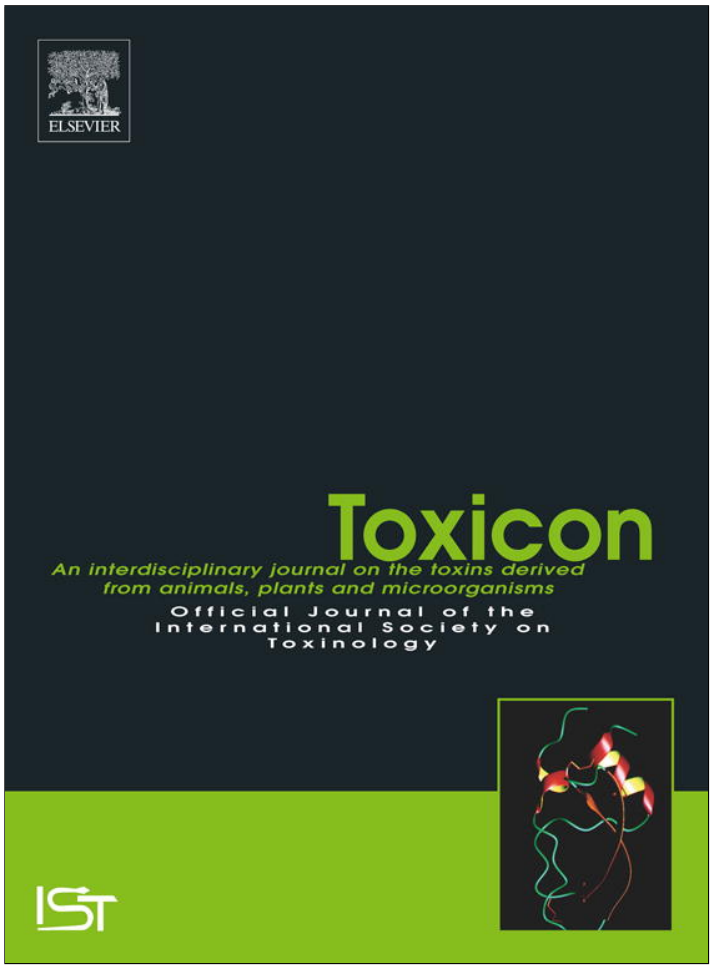

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Short communication

Effect of geographical variation of *Echis ocellatus*, *Naja nigricollis* and *Bitis arietans* venoms on their neutralization by homologous and heterologous antivenoms

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ABSTRACT

Two antivenoms prepared by using *Echis ocellatus*, *Bitis arietans* and *Naja nigricollis* venoms from different locations in sub-Saharan Africa were compared for their neutralizing ability. Both antivenoms were similarly effective in the neutralization of the venoms of the three species from different locations. However in the case of *E. ocellatus* venom, antivenom prepared using venom from Nigerian specimens was more effective than antivenom prepared with venom from Cameroon specimens in the neutralization of coagulant activity.

The only scientifically-validated therapy to confront snakebite envenomation is based on the parenteral administration of antivenoms (WHO, 2010). Antivenoms are formulations of immunoglobulins or its fragments purified from plasma of animals immunized with snake venoms (Gutiérrez et al., 2011). Owing their medical importance and wide geographical distribution, venoms of *Echis ocellatus*, *Naja nigricollis* and *Bitis arietans* have been used as immunogens to produce antivenoms for the treatment of snakebite envenomation in sub-Saharan Africa (Laing et al., 1995; Gutiérrez et al., 2005; Ramos-Cerrillo et al., 2008). These antivenoms have the capability of neutralizing toxins not only of the venoms used as immunogens, but also of other antigenically-related venoms (Calvete et al., 2010; Casewell et al., 2010; Petras et al., 2011; Segura et al., 2010). Nevertheless, due to intraspecific variations on venom phenotypes in populations of species of wide geographical distribution (Chippaux et al., 1991; Fry et al., 2003), antivenoms raised against venoms of specific populations might not be as effective in the neutralization of venoms from other populations within the same species (Gutiérrez et al., 2011). To study how intraspecific geographical variation of venoms affects antivenom efficacy in sub-Saharan Africa, this work explored the neutralization of venoms of *E. ocellatus*, *B. arietans* and *N. nigricollis* from several regions in sub-Saharan Africa by two antivenoms prepared with venoms of these species from different locations.

All procedures involving the use of mice in this study were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of Universidad de Costa Rica (project 82-08) and meet the International Guiding Principles for Biomedical Research Involving Animals (CIOMS, 1985).

All venoms in this study were obtained from adult individuals, and correspond to pools from many specimens. The venoms from Cameroon (*E. ocellatus* batch 522.060 and *N. nigricollis* batch 406.090) and from East Africa (*B. arietans* batch 128.090) were from Latoxan. Venoms of Nigerian snakes were collected from specimens captured in this country, and maintained in captivity at the herpetarium of the Liverpool School of Tropical Medicine, U.K. All venoms were stabilized by lyophilization and stored at −20 °C. Solutions of venoms in 0.12 M NaCl, 0.04 M phosphate, pH 7.2

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buffer (PBS) were prepared immediately before use. Two antivenoms manufactured at Instituto Clodomiro Picado were used: antivenom EchiTAb-Plus-ICP (batch 5290513 PALQ), prepared by immunizing horses with a mixture of the venoms of *E. ocellatus*, *B. arietans* and *N. nigricollis* from Nigeria (Gutiérrez et al., 2005); and antivenom Anti-C/EA, produced by immunizing a group of four horses with a mixture of venoms of *E. ocellatus* and *N. nigricollis* from Cameroon, and venom of *B. arietans* from East Africa. Both antivenoms were prepared following a procedure similar to that described by Gutiérrez et al. (2005). Antivenom immunoglobulins from plasma of the immunized animals were purified by caprylic acid precipitation (Rojas et al., 1994), and formulated at 5.1 g/dL protein content, 0.2 g/dL phenol, 0.85 g/dL NaCl, and pH 7.0.

The toxic activities of the venoms were compared. Lethality was determined by injecting groups of six mice (20–22 g; CD-1 strain) intravenously (i.v.) with different amounts of venom dissolved in PBS. Deaths occurring within 48 h were recorded, and the Median Lethal Dose (LD_{50}) was estimated according to the Spearman–Karber procedure (WHO, 1981). Hemorrhagic activity was assessed by triplicate in groups of four mice (18–20 g) by the intradermal injection of different amounts of venom dissolved in PBS, followed by the measurement of the diameter of the hemorrhagic lesion in the inner side of the skin 2 h after injection. The Minimum Hemorrhagic Dose (MHD) was defined as the amount of venom that induced a hemorrhagic lesion of 10 mm diameter (Gutiérrez et al., 1985). The coagulant activity was determined by triplicate, adding various doses of venom dissolved in 100 μL PBS, to 200 μL of human citrated plasma at 37 °C, followed by the determination of the clotting time. The Minimum Coagulant Dose (MCD) was defined as the dose of venom that causes the coagulation of plasma in 60 s (Theakston and Reid, 1983).

Neutralization studies were performed by: 1) mixing a constant 'challenge dose' of venom with several dilutions of antivenom, 2) incubating the mixtures for 30 min at 37 °C, and 3) determining residual toxicity of mixtures in mice or in plasma, as described above (Segura et al., 2010). In the control groups, PBS was used instead of antivenom. The 'challenge doses' used were: 5 LD_{50}s for lethal effect of *E. ocellatus* and *B. arietans* venoms; 3 LD_{50}s for lethal effect of *N. nigricollis* venom; 5 MHDs for hemorrhagic effect, and 2 MCDs for coagulant effect. The capacity of antivenoms to neutralize lethal and hemorrhagic activities was expressed as the Median Effective Dose (ED_{50}), defined as the volume of antivenom per 'challenge dose' of venom and as the ratio of mg venom neutralized/mL antivenom in which the activity of venom was reduced by 50% (WHO, 1981, 2010). The capacity of antivenoms to neutralize coagulant activity was expressed as the Effective Dose (ED), defined as the volume of antivenom required per 'challenge dose' of venom and as the ratio of mg venom/mL antivenom in which the clotting time was prolonged three times as compared with the clotting time of plasma incubated with venom alone (Gené et al., 1989). Neutralization of hemorrhagic and coagulant effects was performed by triplicate. The significance of the differences in LD_{50} and ED_{50} values for lethality was assessed by the overlap of 95% confidence intervals; values that do not overlap were considered significantly different. In the case of neutralization of hemorrhagic and coagulant activities, the significance of the differences between mean values of two experimental groups was assessed by non-parametric Mann–Whitney U test. Differences were considered statistically significant at values of $P < 0.05$.

Intraspecific variations on the venom activities of *E. ocellatus* snakes from Cameroon and Nigeria were observed. Compared to the venom from Cameroon, the venom of specimens from Nigeria showed higher lethal and coagulant ($P < 0.05$) activities, but similar hemorrhagic activity ($P = 0.1$; Table 1). Both antivenoms were similarly effective in the neutralization of the lethality and hemorrhage ($P = 0.1$) of the venom of *E. ocellatus* from Cameroon. However, the coagulant activity of this venom was better neutralized by the antivenom raised against venom of Nigerian specimens ($P < 0.05$; Table 2). Similarly, when tested against the venom of *E. ocellatus* from Nigeria, the antivenom raised against venoms of Nigerian specimens (EchiTAb-Plus-ICP) showed a significantly higher neutralization against coagulant effect ($P < 0.05$) than the anti C/E antivenom, although both neutralized lethality and hemorrhage ($P = 0.1$) with a similar ED_{50} (Table 2). These observations are relevant in the light of the high incidence and medical impact of envenomings by *E. ocellatus* in West sub-Saharan Africa (Habib and Warrell, 2013). Our data suggest that antivenom prepared with venom from Nigeria neutralize the coagulant activity of venoms from Nigeria and Cameroon more effectively than antivenom prepared using venom from Cameroon in the immunization. These observations are likely to underscore variations in the immunological properties of snake venom metalloproteinases, responsible for coagulant activity, in the venoms of *E. ocellatus* from these localities.

Venoms of *N. nigricollis* from Nigeria and Cameroon showed similar toxicity, i.e. LD_{50} (Table 1), and were devoid of hemorrhagic and coagulant activities, as described in earlier studies (Theakston and Reid, 1983; Gutiérrez et al., 2005; Segura et al., 2010). In contrast, previous observations described variation in *N. nigricollis* venoms from Nigeria and Cameroon in their reverse phase HPLC

Table 1
Toxic activities induced by venoms of *E. ocellatus*, *B. arietans* and *N. nigricollis*.

Species	Effect	Activity	Venom origin	
			Cameroon (*E. ocellatus* and *N. nigricollis*) or East Africa (*B. arietans*)	Nigeria
E. ocellatus	Lethal[a]	LD_{50}*	27.7 (23.9–32.1) μg	5.8 (4.6–6.9) μg
	Hemorrhagic[b]	MHD	0.30 ± 0.06 μg	0.11 ± 0.02 μg
	Coagulant[b]	MCD*	1.31 ± 0.14 μg	0.50 ± 0.02 μg
N. nigricolis	Lethal	LD_{50}	18.6 (15.9–21.2) μg	19.2 (16.7–23.2) μg
	Hemorrhagic	MHD	NA[c]	NA
	Coagulant	MCD	NA	NA
B. arietans	Lethal	LD_{50}*	22.4 (19.1–30.7) μg	13.6 (11.6–15.5) μg
	Hemorrhagic	MHD	0.24 ± 0.03 μg	0.15 ± 0.01 μg
	Coagulant	MCD	NA	NA

*Activities in which venoms from both localities differ significantly ($P < 0.05$).
[a] Lethal activity is expressed as Median Lethal Dose (LD_{50}, μg per mouse), with the 95% confidence limits in parentheses.
[b] Hemorrhagic and coagulant activities are expressed as Minimum Hemorrhagic Dose (MHD) and Minimum Coagulant Dose (MCD), respectively (see text for details); results are presented as mean ± SD of triplicates.
[c] NA: Activity not induced by the venom.

Table 2
Neutralization of toxic activities induced by venoms of African snakes, by homologous and heterologous antivenoms.[a]

Venom origin	Snake species	Toxic activity	Neutralization[b]			
			Antivenom anti-C/EA[a]		Antivenom EchiTAb-Plus-ICP[a]	
			μL antivenom/challenge dose	mg venom/mL antivenom	μL antivenom/challenge dose	mg venom/mL antivenom
Cameroon	*E. ocellatus*	Lethality[c]	47.8 (33.8–65.9)	2.9 (2.1–4.1)	42.0 (31.4–53.3)	3.3 (2.6–4.4)
		Hemorrhagic[d]	0.70 ± 0.14	2.2 ± 0.4	0.52 ± 0.03	2.9 ± 0.1
		Coagulant[e*]	0.75 ± 0.05	3.5 ± 0.2	0.21 ± 0.01	12.5 ± 0.7
	N. nigricollis	Lethality	93.0 (62.0–186.0)	0.6 (0.3–0.9)	79.7 (62.0–93.0)	0.7 (0.6–0.9)
		Hemorrhagic	NA[f]	NA	NA	NA
		Coagulant	NA	NA	NA	NA
East Africa	*B. arietans*	Lethality	19.3 (12.9–29.5)	5.8 (3.8–8.7)	29.5 (24.3–38.6)	3.8 (2.9–4.6)
		Hemorrhagic	0.25 ± 0.02	4.8 ± 0.4	0.28 ± 0.03	4.4 ± 0.5
		Coagulant	NA	NA	NA	NA
Nigeria	*E. ocellatus*	Lethality	9.0 (3.0–18.1)	3.2 (1.6–9.6)	9.7 (7.8–13.2)	3.0 (2.2–3.7)
		Hemorrhagic	0.77 ± 0.26	0.8 ± 0.2	0.23 ± 0.03	2.7 ± 0.4
		Coagulant*	1.26 ± 0.04	0.8 ± 0.03	0.17 ± 0.004	6.0 ± 0.1
	N. nigricollis	Lethality	64.0 (41.1–96.0)	0.9 (0.6–1.4)	115.2 (82.3–192.0)	0.5 (0.3–0.7)
		Hemorrhagic	NA	NA	NA	NA
		Coagulant	NA	NA	NA	NA
	B. arietans	Lethality	22.6 (14.8–32.4)	3.0 (2.1–4.6)	14.5 (11.5–17.9)	4.3 (3.8–5.9)
		Hemorrhagic	0.26 ± 0.01	2.8 ± 0.2	0.25 ± 0.02	3.0 ± 0.3
		Coagulant	NA	NA	NA	NA

*Activities in which antivenoms significantly differ in their neutralizing ability ($P < 0.05$).

[a] Antivenom towards Cameroon and East African venoms was prepared from plasma of horses immunized with a venom mixture of *E. ocellatus* and *N. nigricollis* from Cameroon, and *B. arietans* from East Africa. EchiTAb-Plus-ICP antivenom was prepared from plasma of horses immunized with a venom mixture of *E. ocellatus*, *N. nigricollis* and *B. arietans* from Nigeria.

[b] Neutralization is expressed in two different ways: a) mg venom/mL antivenom and b) μL antivenom/challenge dose of venom.

[c] Neutralization of lethal activity is expressed as Median Effective Dose (ED_{50}), with the 95% confidence limits in parentheses. Challenge doses correspond to 5 LD_{50}s for the venoms of *E. ocellatus* and *B. arietans*, and 3 LD_{50}'s for the venom of *N. nigricollis*.

[d] Neutralization of hemorrhagic activity is expressed as Median Effective Dose (ED_{50}) and presented as mean ± SD of triplicate determinations. Challenge dose correspond to 5 MHDs.

[e] Neutralization of coagulant activity was expressed as Effective Dose (ED) and presented as mean ± SD of triplicate determinations. Challenge dose of venom corresponds to 2 MCDs.

[f] NA: Activity not induced by the venom.

profile (Petras et al., 2011). The two antivenoms had a similar ability to neutralize the lethality induced by venoms from both countries (Table 2), thus suggesting that differences in HPLC profiles do not translate into immunological variation in toxins responsible for lethality between venoms from Nigeria and Cameroon.

The venoms of *B. arietans* were devoid of *in vitro* coagulant activity, in agreement with previous studies (Gutiérrez et al., 2005; Segura et al., 2010). On the other hand, venom from East African specimens is less lethal than venom from Nigerian snakes, but did not differ in hemorrhagic activity (Table 1). Intraspecific variations in the venoms of *B. arietans* from various geographical locations have been previously described (Currier et al., 2010). However, this heterogeneity in *B. arietans* venoms used in this study does not translate into differences in neutralization, since both antivenoms were similarly effective in the neutralization of lethal activity of the venoms. Likewise, both antivenoms were similarly effective in the neutralization of the hemorrhagic activity of venoms from East Africa ($P = 0.2$; Table 2) and Nigeria ($P = 0.7$; Table 2).

In conclusion, the issue of intraspecific variation in venom composition and activities is particularly relevant in species of wide geographical distribution and high medical impact, as in the three species studied in this work. These studies are important for selecting the most appropriate venom pools for antivenom production. The variations observed in the venoms of *B. arietans* and *N. nigricollis* from different locations did not significantly impact the neutralizing ability of antivenoms generated. In contrast, in the case of *E. ocellatus* venom, our results suggest that, depending on the geographical origin of the snakes whose venoms are used in immunization, there are significant variations in the ability of antivenoms to neutralize coagulant effects. Our results stress the need to test antivenoms aim to neutralize the venom of *E. ocellatus* against venoms from different countries in West sub-Saharan Africa, in order to define the geographical spectrum of efficacy of antivenoms.

Ethical statement

This manuscript presents an experimental study performed following the standard procedure of scientific ethics, including the use and care of experimental animals.

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