved when a challenge dose of 3LD₅₀ of venoms were used in mice.

Conclusion: The antivenoms study showed the potential of FAV-AFRIQUE as a primary treatment for snake envenomation in Ethiopia but the low cost and availability of VECSERA antivenom make the latter more practical.

Keywords: *Viperidae*; *Bitis arietans somalica*; *Bitis parviocula*; *Echis pyramidum*; *Causus rhombeatus*; antivenom

Introduction

Snake bite envenoming is a public health hazard that many people in the rural tropics face on a daily basis (Warell 2010). In these areas, snake bite envenoming is a common cause of occupational injury affecting agricultural and plantation workers with important socioeconomic implications. During the rainy season in Ethiopia, there is high incidence of envenomation when there is an intense agricultural activity (Trishnananda 1979). The type of snakebite encounter in Ethiopia varies according to the geographic location and distribution of the snakes (Spawls 2004). There are approximately 21 snake species which occur within Ethiopia that may deliver medically significant bites as reported by Lagren and Spawls (2010), one of which is endemic to the country (*B. parviocula*). Others are believed to be highly venomous snakes by most herpetologists. With the exception of *B. parviocula*, none of them have detailed information for their lethality and venom composition. *Bitis* and *Echis* species are responsible for the majority of envenomation in the eastern and western parts of Ethiopia. A retrospective national epidemiological incidence study of snake

bite in 2014 revealed approximately 1000 envenomation per year (Aga et al. 2014). However, this significant public health importance has been largely ignored by medical professionals within the country.

The toxin components of these species have pronounced toxicity and can damage endothelial vessels leading to alteration of vessel permeability and extravasations of blood (Borkow 1994). The toxic composition of snake venom varies among species; this variation can show different clinical effects in humans which result major medical implications for the treatment of human snakebite victims (Calvet 2007). Variation in venom effects has been observed in clinical presentation following envenomation especially in eastern part of Ethiopia (Calvete 2007). The identification of snake species was crucial for optimal clinical management. In Dire Dawa hospital, the most common reported envenomation which have public health important are the Puff adder (*B.a.somalica*), Carpet viper (*E. pyramidum*) and Spitting cobra (*Naja pallid*) (Manyazawal 2014). The endemic venomous snake *B.parviocula* considered highly dangerous in

mountains regions specifically in Bale (Dinsho) and south eastern part of Ethiopia. Therefore, venomous snake species diversity is a significant public health concern.

Venomous snakebite in Ethiopia is a public health problem and the lack of antivenom makes things worse (Theakston and Warrell 2000). There are also difficulties in accurate snake species identification. Additionally, those antivenoms imported from outside the country are very expensive (60-70 USD per vial) in Ethiopia; and depending on the degree of envenomation a patient may need several vials of antivenom for effective treatment. Lastly, the availability of antivenom and its usage in the hospitals are very limited (Williams 2011). The practical approach for therapeutic treatment of envenomation patients is to choose the appropriate polyvalent antivenom preferably based on the extended neutralization spectrum of heterologous antivenom and cost (Solano 2010). At present, only two types of polyvalent antivenoms available against venomous snake species in Ethiopia and, they are imported from France (FAV-AFRIQUE) and Egypt (VECSERA). In this paper, we evaluated the cross-neutralization potential of the two polyvalent antivenoms against homologous and heterologous venoms of medically important snake species especially in the genera *Bitis* and *Echis* (Segura 2014).

Materials and Methods

Specimens collection sites: Specimens of *B. parviocula* were collected in southwestern part of Ethiopia (6° 43'01"N, 35° 31'01"E), *B. a.somalica* (9° 4' 59.16"N, 39° 59'59.80"E), *E. pyramidum* (10° 32'4.94"N, 40° 52'4.36"E), *C. rhombeatus* (9°2'26.54"N, 43°10'47.04"E) were collected in the eastern part of Ethiopia and transported to snake facility in Addis Ababa. Snakes were enclosed and kept at 18-22°C. White laboratory mice (Swiss albino) were given as food every week and fresh water available *ad libitum*. Those species were wild animals and considered adults.

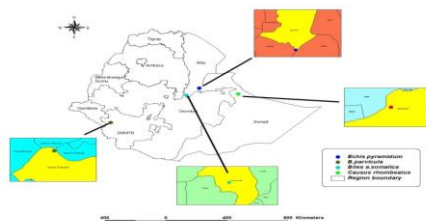


Figure 1: Map shows collection locations of *Echis pyramidum*, *B. parviocula*, *B. a.somalica* and *C. rhombeatus* specimens.

Venom collection and Antivenom: Venoms were extracted individually by allowing the snakes to bite

into a sterile disposable beaker covered with Para film and freeze-dried using a mini Lyotrap lyophilizes later. The venoms of four species in the family *Viperidae* (*B. parviocula*, *B. a.somalica*; *Echis pyramidum* and *C. rhombeatus*) were collected from east and southwestern part of Ethiopia (Figure1). The antivenoms tested were: Polyvalent equine F (ab')₂ antivenom for Sub-Saharan African snakes (Ref. 69007, expiry: 21st October 2016, manufacturer: SANOFI PASTEUR SA, Lyon, France); Snake Venom Antiserum Polyvalent (equine) Liquid for Sub-Sahara African snakes (Ref. 51, expiry: Manufacture: Wezaret El Zeras St., Agouza, Giza, Egypt. Antivenoms were purified equine F(ab)₂ products and in the volume of 10ml per vial according the manufacturers recommendation.

Animals Use and Supply: White Swiss albino (ICR strain weighing 18-20g of 4-6 weeks old mice were supplied by the Animal Experimental unit of Ethiopian Public Health Institute. During the 10 days prior to the experiment, the mice were fed and received water and pellate (usable food for mice).

Lethality study: Different concentrations (dosages) of the venoms of four species of snakes were injected into five groups of mice via intravenous and intraperitoneal routs. The venom was dissolved in 0.85% saline at the highest test dose per mouse of each species and then 2-fold serial dilutions were made using saline to produce four additional concentrations. All solutions during the experiment were stored at 0°C just before being injected into mice. The lethal toxicity was determined by injecting 0.2ml of venom (containing dosages to cover the entire range of 0%-100%) into intravenous and intraperitoneal routs of (18-20g) female mice. The survival ratio for mice at each dose was recorded after 48 hours of observation. The median lethal doses were determined by the method Prism 5Graphpad, Inc. A non-linear regression was generated then constraint was imposed to get minimum and maximum values. The LD₅₀ value is defined as the amount of a substance (or venom) causing death of 50% injected mice at different routs of administration (Mories 2010).

Correlation analysis: The LD₅₀ of each of the four venoms was determined individually using the method adapted by Molinengein (1979), for assessing the toxicity of venoms (LD₅₀). Male Swiss albino mice (20 ± 2g body weight) were given intraperitoneally injections of weight-proportional dose (D) of the four venoms dissolved in 0.85% saline to the total volume of 30ml prior to injection. One animal was used for each dose. The LD₅₀ by intravenously injection in male Swiss albino mice of

the four venoms values were determined. The time between injection and death indicating the survival time (T) was recorded for each dose of venom. A plot of D/T (abscissa) versus D (ordinate) was constructed and the point where the regression line intersected the ordinate depicted the LD₅₀, defined by this method as the smallest dose of volumes that kills 50% of experimental animals in unlimited time. The data was analyzed using basic program estimating LD50 values in IBM-PC developed by Travers in 1986.

Neutralization Study: The objective of this experiment was to determine the neutralization efficacy of the two polyvalent antivenoms tested in the present study. For this purpose, a fixed dose of the venom (challenge dose) was used. The fixed challenge dose was determined based on the LD₅₀ result obtained from previous experiments. The strength of the challenge dose was determined to be 3LD₅₀. The accepted potency of FAV-AFRIQUE and VECSERA efficacy, calculated as the volume necessary to neutralize the lethality of standard *B.a.somalica*, and is 1ml antivenom/5 mg venom. This value was used as a reference to design the neutralization protocols, where by this proportion was sufficient to protect more than 50% of mice. The challenge (3LD₅₀) doses of 103µg for *B.a.somalica*, 120µg for *B.parviocula* and 107µg for *Echis*

pyramidum venoms were incubated with FAV-AFRIQUE 1, 2, 3 and 4 times and also with VECSERA 1, 2, 3, 4 and 5 times the potency reference value (1mL/5mg venom) (Gutierrez 2012). The venom-antivenom mixtures were injected intravenously into eight mice in each group with the total of five groups per species. Control groups injected with venom incubated with PBS saline without antivenom were used. The venom lethal (LD₅₀) and antivenom effective doses (ED₅₀) were determined in 18–20 g, female mice. The result indicated for three species represent the value that obtained in independent experiments and are expressed in % neutralization considering the number of mice that survived after 48 hours was recorded for antivenom efficacy and potency estimation.

Lethality dose determination: The venom toxicity analysis was analyzed by Meier and Theakson 1986 method. Data were computed using 95% CI, Graph Pad Prism 5 software and Statistical Analysis Program (SPSS). Ethical approved was obtained from Scientific and Ethical Review Office of Ethiopian Public Health Institute.

Results

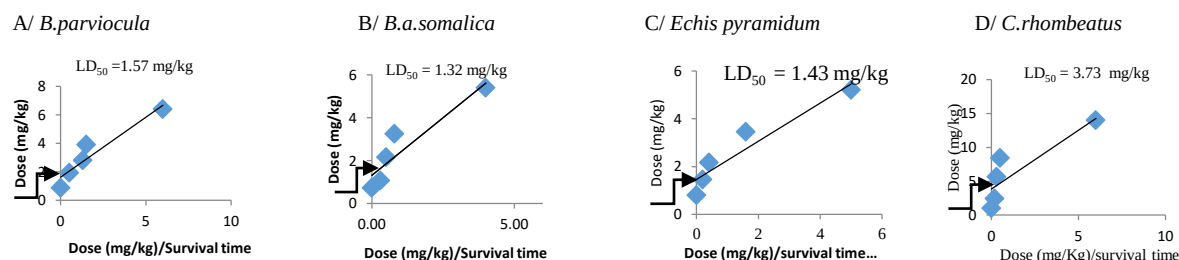
The result in Table 1 clearly shows the impact of the injection route on the venom-induced lethal effects.

Table 1: LD₅₀ of four venoms of snakes in Ethiopia with comparison of intravenous (IV) and intraperitoneal (IP) administration routes

Routs of injection	<i>B. parviocula</i> (LD ₅₀)	<i>B.a.somalica</i> (LD ₅₀)	<i>E.pyramidum</i> (LD ₅₀)	<i>C.rhombeatus</i> (LD ₅₀)
IV(µg/mouse)	8.3 (7.5-8.8)	5.8 (5.3-6.3)	6.5 (6.3-7.2)	15.2 (14.2-16.4)
IV (mg/Kg)	1.65	1.35	1.41	3.56
IP (µg/mouse)	21.5 (21.4-21.8)	14.3 (14.1-15.2)	16.4 (16.1-16.9)	37.5 (37.01-37.7)
IP (mg/Kg)	4.3	2.86	3.28	7.5
IP/IV ratio	2.59	2.5	2.52	2.5

The plot of dose (mg/kg)/time (min) (abscissa versus D ordinate) was constructed at five different doses that kill mice within minutes to each of the four

venoms (Figure 2). The LD₅₀ values depicted by the point insertion of the regression line to the ordinate were determined from the respective plots.



B.parviocula venom doses used: 0.86, 1.92, 2.8, 3.9 and 6.4 mg/kg; *B.a.somalica* venom doses used: 0.72, 1.06, 2.16, 3.24 and 5 mg/kg; *Echis pyramidum* venom doses used: 0.8, 1.46, 2.41, 3.44 and 5.2 mg/kg and *C. rhombeatus* venom doses used: 1, 2.4, 5.6, 8.4 and 14 mg/kg dissolved in 0.9 % saline to total venom of 30 µL each doses prior to injection. One animal was used for each dose

Figure 2: Detection of the LD₅₀ of venoms of the four common venomous snakes in Ethiopia.

The point of interception in the four plots indicates the LD₅₀ of each snake species. *B.a.somalica* venom

was the most toxic venom followed by *Echis pyramidum*, *B.parviocula* and *C. rhombeatus* venoms. The

survival time of 50% of mice at 2.16 mg/kg *B.a.somalica* venom dose was 1.5mins. After 2 mins time, *B.parvicula* and *Echis pyramidum* responded to dose of 2.8 mg/kg and 2.41 mg/kg, respectively.

Neutralization potential: The neutralization of 3LD₅₀ doses of *B. parvicula* venom (120µg) was achieved when the proportion of 2ml antivenom/5mg venom was used in FAV-AFRIQUE, 3ml antivenom/5mg venom in VECSERA whereas in *Echis pyramidum* venom (107µg) and *B.a.somalica* venom (103µg) were achieved when the proportion of 3ml-4ml antivenom/5mg venom was used FAV-AFRIQUE, 4ml-5ml antivenom/5mg venom in VECSERA antivenom respectively (Figure 2). The effective doses of 50% was achieved when 3ml-4ml

antivenom/5gm venoms of FAV-AFRIQUE in *Echis pyramidum* and *B.a.somalica* venoms respectively. In VECSERA antivenom the effective dose of 50% for *B. parvicula*, *Echis pyrsmidum* and *B.a.somalica* venoms was achieved when the antivenom levels were 2ml, 2ml and 4ml antivenom/5gm venoms were used (Figure 3). Most of the standard protocols to assess antivenom potency use a fixed LD₅₀ value to challenge experimental mice. Therefore, our results showed that the reference assay used to compare the antivenom efficacy against three Ethiopian snake venoms was effective in the neutralization of clinically important viper snake venoms. In fact several studies reported that some venom with higher LD₅₀ values need higher concentration of commercial antivenoms (Figure 3).

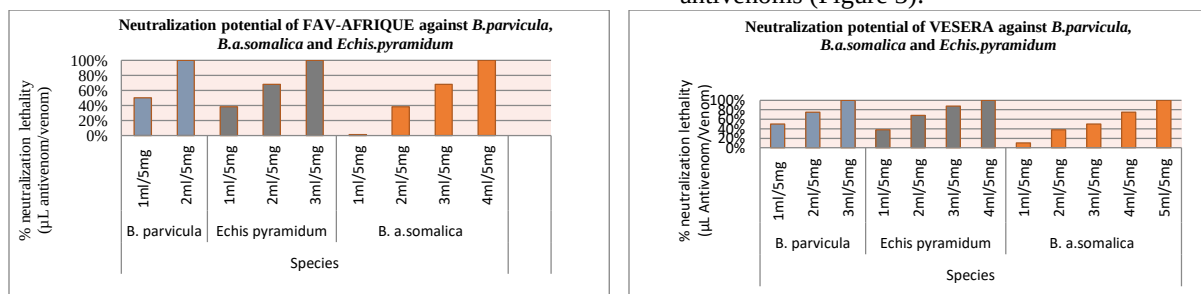


Figure 3: Neutralizing potential of FAV-AFRIQUE and VECSERA antivenoms against the major toxic activity of fixed dose of three viper snake venoms.

FAV-AFRIQUE antivenom was able to effectively neutralize 3LD₅₀ doses of the venoms of *B.parvicula*, *B.a.somalica* and *Echis pyramidum*. VECSERA antivenom was able to neutralize the venoms of *B.parvicula*, *B.a.somalica* and *Echis pyramidum*, but with less efficiency.

Discussion

The quantity and quality of venoms from a snake can be influenced by age, diet, geographical location as well as other factors which in turn affect the lethality of the venom (Mintons and Weinsteins 1986). Also the methodological aspects like application of routes, volume, temperature and solvent used bring lethality of snake venom different from that of the same venomous species. Viper species (*B.a.somalica* and *Echis pyramidum*) are more toxic than *B.parvicula* and *C.rhombeatus* venoms. In our comparative study, the injection route has a significant impact on the LD₅₀ value and affect on the relative toxicity of viper venoms. The LD₅₀ values obtained in mice injected intravenously were lower than those obtained by intraperitoneal injection meaning more lethal potency. Viper venoms are approximately 2.5 less toxic when injected intraperitoneal than that of intravenous. This result may imply an important role of injection route in venom toxicokinetic parameters. Additionally the bio-distribution of viper venoms is relatively different to its various targets of injection site. The determined lethality of four clinically important Ethiopian venomous snakes in rank-order

supports that of previously published by Sanchies (2011). The lethal potency of *C.rhombeatus* in Ethiopia is different compared with that of South African snake published by Spawls (1995). The lethal dose toxic value of *B.a.somalica* is relatively similar to that the Tanzanian *B.ariatans* (Thromb 2007) and *Echis pyramidum* on which both of the lethal dose toxins were found under the range of WHO (2001) data base. In neutralization tests of the two polyvalent antivenoms, FAV-AFRIQUE showed a distinctly higher neutralizing potency. During mice challenge venom and antivenom experiments, the reaction of toxins were detoxified or cleared from the solution at different concentration of the imported commercial antivenoms. The lethality neutralization test provided complementary information for understanding of pre-clinical efficacy of antivenom and is a useful tool for the prediction of in vivo activity (Gutierrez 2013). Both antivenoms showed different neutralizing efficacy against the venoms of the three species of viper species.

Conclusions

In this study, the lethality and relative toxicity of snake venoms varies significantly with routes of

administration. The polyvalent antivenoms currently used in the eastern and in the southwestern part of Ethiopia can effectively neutralize venoms of *B. parviocula*, *B. arietans somalica*, and *Echis pyramidum* venoms. Antivenoms studied using Swiss albino mice showed their effective neutralization and protection against common Ethiopian viper venom-induced cytotoxic and hemotoxic effects of the major lethal toxins (metalloproteinases and serine protease).

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