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Study of the design and analytical properties of the lethality neutralization assay used to estimate antivenom potency against *Bothrops asper* snake venom

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ABSTRACT

The lethality neutralization assay performed in mice is the standard recommended by the World Health Organization to estimate antivenom potency. The interpretation of its results without considering its analytical capacity may lead to erroneous conclusions. Therefore, laboratories that manufacture or control antivenoms must demonstrate the appropriateness of their models. A study of the method used at Instituto Clodomiro Picado, Costa Rica, to estimate the potency of antivenoms against *Bothrops asper* snake venom was performed. Results show that venom doses ranging from 2 to 6 Median Lethal Doses (LD_{50}) are appropriate to be used as challenge in this test. Variables such as the injection route, number of mice used per venom/antivenom level, and weight of the animals are critical in the estimation of the Median Effective Dose (ED_{50}), whereas incubation time is not. The assay has an acceptable selectivity, linearity, and limits of detection and quantification. Accuracy of the lethality neutralization assay, expressed as percentage recovery, was between 71% and 127%. Intermediate precision, expressed as relative standard deviation, was $\leq$17%. It is concluded that the analytical characteristics of this assay are adequate enough to prove product compliance and to have statistical control over an industrial line of antivenom serial production.

1. Introduction

Antivenoms are therapeutic preparations of immunoglobulins purified from the plasma of animals immunized with venoms. Parenteral antivenom administration is the central procedure in the treatment of snakebite envenomation [1]. The effectiveness of this immunotherapy depends on antivenom potency and specificity [2–5], as well as on other factors such as the time lapse between envenomation and antivenom administration [6], the route of antivenom administration [7] and the use of a correct antivenom dose [8].

Antivenom neutralizing potency is the capacity of antivenoms to neutralize the toxic effects induced by snake venoms [2–4,9]. Since venom composition varies between species, and even between different populations of a single species [10–12], antivenoms can neutralize only venoms that are antigenically related to the venoms used in the immunization process [13,14]. This characteristic of antivenoms, known as specificity, was described by Vital Brazil at the beginning of the last century [15,16].

Lethality is the most relevant toxic effect induced by snake venoms. Therefore, since the first formulations produced by Calmette, its neutralization in different animal species has been used to estimate antivenom potency [9,17]. At the end of the 1930s, the use of mice was introduced to assess the neutralization of lethality [18]. Although murine models do not exactly reproduce what occurs in human accidental envenomations, they have been widely adopted in the quality control of antivenoms. Today, lethality neutralization assay in mice is the gold standard recommended by the World Health Organization to estimate antivenom potency [3–5]. Usually, these tests are performed by incubating a fixed dose of venom ('challenge dose') and variable dilutions of antivenom, in order to achieve several venom/antivenom ratios. Then, aliquots of the mixtures are injected in animals and lethality is observed.

Alternatively, it has been proposed that injecting venom and antivenom separately in mice is a protocol that more closely simulates what occurs in accidental envenomations. In this model, the influence of venom toxicokinetics, antivenom pharmacokinetics

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and envenomation dynamics are considered [19–25]. However, the difficulty in standardizing the lapse between envenomation and antivenom administration largely prevents laboratories from using this model in order to routinely evaluate antivenom performance. Thereby, protocols in which venom and antivenom are mixed and pre-incubated are preferred and widely used in antivenom production and quality control laboratories.

According to the World Health Organization, venom lethality and the capacity of antivenom to neutralize it are expressed as Median Lethal Dose (LD_{50}) and Median Effective Dose (ED_{50}), respectively. Venom LD_{50} is defined as the minimum amount of venom causing death in 50% of the mice injected, while antivenom ED_{50} is defined as the volume of antivenom that protects 50% of mice injected with a mixture of a constant amount of venom and several volumes of antivenom [3,4]. Although this procedure is followed by most groups, some laboratories perform this determination by mixing different amounts of venom with a constant volume of antivenom [3,4,26,27]. Moreover, some laboratories use the Minimal Lethal Dose (MLD) instead of LD_{50} when expressing venom toxicity [28].

Regardless of methodological differences in these procedures, venom-antivenom mixtures are incubated at particular conditions during a period of time and then are injected into groups of mice, usually by the intravenous or intraperitoneal routes. The number of deaths occurring during the following days is recorded, and the neutralizing capacity of antivenom is usually estimated with Probits, Spearman-Karber or non-linear regression procedures [2–4,29].

The lethality neutralization assay has been used to verify that snake antivenoms from industrial serial production fulfill the specifications [30], to estimate the capacity of antivenoms to neutralize homologous and heterologous snake venoms [14,31–33], and to establish preliminary doses of new antivenom formulations to start human dose-finding/safety phase 1 clinical trials [34]. In all cases, interpretation of results without considering the analytical performance of the assay might lead to erroneous conclusions regarding the neutralizing potency of antivenoms.

The suitability of murine models to estimate antivenom potency has been partially proved [9,27,32,35,36], and the effect of several factors on the results obtained has been previously studied [37]. Nevertheless, to the best of our knowledge there are no published studies that characterize the analytical properties of the lethality neutralization assay in order to satisfy the current requirements of the pharmaceutical industry. Thus, following the recommendations of the World Health Organization [4], a study on the design and analytical properties of the lethality neutralization assay used in Costa Rica to evaluate antivenoms against *Bothrops asper* venom was performed. Results indicate that the analytical characteristics of this assay are adequate enough to prove product compliance and to have statistical control over an industrial line of antivenom serial production.

2. Materials and methods

2.1. Animals

CD-1 mice of both sexes and different weight ranges were used. All procedures used 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 [38].

2.2. Venom

Venom was collected from Costa Rican adult specimens of *B. asper* maintained in captivity at the Serpentarium of Instituto Clodomiro Picado. Venom was stabilized by lyophilization and stored at −20 °C. Solutions of venom were prepared immediately before use.

2.3. Antivenom and normal equine immunoglobulins (NEI)

The antivenom used was a liquid formulation of whole equine IgG, purified by caprylic acid fractionation of plasma of animals immunized with the venoms of *B. asper*, *Crotalus simus* and *Lachesis stenophrys* [39]. The NEI used as placebo was prepared by caprylic acid fractionation of plasma obtained from non-immunized horses. NEI was formulated at a protein concentration of 2.4 g/dL.

2.4. Venom lethality determination

Groups of eight mice were injected with different amounts of venom dissolved in 0.12 M NaCl, 0.04 M phosphate buffer, pH 7.2 (PBS). Volume of injection was 0.5 mL for intraperitoneal (IP) route or 0.2 mL for intravenous (IV) route. Assays were performed using five levels (dilution factor 1/1.2). During the following 48 h, the number of deaths was recorded [40]. The median lethal dose (LD_{50}) was calculated by Probits [41,42]. Experiments were performed in triplicates and results expressed as mean ± SD.

2.5. Lethality neutralization assay

The neutralization of lethality was assessed by mixing a constant amount of venom, dissolved in PBS, with different volumes of antivenom. Each venom (mg)/antivenom (mL) ratio is referred to as a 'level'. Assays were performed using five levels (dilution factor 1/1.5). Depending on the experiment, different numbers of LD_{50} were used as challenge dose. Mixtures were incubated at 37 °C for 30 min. Then, groups of mice were injected with aliquots of venom/antivenom mixtures. Injection volume was 0.5 mL or 0.2 mL depending on whether the route used was IP or IV, respectively. In the control groups, venom was incubated with PBS instead of antivenom. Deaths were recorded during 48 h, and neutralizing activity, expressed as Median Effective Dose (ED_{50}), was calculated using Probits [32,41].

2.6. Study of various parameters of the lethality neutralization assay

2.6.1. Challenge dose

The effect of the challenge dose in the lethality neutralization assay was determined by injecting groups of eight mice (16–18 g) by the IP route with several challenge doses that were incubated during 30 min with various volumes of antivenom. ED_{50} values obtained for the same antivenom sample using challenge doses of 2, 3, 4, 5, and 6 LD_{50} were compared. Experiments were performed in triplicate and results were expressed as mean ± SD.

2.6.2. Incubation time

To study the effect of incubation time on the lethality neutralization assay, ED_{50} values of the same antivenom were assessed by injecting groups of eight mice (16–18 g) with samples incubated for 0, 30, 60 or 120 min. Injections were performed by the IP route, and 4 LD_{50} were used as challenge dose. Experiments were performed in triplicate and results were expressed as mean ± SD. Additionally, mixtures of different venom/antivenom levels were incubated at 37 °C during 120 min. Turbidity of the mixtures was measured every 10 min using a turbidimeter (La Motte, model 2020, Chestertown, MD) and recorded as nephelometric turbidity units (NTU).