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Expanding the neutralization scope of the EchiTAb-plus-ICP antivenom to include venoms of elapids from Southern Africa

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ABSTRACT

EchiTAb-plus-ICP is an antivenom prepared from plasma of horses hyperimmunized with the venoms of the carpet viper (*Echis ocellatus*), the puff adder (*Bitis arietans*) and the black-necked spitting cobra (*Naja nigricollis*). Therefore, the use of this antivenom has been limited to Western Africa. In order to expand the neutralization scope of EchiTAb-plus-ICP, we supplemented the immunogenic mixture with the venoms of *B. arietans*, the black mamba (*Dendroaspis polylepis*), the Mozambique spitting cobra (*Naja mossambica*), the snouted cobra (*N. annulifera*), and the rinkhals (*Hemachatus haemachatus*) from Swaziland. The ability of the expanded-scope antivenom, hereby named EchiTAb + ICP, to neutralize the venoms of *B. arietans*, *D. polylepis*, *N. mossambica* and *H. haemachatus* was similar to those of FAV Afrique and the SVA African antivenoms. In comparison to the SAIMR antivenom, the expanded-scope EchiTAb + ICP had lower ability to neutralize the venom of *B. arietans*, but similar ability to neutralize the venoms of *D. polylepis*, *N. mossambica* and *H. haemachatus*. Owing to its low protein concentration, the expanded-scope EchiTAb + ICP had lower ability to neutralize the venom of *N. annulifera* than FAV Afrique and the SAIMR antivenoms. However, when formulated at a protein concentration as high as FAV Afrique and SAIMR antivenoms, the expanded-scope EchiTAb + ICP showed similar capacity to neutralize this poorly immunogenic venom. Our results encourage the transition to the new EchiTAb + ICP antivenom, with an expanded neutralization scope that includes venoms of some of the most medically important elapids from Southern Africa. Clinical trials are required to determine the minimum effective-safe dose of the new EchiTAb + ICP for each type of envenomation.

1. Introduction

Snakebite envenomation is a neglected public health problem in most of the tropical and sub-tropical regions of the world (WHO, 2010). In sub-Saharan Africa, this problem has been worsened by the shortage of specific antivenoms, the inadequate strategy for antivenom storage and distribution, and the lack of training of medical personnel in charge of taking care of snakebites victims (Chippaux and Habib, 2015; Chippaux et al., 2015a).

In order to improve the supply of antivenoms for sub-Saharan Africa, several formulations have been developed by laboratories in the United Kingdom (Laing et al., 1995; Meyer et al., 1997; Al-Abdulla et al., 2003), Colombia (Laing et al., 2003), Costa Rica (Gutiérrez et al., 2005), México (Ramos-Cerrillo et al., 2008), Spain (Chippaux et al., 2015b) and Brazil (Guidolin et al., 2016). Several of these formulations are currently used in hospitals of some African countries. However, at the same time, Sanofi-Pasteur discontinued the production of the FAV Afrique F $(ab')_2$ antivenom, a long-established product considered as reference in the continent (Wolf et al., 2011). The new antivenoms, along with the products traditionally manufactured by the South African Vaccine Producers (i.e. a division of the National Health Laboratory Service, formerly known as the South African Institute for Medical Research; Hawgood, 2001) and by several producers in India (Kanthawala,

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2009), do not meet the current demand for antivenoms in sub-Saharan Africa.

The antivenom produced in Costa Rica (EchiTAb-plus-ICP) is a formulation of immunoglobulins purified from plasma of horses immunized with an immunogenic mixture composed by venoms of the carpet viper (*Echis ocellatus*), the puff adder (*Bitis arietans*) and the black-necked spitting cobra (*Naja nigricollis*) (Gutiérrez et al., 2005). This antivenom recognizes and neutralizes the homologous venoms (i.e. venoms included in the immunogenic mixture) and some heterologous venoms (i.e. venoms not included in the immunogenic mixture) of saw scaled vipers (*Echis* sp), African adders (*Bitis* sp), and African spitting cobras (*Naja* sp) of Western Africa (Calvete et al., 2010; Segura et al., 2010; Petras et al., 2011; Sánchez et al., 2015; our unpublished results). A clinical trial in Nigeria demonstrated that this antivenom is safe and effective in envenomations by *E. ocellatus* (Abubakar et al., 2010). Therefore, it has been successfully used, at a clinical level, during the last years in countries such as Mali, Burkina Faso and Nigeria.

Hitherto, the immunogenic mixture used to prepare the EchiTAb-plus-ICP antivenom has not included venoms of snakes inducing high levels of morbidity, disability and mortality in Southern Africa, such as mambas (*Dendroaspis* sp), and spitting and neurotoxic cobras (*Naja* sp). Hence, until now, the use of EchiTAb-plus-ICP has not been recommended for the treatment of snakebite envenomations in this region.

The expansion of the neutralization scope of the EchiTAb-plus-ICP antivenom requires the incorporation of several elapid venoms from Southern Africa in the immunogenic mixture. However, this modification in the production process must be cautiously addressed. Previously, it has been demonstrated that some snake venoms have immunomodulatory properties that affect the antibody response induced by other venoms used as co-immunogens (Arroyo et al., 2015). Accordingly, as a consequence of the inclusion of venoms of elapids from Southern Africa in the immunization mixture, a reduced neutralizing activity of the new antivenom towards the venoms that constitute the standard immunogenic mixture of EchiTAb-plus-ICP (i.e. *E. ocellatus*, *B. arietans* and *N. nigricollis*) may occur.

In this work, we assessed the supplementation of the immunogenic mixture used to produce the EchiTAb-plus-ICP antivenom with venoms of *B. arietans*, *D. polylepis*, *N. mossambica*, *N. annulifera* and *H. haemachatus* from Swaziland. The ability of the expanded-scope antivenom to neutralize venoms of Southern African snakes was compared with those of other antivenoms that are available in the region. Moreover, the effect of this procedure on the pre-clinical neutralization profile of venoms of *E. ocellatus*, *B. arietans* and *N. nigricollis* from Nigeria was determined.

2. Materials and methods

This study meets the International Guiding Principles for Biomedical Research Involving Animals (CIOMS, 1985). All procedures involving animals were approved by the Institutional Committee for the Care and Use of Laboratory Animals of Universidad de Costa Rica (approval number CICUA 82-08).

2.1. Snake venom

Venoms of *E. ocellatus*, *B. arietans* and *N. nigricollis* from Nigeria were obtained from adult snakes maintained in captivity at the Herpetarium of the Liverpool School of Tropical Medicine, U.K. Venoms of *B. arietans*, *D. polylepis*, *N. mossambica*, *N. annulifera* and *H. haemachatus* from Swaziland were obtained from adult specimens collected in Swaziland and maintained in captivity at the Herpetarium of African Reptiles & Venom Company, South Africa. Samples of venom from many specimens were mixed by species, stabilized by lyophilization, and stored at −20 °C. Solutions of venoms in 0.12 M NaCl, 0.04 M phosphate, pH 7.2 buffer (PBS) were prepared immediately before use.

2.2. Production of the expanded-scope antivenom

Three horses were used (5–6 years old; 400–450 kg), previously immunized with venoms of *E. ocellatus*, *B. arietans* and *N. nigriollis* from Nigeria (using the immunization schedule described by Gutiérrez et al., 2005) and used for antivenom production during the last 2 years. These horses were immunized with venoms of *B. arietans*, *D. polylepis*, *N. mossambica*, *N. annulifera* and *H. haemachatus* from Swaziland, in addition to the three venoms from Nigeria. Briefly, horses were injected with venoms by the subcutaneous route, in one single injection, at different time intervals. In the first injection, the immunogen (10 mg of a mixture of equal parts of venoms of *B. arietans*, *D. polylepis*, *N. mossambica*, *N. annulifera* and *H. haemachatus*) was emulsified in Freund's complete adjuvant. In the second booster, the same immunogen was emulsified in Freund's incomplete adjuvant. The rest of the injections, consisting of 10 mg of a mixture of equal parts of the eight venoms dissolved in sterile saline solution, were applied biweekly within the following two months. The horses were bled thirteen days after the last booster, and plasma was separated from red cells and stored at 2–8 °C until use. For the preparation of the antivenom, immunoglobulins were purified by the caprylic acid precipitation method (Rojas et al., 1994). The antivenom was formulated at 9 g/L NaCl, 2.5 g/L phenol and pH 7.0; it was then sterilized by filtration and dispensed in 10 mL glass vials. This novel antivenom with expanded coverage is hereby named EchiTAb + ICP in order to differentiate it from the traditional EchiTAb-plus-ICP antivenom.

2.3. Antivenoms used as reference

The following antivenoms were used as reference: (a) standard EchiTAb-plus-ICP (Instituto Clodomiro Picado, batch number 5141112PALQ), (b) FAV Afrique antivenom, manufactured by Sanofi-Pasteur, batch number K8453; (c) Snake Venom Antiserum (SVA African), manufactured by VINS Bioproducts Ltd., batch number 06AS12001; (d) ASNA-C Antivenom, manufactured by Bharat Serum and Vaccines Ltd., batch number A2610002, and (e) SAIMR Polyvalent Snake Antivenom, manufactured by the South African Vaccine Producers, batch number BD01946. Table 1 summarizes several immunochemical characteristics of the antivenoms used in this study. All antivenoms were used within their valid shelf life.

2.4. Lethal activity and lethality neutralization assays

Lethality of each venom was determined by injecting groups of five mice (18–20 g; CD-1 strain) intravenously (i.v.) with different amounts of venom dissolved in 0.2 mL PBS. Deaths occurring within 24 h were recorded. The Median Lethal Dose (LD_{50}; i.e. the amount of venom that kills half of the challenged animals) and their 95% confidence interval were estimated using Probits analysis. The neutralization of lethality was assessed injecting the mice with 0.2 mL of venom/antivenom mixtures containing 5 LD_{50}s (for venoms of *E. ocellatus* and *B. arietans*) or 3 LD_{50} (for venoms of *D. polylepis*, *N. nigricollis*, *N. mossambica*, *N. annulifera* and *H. haemachatus*) as challenge dose. Deaths occurring within 24 h were recorded. The neutralizing activity of antivenoms was expressed as Median Effective Dose (ED_{50}; i.e. the antivenom/venom ratio in which the lethality of venom was inhibited by 50%) and their 95% confidence interval.