Toxicon 119 (2016) 280–288

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

# Preclinical evaluation of three polyspecific antivenoms against the venom of *Echis ocellatus*: Neutralization of toxic activities and antivenomics

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ARTICLE INFO

*Article history:*
Received 10 May 2016
Received in revised form
23 June 2016
Accepted 30 June 2016
Available online 1 July 2016

*Keywords:*
Africa
*Echis ocellatus* venom
Antivenoms
Neutralization
Antivenomics
Lethality

ABSTRACT

Snakebite envenoming has a heavy burden in the public health in sub-Saharan Africa. The viperid species *Echis ocellatus* (carpet viper or saw-scaled viper) is the medically most important snake in the savannahs of western sub-Saharan Africa. Several antivenoms are being distributed and used in this region for the treatment of envenomings by *E. ocellatus*, but the preclinical efficacy of some of these antivenoms has not been assessed. The present study evaluated the preclinical efficacy against *E. ocellatus* venom of three polyspecific antivenoms: (a) Snake Venom Antiserum (Pan Africa), manufactured by Premium Serums and Vaccines (India); (b) Snake Venom Antiserum (Africa), manufactured by VINS Bioproducts (India); and (c) Antivipmyn® Africa, manufactured by Instituto Bioclon (Mexico). Antivenomics analysis revealed the ability of the three antivenoms to immunocapture the majority of components of the venoms of *E. ocellatus* from Cameroon, Nigeria and Mali, although their maximal immunocapturing capability varied. Bioclon and Premium Serums antivenoms were effective in the neutralization of lethal, hemorrhagic and *in vitro* coagulant activities of the venom of *E. ocellatus* from Cameroon, albeit with different potencies. VINS antivenom neutralized hemorrhagic activity of this venom, but failed to neutralize lethality at the highest antivenom dose tested, and had a low neutralizing efficacy against *in vitro* coagulant effect.



## 1. Introduction

Sub-Saharan Africa suffers a very high burden of snakebite envenomings (WHO, 2007, 2010). The annual number of envenomings has been estimated in 320,000, resulting in 6000 fatalities and between 6000 and 15,000 amputations, according to a meta-analysis (Chippaux, 2011). A study based on a variety of sources provided estimates of snakebite cases in sub-Saharan Africa ranging from 90,622 to 419,639, with a range of fatal cases from 3529 to 32,117 (Kasturiratne et al., 2008). However, it is very likely that these numbers largely underestimate the actual magnitude of this highly neglected tropical disease, as there is a high extent of underreporting of snakebite cases in this region. The situation is further complicated by factors such as the low percentage of affected people that attend health services, the often weak public health systems, especially in the rural settings of those countries, the poor availability and accessibility of antivenoms, and the deficiencies in the knowledge that many health practitioners have in the topic of snakebite envenoming management (Chippaux, 2010).

The deficient antivenom provision in many countries, a situation that has been considered an "antivenom crisis" (Theakston and Warrell, 2000; WHO, 2007; Williams et al., 2011; Williams, 2015; Schiermeier, 2015), is further complicated by the poor regulatory

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http://dx.doi.org/10.1016/j.toxicon.2016.06.022
0041-0101/

control of the antivenoms that reach these countries, some of which have a poor safety and efficacy profiles (Visser et al., 2008; Warrell, 2008). A number of antivenom manufacturers are producing polyspecific and monospecific antivenoms for use in sub-Saharan Africa (Alirol et al., 2015; and see http://apps.who.int/bloodproducts/snakeantivenoms/database/). Nevertheless, there is very limited information on the preclinical efficacy of some of these antivenoms against medically-relevant African snake venoms. It is therefore necessary to evaluate the ability of these antivenoms to neutralize the most relevant toxic activities of viperid and elapid venoms from this region, in order to provide information for health authorities and decision makers in the public health arena.

In West sub-Saharan Africa, the viperid species *Echis ocellatus*, known as carpet viper or saw-scaled viper, is responsible for the highest burden of snakebite envenomings, both in terms of morbidity and mortality (Warrell, 1995; WHO, 2010; Habib, 2013). Human envenomings by this species are characterized by local necrosis, edema, hemorrhage and blistering, and by systemic effects such as coagulopathies and extensive bleeding which might result in severe anemia, internal hemorrhages, cerebrovascular accident, and cardiovascular shock (Warrell et al., 1974; Warrell, 1995; Abubakar et al., 2010; WHO, 2010). Owing to the medical relevance of this species, it is necessary to assess the preclinical efficacy of antivenoms distributed in West sub-Saharan Africa. A previous study evaluated three polyspecific and one monospecific antivenoms (Sánchez et al., 2015). In the present work we report the preclinical efficacy of three additional polyspecific antivenoms against the venom of *E. ocellatus*, by combining antivenomics and the assessment of neutralization of lethal, hemorrhagic, and *in vitro* coagulant activities.

## 2. Materials and methods

### 2.1. Venoms

Pools of freeze-dried venoms of *E. ocellatus* were obtained from various localities. The venoms of specimens from Mali and Cameroon were from Latoxan, whereas the venom of specimens from Nigeria was provided by the Liverpool School of Tropical Medicine, UK. Venoms were stored at −20 °C until used. Prior to their use, venoms were dissolved in 0.12 M NaCl, 0.04 M phosphate, pH 7.2 (PBS).

### 2.2. Antivenoms

The following antivenoms were used in this study: (a) Snake Venom Antiserum (Pan Africa), manufactured by Premium Serums and Vaccines Pvt. Ltd., India, batch number 062903 (Premium Serums antivenom); (b) Snake Venom Antiserum (Africa), manufactured by VINS Bioproducts Ltd., India, batch number 07AS15002 (VINS antivenom); and (c) Antivipmyn® Africa, manufactured by Instituto Bioclon S.A. de C.V., Mexico, batch number DFB-150903 (Bioclon antivenom). All antivenoms were used within their shelf-lives. The total protein concentration of antivenoms was quantified by a modification of the Biuret method (Parvin et al., 1965). Table 1 summarizes the characteristics of the three antivenoms used.

### 2.3. Analysis of the preclinical neutralizing profile of antivenoms

For the neutralization of toxic activities, only the venom of *E. ocellatus* from Cameroon was used, on the basis of the previous study that showed similar patterns of neutralization of antivenoms against the venoms from Cameroon, Nigeria and Mali (Sánchez et al., 2015), and as an effort to reduce the number of mice used in the study. The protocols described in the previous publications by Segura et al. (2010) and Sánchez et al. (2015) for the study of the neutralization of lethal, hemorrhagic and *in vitro* coagulant effects were followed. Mice of the CD-1 strain were used throughout the study. The protocols which involved the use of mice were approved by the Institutional Committee for the Care and Use of Laboratory Animals (CICUA) of the University of Costa Rica.

#### 2.3.1. Lethal activity

Mixtures containing a fixed dose of venom and various dilutions of antivenom were prepared, and incubated at 37 °C for 30 min. Aliquots of 0.2 mL of each mixture, containing a dose of venom corresponding to 5 $LD_{50}$s, were then injected i.v. into groups of six mice (18–20 g). Mixtures corresponding to various ratios of mg venom/mL antivenom (or mg venom/g antivenom protein) were used. A control group was injected with 5 $LD_{50}$s of venom incubated with PBS instead of antivenom. Deaths occurring during 24 h were recorded, and the neutralizing ability of antivenom was expressed as the Median Effective Dose ($ED_{50}$), i.e. the venom/antivenom ratio at which half of the population of injected mice is protected, estimated by probits.

#### 2.3.2. Hemorrhagic activity

Mixtures containing a fixed dose of venom and various dilutions of antivenom were prepared, and incubated at 37 °C for 30 min (Gutiérrez et al., 1985). Then, aliquots of 0.1 mL of each mixture, containing a dose of venom corresponding to 5 Minimum Hemorrhagic Doses (MHDs), were injected intradermally into groups of five mice (18–20 g) (Sánchez et al., 2015). Mixtures corresponded to various ratios of mg venom/mL antivenom (or mg venom/g antivenom protein). A control group of mice was injected with the same dose of venom incubated with PBS instead of antivenom. Mice were sacrificed by $CO_2$ inhalation 2 h after injection, and the area of the hemorrhagic lesion was measured. Neutralizing ability was expressed as the Median Effective Dose ($ED_{50}$), corresponding to the ratio venom/antivenom at which the diameter of the hemorrhagic spot is reduced by 50% when compared to the diameter of the hemorrhagic lesion in mice injected with venom incubated with no antivenom.

#### 2.3.3. Coagulant activity

Mixtures containing a fixed dose of venom and various dilutions of antivenom were prepared, and incubated at 37 °C for 30 min (Gené et al., 1989). Then, aliquots of 0.1 mL of each mixture, containing a venom dose corresponding to 2 Minimum Coagulant Doses (MCDs), were added to 0.2 mL of human citrated plasma, previously incubated at 37 °C. Mixtures corresponded to various ratios of mg venom/mL antivenom (or mg venom/g antivenom protein). A control group of plasma incubated with venom that was previously incubated with PBS instead of antivenom was also included. Clotting times were recorded and neutralization was expressed as Effective Dose (ED), corresponding to the ratio of venom/antivenom in which the clotting time is prolonged three times as compared to the clotting time of plasma incubated with venom alone (Gené et al., 1989; Sánchez et al., 2015).

### 2.4. Antivenomics

#### 2.4.1. Second-generation immunoaffinity chromatography-based antivenomics

The protocol described by Pla et al. (2012) was applied to assess the immunoreactivity of the three equine $F(ab')_2$ antivenoms towards the venoms of *E. ocellatus* from Mali, Cameroon, and Nigeria. Venoms and analytical conditions described below were essentially