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Active immunization of cattle with a bothropic toxoid does not abrogate envenomation by *Bothrops asper* venom, but increases the likelihood of survival

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ABSTRACT

This study assessed the protective effect of active immunization of cattle to prevent the envenomation induced by *B. asper* venom. Two groups of oxen were immunized with a bothropic toxoid and challenged by an intramuscular injection of either 10 or 50 mg *B. asper* venom, to induce moderate or severe envenomations, respectively. Non-immunized oxen were used as controls. It was found that immunized oxen developed local edema similar to those observed in non-immunized animals. However, systemic effects were totally prevented in immunized oxen challenged with 10 mg venom, and therefore antivenom treatment was not required. When immunized oxen were challenged with 50 mg venom, coagulopathy was manifested 3–16 h later than in non-immunized oxen, demonstrating a delay in the onset of systemic envenomation. In these animals, active immunization did not eliminate the need for antivenom treatment, but increased the time lapse in which antivenom administration is still effective. All experimentally envenomed oxen completely recovered after a week following venom injection. Our results suggest that immunization of cattle with a bothropic toxoid prevents the development of systemic effects in moderate envenomations by *B. asper*, but does not abrogate these effects in severe envenomation.

1. Introduction

From a human public health perspective, *Bothrops asper* is the most important venomous snake from northern Central America to northern South America [1]. Therefore, this snake is most likely involved in the majority of envenomations suffered by cattle and other domestic species in the region. Our unpublished observations suggest that, annually, around 10,000 cases of *B. asper* envenomed cattle may occur just in the Central Pacific region of Costa Rica. However, the lack of reliable records prevent an accurate assessment of the real magnitude of this problem in the whole region.

Bites of *B. asper* in cattle are commonly inflicted on the legs. Nonetheless, bites in the face are also frequent [2]. In the earliest stages of envenomation, most cattle develop local swelling and hemorrhage at the anatomical site of the bite, and systemic manifestations such as lethargy and coagulopathy, in the case of moderate envenomations. In the case of severe envenomations, symptoms such as bleeding from nose, ears, skin and urinary and digestive tracts can develop [2]. In the absence of an adequate treatment, death could occur within 12 h following envenomation [2,3].

The only scientifically validated treatment for cattle envenomated by *B. asper* is the parenteral administration of antivenoms [2]. Antivenoms are solutions of immunoglobulins purified from plasma of animals (e.g. horses or sheep) immunized with snake venoms [4]. After intravenous administration, anti-venom immunoglobulins reach the different body compartments of cattle [5], where they bind to the venom toxins and neutralize their capacity to induce toxicity.

Efficacy of the antivenom treatment depends on the time elapsed between envenomation and antivenom administration

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[2,6]. When administered within 6 h following envenomation, antivenoms are effective in controlling the main clinical manifestations of envenomation. When animals are envenomated during the workday, they are treated timely with antivenom, and the vast majority survives without physical aftermaths [2].

However, a significant number of animals are bitten after working hours, and therefore they are not treated until the following day. Those animals remain untreated for 8–12 h. During this time, mild or moderate envenomations may evolve to severe envenomations, and around 73% of these animals die despite a delayed antivenom administration [2].

Previous experiments performed in mice suggest that the resistance to envenomation by *B. asper* or *Crotalus atrox* bites could be increased by active immunization with the corresponding venom toxins [7,8]. Since active immunization is performed before accidental snakebite, its efficacy is not affected by the delay in the diagnosis of envenomation, as is the case with the administration of antivenoms. However, the effectiveness of some commercial antiophidic vaccines offered to protect animals from envenomation inflicted by snakes remains controversial [9]. This study was carried out to assess whether immunization with a bothropic toxoid prevents envenomation of cattle by *B. asper* venom.

2. Materials and methods

All procedures involving animals used in this study were approved by the Institutional Committee for the Care and Use of Laboratory Animals of Universidad de Costa Rica (Approval CICUA-37-11), and meet the International Guiding Principles for Biomedical Research Involving Animals [10], and the items of the ARRIVE guidelines. All procedures were performed under strict veterinary supervision.

2.1. Venom and antivenom

The venom was collected from adult specimens of *B. asper*, captured in the Pacific versant of Costa Rica and maintained in captivity at the Serpentarium of the Instituto Clodomiro Picado. Venom was stabilized by lyophilization and stored at −20 °C. Solutions of venom were prepared immediately before use. The antivenom used was PoliVet-ICP (Instituto Clodomiro Picado; batches 5000512POLQ and 5090912POLQ); it is a liquid formulation of IgG molecules purified from the plasma of horses immunized with venoms of *B. asper*, *Crotalus simus* and *Lachesis stenophrys* snakes [11] using the caprylic acid method [12]. The residual caprylic acid in this formulation has been determined as lower than 250 μg/mL [13]. Each milliliter of the antivenom contains approximately 20 mg/mL of antibodies towards the venom of *B. asper* [14] and neutralizes 3.0 mg of this venom, as demonstrated by a mouse neutralization assay [15].

2.2. Passive immunization of oxen by intravenous administration of antivenom

Four non-immunized oxen (Pardo/Zebu strain) weighing 600 ± 50 kg were injected intravenously with 200 mL of equine-derived antivenom. A blood sample was collected 1 h after antivenom administration to estimate the serum concentration of equine anti-venom antibodies. Immediately after the collection of the blood sample, animals were submitted to experimental envenomation.

2.3. Determination of equine anti-venom antibodies in passively immunized oxen

ELISA was performed at room temperature. Polystyrene plates (Costar 9017, Corning) were coated overnight with 100 μL of a solution of *B. asper* venom (3 μg/100 μL) in 0.14 M NaCl, 0.04 M phosphate, pH 7.2 solution (PBS). The plates were washed 10 times with distilled water. Then, 100 μL of either equine antibody standard towards *B. asper* venom or serum samples of passively immunized oxen (diluted 1:1000) were added and the plates incubated for 1 h. Both standards and samples were diluted in PBS containing 1% of human serum albumin (PBS-1% HSA). The plates were washed 10 times with distilled water and 100 μL of rabbit anti-equine IgG conjugated with alkaline phosphatase (A6063; Sigma-Aldrich), diluted 1:3000 with PBS-1%HSA, were added to each well and incubated for 1 h. Then, the plates were washed 10 times with distilled water and the color was developed by the addition of *p*-nitrophenyl phosphate (N9389; Sigma-Aldrich). Absorbance was recorded at 410 nm using a microplate photometer (Multiskan FC; Thermo Scientific). Finally, the concentration of equine anti-venom antibodies in the serum of passively immunized oxen was calculated by interpolating the absorbance of samples in a calibration curve constructed by plotting the absorbance of standards as a function of their concentration of equine anti-venom antibodies.

2.4. Active immunization of oxen by injection of bothropic toxoid

For the preparation of the bothropic toxoid, 60 mL of a 10 mg/mL venom solution were sterilized by filtration through a 0.22 μm membrane. Afterwards, venom was mixed with 60 mL of calcium phosphate adjuvant (Brenntag Biosector, Denmark, batch 2011-50) and then inactivated by incubation at 70 °C for 45 min [16]. No preservatives were added to the preparation. The bothropic toxoid was aseptically dispensed in sterile vials of borosilicate and stored at 2–8 °C until use. A group of 14 oxen (Pardo/Zebu strain), weighing 600 ± 50 kg, were immunized subcutaneously with three injections administered at intervals of three weeks (each one constituted by 5 mg of bothropic toxoid contained in 1 mL of injection). Blood samples were collected before each booster and three weeks after the last one, in order to evaluate the development of the antibody response. The 14 oxen were used to evaluate the increase of anti-venom antibodies induced by the active immunization, but only eight of them (randomly selected) were submitted to experimental envenomation, three weeks after the last booster.

2.5. Determination of anti-venom antibodies in oxen actively immunized

ELISA was performed at room temperature. Polystyrene plates (Costar 9017, Corning) were coated overnight with 100 μL of a solution of *B. asper* venom (3 μg/100 μL) in PBS. Then, the plates were washed 10 times with distilled water, and 100 μL of standards of bovine antibodies towards *B. asper* venom or serum samples of actively immunized oxen (diluted 1:1000) were added and incubated for 1 h. Both standards and samples were diluted in PBS-1% HSA. The plates were washed 10 times with distilled water and 100 μL of rabbit anti-bovine IgG conjugated with alkaline phosphatase (A0705; Sigma-Aldrich), diluted 1:3000 with PBS-1%HSA, were added to each well and incubated for 1 h. Afterwards, the plates were washed 10 times with distilled water and color was developed by the addition of *p*-nitrophenyl phosphate (N9389; Sigma-Aldrich). Absorbance was recorded at 410 nm using a microplate photometer (Multiskan FC; Thermo Scientific). Finally, the concentration of bovine anti-venom antibodies in the serum of actively immunized oxen was calculated by interpolating the absorbance of samples in a calibration curve constructed by plotting the absorbance of standards as a function of their concentration of anti-venom antibodies.