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Evaluation of the preclinical efficacy of four antivenoms, distributed in sub-Saharan Africa, to neutralize the venom of the carpet viper, *Echis ocellatus*, from Mali, Cameroon, and Nigeria

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ABSTRACT

Snakebite envenoming causes a heavy toll in sub-Saharan Africa in terms of mortality and sequelae. In the West African savannah, the viperid *Echis ocellatus* is responsible for the vast majority of bites. In the last decades, several new antivenoms have been introduced for the treatment of these envenomings, although the assessment of their preclinical efficacy against the venom of *E. ocellatus* has been studied only for some of them. This work analyzed comparatively the ability of four antivenoms (FAV Afrique, EchiTAb G, EchiTAB-Plus-ICP®, and Inoserp™ Panafricain) to neutralize lethal, hemorrhagic, and *in vitro* coagulant activities of the venoms of *E. ocellatus* from Mali, Cameroon, and Nigeria. In addition, an immunoaffinity chromatography antivenomic protocol was used to assess the ability of the four antivenoms to bind to the proteins of these venoms. Results showed that all the antivenoms were effective in the neutralization of the three effects investigated, and were able to immunocapture, completely or partially, the most abundant components in the *E. ocellatus* venoms from the geographical origins sampled. Our observations also highlighted quantitative differences between antivenoms in their neutralizing and antivenomics profiles, especially regarding neutralization of *in vitro* coagulant activity, suggesting that different doses of these antivenoms are probably needed for an effective treatment of human envenomings by this species.

1. Introduction

Snakebite envenomings constitute a highly relevant, albeit largely neglected, public health problem in sub-Saharan Africa (Warrell, 1995; Chippaux, 2010; WHO, 2007). One of the snake species that inflicts a high number of envenomings, especially in Western sub-Saharan Africa, is the saw-scaled or carpet viper, *Echis ocellatus* (Warrell, 1995; WHO, 2010). Envenomings caused by this viperid species are characterized by local tissue damage, i.e. soft tissue necrosis, edema, hemorrhage and blistering, and by systemic manifestations associated mainly with coagulopathies and profuse bleeding which might lead to cerebrovascular accident and cardiovascular shock (Warrell et al., 1974; Warrell, 1995; Abubakar et al., 2010).

The only scientifically-validated treatment for snakebite envenoming is the parenteral administration of animal-derived antivenoms (Warrell, 2010; Gutiérrez et al., 2011). Despite the fact that various antivenom manufacturers produce antivenoms for sub-Saharan Africa (see http://apps.who.int/bloodproducts/snakeantivenoms/database/), the availability of some of these products is very limited and often discontinuous. In addition, some manufacturers that used to produce antivenoms for sub-

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Saharan Africa have ceased production (Stock et al., 2007; Brown, 2012). This has prompted a crisis in the availability and accessibility of antivenoms for this region, characterized by a vicious cycle that greatly compromises the treatment of this tropical disease in many African countries (Chippaux, 2010; Williams et al., 2011; Brown, 2012). Various international initiatives have emerged in the last decade aimed at developing new antivenoms for Africa; some of these efforts have resulted in the generation of antivenoms manufactured in the United Kingdom, Costa Rica, and Mexico (Gutiérrez, 2012; Gutiérrez et al., 2014). In addition, two other antivenoms of demonstrated clinical efficacy and safety, IPSER Afrique (Chippaux et al., 1998) and FAV Afrique (Chippaux et al., 1999), which differ by the presence of *E. ocellatus* in the immunizing mixture and by the introduction of a chromatographic step of purification for the latter, have been manufactured in France for years, but their production has been discontinued in 1998 and 2014, respectively.

One of the aspects that limits the distribution and appropriate use of antivenoms in sub-Saharan Africa is the lack of rigorous knowledge on the spectrum of preclinical efficacy of currently available antivenoms. In the case of Western sub-Saharan Africa, where the most important snake from a medical standpoint is *E. ocellatus*, there is limited information on the ability of antivenoms to neutralize the main toxic activities induced by the venom of this species. Moreover, there is little information on the variation in the composition of the venom of *E. ocellatus* from various locations, and whether such variations have an impact in the preclinical neutralizing ability of antivenoms. Owing to the medical impact of this species in the African savannah, there is an urgent need to perform preclinical analysis of antivenom efficacy against the venom of *E. ocellatus*.

This study was designed to assess the preclinical efficacy of four antivenoms, available in Western sub-Saharan Africa, to neutralize the main toxic activities of venoms of *E. ocellatus* from various geographical origins. Assessment of efficacy has been performed by combining the study of the neutralization of three key toxic activities of the venoms, i.e. lethality, hemorrhagic activity and coagulant activity, with an antivenomics analysis of the ability of these antivenoms to recognize the various components present in these venoms. The integration of these methodological platforms now provides novel evidence on the preclinical efficacy of these antivenoms.

2. Materials and methods

2.1. Venoms

Samples of pools of freeze-dried venoms of *E. ocellatus* from different countries were obtained. The venoms of specimens from Mali and Cameroon were purchased from Latoxan. The venom of specimens from Nigeria was obtained from the Liverpool School of Tropical Medicine, UK. These venoms correspond to pools of many specimens. Venoms were stored at −20 °C until used.

2.2. Antivenoms

Samples of the following antivenoms were tested in the study: (a) FAV Afrique F(ab′)$_2$ antivenom, manufactured by Sanofi-Pasteur, France, batch number K8453; (b) EchiTAb-Plus-ICP® IgG antivenom, manufactured by Instituto Clodomiro Picado, Costa Rica, batch number 5370114PALQ; (c) Inoserp™ Panafricain F(ab′)$_2$ antivenom, manufactured by Inosan Biopharma, S.A., Spain, batch number 2VT08001; and (d) EchiTAb G IgG antivenom, manufactured by Micropharm, UK, batch number EOG 000950. All antivenoms were used within their valid shelf-life. The total protein concentration of antivenoms was quantified by a modified Biuret method (Parvin et al., 1965). Table 1 summarizes the characteristics of the four antivenoms used.

2.3. Analysis of the preclinical neutralizing profile of antivenoms

The protocols previously used in the study of the preclinical efficacy of antivenoms were followed (Segura et al., 2010). The study included the analysis of the neutralization of the three most important toxic activities of *E. ocellatus* venom, i.e. lethal activity, hemorrhagic activity and *in vitro* coagulant activity. The protocols which involve 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

The Median Lethal Dose (LD_{50}) of each venom, by the intravenous route, was initially determined (Segura et al., 2010). For this, groups of five CD-1 mice (18–20 g body weight) were injected intravenously, in the caudal vein, with various doses of venom, dissolved in 0.12 M NaCl, 0.04 M phosphate, pH 7.2 (PBS), in a volume of 0.2 mL. Deaths occurring during 24 h were recorded, and the value of LD_{50} was estimated by probits.

For the neutralization of lethal activity, mixtures containing a fixed dose of venom and various dilutions of antivenom were prepared, and incubated at 37 °C for 30 min. Then, aliquots of 0.2 mL of each mixture, containing a dose of venom corresponding to 5 LD_{50}s, were injected i.v. into groups of five mice. 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. Deaths were recorded for 24 h 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, as estimated by probits.

2.3.2. Hemorrhagic activity

The Minimum Hemorrhagic Dose (MHD) of each venom was initially determined (Gutiérrez et al., 1985). For this, groups of five CD-1 mice (18–20 g) were injected intradermally, in the abdominal region, with various doses of venom, dissolved in PBS, in a volume of 0.1 mL. Two hours after injection, mice were sacrificed by CO_2 inhalation, their skin removed, and the area of the hemorrhagic spot in the inner side of the skin measured. The MHD corresponds to the dose of venom that induces a hemorrhagic spot of 10 mm diameter (Gutiérrez et al., 1985).

For the neutralization of 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 MHDs, were injected intradermally into groups of five mice, as described above. 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. After 2 h, mice were sacrificed by CO_2 inhalation, and the area of the hemorrhagic spot 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 spot in mice injected with venom incubated with no antivenom.

2.3.3. Coagulant activity

The Minimum Coagulant Dose (MCD) of each venom was