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Neutralization of toxicological activities of medically-relevant *Bothrops* snake venoms and relevant toxins by two polyvalent bothropic antivenoms produced in Peru and Brazil

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ABSTRACT

Snakebite envenoming is a neglected public pathology, affecting especially rural communities or isolated areas of tropical and subtropical Latin American countries. The parenteral administration of antivenom is the mainstay and the only validated treatment of snake bite envenoming. Here, we assess the efficacy of polyspecific anti-*Bothrops* serum (α-BS) produced in the Instituto Nacional de Salud (INS, Peru) and at the Fundação Ezequiel Dias (FUNED, Brazil), to neutralize the main toxic activities induced by five medically-relevant venoms of: *Bothrops atrox, B. barnetti,* and *B. pictus* from Peru, and the Brazilian *B. jararaca* and *B. leucurus*, all of them inhabiting different geographical locations. Protein electrophoretic patterns of these venoms showed significant differences in composition, number and intensity of bands. Another goal was to evaluate the efficacy and safety of lyophilized α-BS developed at INS to neutralize the detrimental effects of these venoms using *in vivo* and *in vitro* assays. The availability of lyophilized α-BS has relevant significance in its distribution to distant rural communities where the access to antivenom in health facilities is more difficult. Despite the fact that different antigen mixtures were used for immunization during antivenom production, our data showed high toxin-neutralizing activity of α-BS raised against *Bothrops* venoms. Moreover, the antivenom cross-reacted even against venoms not included in the immunization mixture. Furthermore, we have evaluated the efficacy of both α-BS to neutralize key toxic compounds belonging to the predominant protein families of *Bothrops* snakes. Most significantly, both α-BS cross-specifically neutralized the main toxicological activities *e.g.* lethality and hemorrhage induced by these venoms. Thus, our data indicate that both α-BS are equally effective to treat snake bite victims inflicted by *Bothrops* snakes particularly *B. atrox,* responsible for the largest numbers of human envenomations in the Amazon regions of some South American countries including Peru and Brazil.

1. Introduction

Among the neglected public health problems, snake bite envenoming belong to the top reasons of mortality and morbidity that predominantly affects rural and poorest communities of developing tropical countries in Asia, Africa, and Latin America. Like dengue, malaria, tuberculosis and parasitic diseases, the risk of snake bite is always present in these rural regions where envenomation by viperid snakes causes deaths and morbidity of surviving victims (Williams et al., 2010; WHO, 2009). Data based on hospital-records indicate that in the Amazon region of Latin America and the Caribbean, approximately 70,000 people are bitten by snakes each year (Williams et al., 2010; Gutierrez, 2014), although this information is certainly underestimate due to the difficulties of patients in accessing healthcare services in the large

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territory marked by natural geographic barriers (Kasturiratne et al., 2008). The vast majority of human snake bite envenomings which occur in Latin America are caused by the neotropical pit vipers of the genus *Bothrops,* commonly referred to as lanceheads, which are widely spread throughout the tropical and non-tropical regions of Central and South America (Campbell and Lammar, 2004; Warrell, 2004). In Peru, *B. atrox,* (Linnaeus, 1758; Jergon, Jergon de la selva) which is found in the tropical lowlands and rainforest areas up to 1200 m of northern South America east of the Andes, is by far the main cause of venomous snakebite in many of the lowland forest regions responsible for approx. 87.6% of all ophidian accidents, followed by *Bothriopsis bilineata* (Loro machaco) with 8.5% and *Lachesis muta* (Bushmaster, Shushupe) with 3.3% (Manrique et al., 2001; Oropeza et al., 2000; Rojas et al., 2005). Along the Pacific Ocean dry coastal deserts spread in western Peru and southwestern Ecuador, with scarce rain with rivers falling dry seasonably. These are the habitats or another poisonous pit viper *B. barnetti,* Parker, 1938 (Barnett's lancehead), whereas *B. pictus,* Tschudi, 1845 (Jergon de la costa, Desert lancehead) is commonly found on the western slopes of the Andes and throughout the arid to semiarid coastal foothills, river valleys along the central to northern Pacific coast. Accidents by *B. pictus* have been reported in the northern districts (Cono Norte) of the metropolitan area of Lima city (Maguiña et al., 1998; Campbell and Lammar, 2004). The venoms of *B. barnetti* and specially *B. pictus* are dangerous and have even caused lethal accidents of humans, but they are not taken sufficiently seriously as life-threatening agents of human diseases. Moreover, the scientific insights provided by the scarce epidemiological and clinical data of human snake bites have been ignored for a long time (Ruiz et al., 1993; Maguiña et al., 1998).

Brazil is a tropical country with an estimated number of snakebites of 25,000 accidents per year. The incidence rate in the Amazon region (64.8 accidents/100,000 inhabitants in 2012) exceeds the one in the rest of Brazil even fourfold (Ministerio da Saúde, 2015; Kasturiratne et al., 2008). *B. atrox* lives in tropical lowlands and the rainforest, but is also found in cultivated areas, mainly fields, and around human settlements of South America east of the Andes. It is the leading cause of most human snakebites (approx. 83%) throughout the Amazon region thereby exceeding any other South American snake (Ministerio da Saúde, 2010; Warrell, 2004; Furtado et al., 2010). Despite its wide range of ecological and geographical habitats no subspecies are currently known (Campbell and Lammar, 2004, http://www.reptile-database.org). Moreover, other medically relevant species include *B. jararaca* (Wied-Neuwied, 1824) which is responsible for the majority of snake bites in the most populated regions of southeastern Brazil, northeastern Paraguay and northern Argentina. In Brazil, *B. jararaca* is mainly found in southern Bahia, Espirito Santo, Rio de Janeiro, Minas Gerais, São Paulo, Paraná, Santa Catarina and Rio Grande do Sul. On the other hand, *B. leucurus,* Wagler, 1824 (White-tailed jararaca) is found in eastern Brazil, in remnants of the Atlantic forest in valleys along the Atlantic coast from northern Espirito Santo, north to Sergipe, Alagoas, Ceara and Bahia, where the altitudes of their habitats range from near sea level to about 400 m.

In general, envenomation by *Bothrops* snakes becomes manifest by severe inflammatory reactions with complex tissue damage *e.g.* drastic hemostatic disturbances, hemorrhage, edema and myonecrosis, extending from the site of the bite (Warrell, 2004). The venom composition of several species of *Bothrops*, including *B. atrox* is already known (Kohlhoff et al., 2012: Bernardoni et al., 2014; Sousa et al., 2013; Dias et al., 2013). Based on proteomic studies, biological properties and the immunoreactivity profile toward homologous and heterologous therapeutic and experimental antivenoms of medically relevant snakes, it has become clear that a low number of pathogenic toxin families compose the venom of *Bothrops* species. These are snake venom metalloproteinases (SVMPs, P-I and P-III classes), snake venom serine proteinases (SVSPs) and so-called "ancillary" toxin families *e.g.* L-amino acid oxidases (L-AAOs) and phospholipases A_2 (PLA_2s), being the most abundant and toxic proteins that most frequently correlate with the clinical features of envenomed victims (Sousa et al., 2013; Casewell et al., 2014; Calvete, 2011). A currently available Brazilian bothropic antivenom (α-BS) for *Bothrops* snake bite patients used in this study is produced at FUNED, Belo Horizonte-Brazil, by using as immunogen a mixture of venoms of: *B. jararaca*, *B. moogeni*, *B. neuwiedi*, *B. alternatus* and *B. jararacussu* (Ministerio da Saúde, 1996). Although the pit viper *B. atrox*, is responsible for the majority of snake bites throughout the Amazon region of Peru, Brazil and other South American countries, it is not included in the immunization mixture.

The only specific antidote to the snake venom toxins is hyperimmune globulin from the animals, usually horses that have been immunized with the appropriate venom of one or several species, thus generating monospecific or polyspecific antidote to reverse venom-induced pathological symptoms (WHO, 2010). The therapeutic, 'polyvalent anti-bothropic serum' (α-BS) produced by the Instituto Nacional de Salud (INS) is designed to neutralize venom-induced pathology caused by the most important *Bothrops* species. It is prepared with a pool of *B. atrox*, *B. barnetti*, *B. pictus*, *B. brazili* and *Bothrocophias hyoprora*. Previous research studies have demonstrated a high degree of cross-neutralization of antivenoms produced in various Latin American countries (Rojas et al., 2005; Camey et al., 2002; Furtado et al., 2010; Segura et al., 2010). Furthermore, clinical trials have supported the efficacy of some antivenoms in the treatment of envenomation inflicted by *Bothrops* sp (Pardal et al., 2004). However, there are conflicting data about the efficacy of antivenoms in neutralizing some toxic effects of heterologous venoms (Theakston et al., 1995; Queiroz et al., 2008). In addition, to ensure that the antidote being distributed to rural and agricultural communities, usually far from the healthcare facilities, the INS has produced the polyvalent α-BS in its lyophilized form which is an important advance when the cold chain is vulnerable and not guaranteed constantly.

In the present work we have evaluated two therapeutic *Bothrops* polyvalent antisera (α-BS) manufactured in the INS (Peru) and at FUNED (Brazil), which are currently in use in both countries, aimed at neutralizing the detrimental effects of the major venom toxins of *Bothrops* snakes, including those whose venom was not used for animal immunization. The pit viper species included in this study inhabit different geographical regions of Peru and Brazil, such as the distantly separated Amazon regions (*B. atrox* complex), which occupy different habitats such as dry climate regions or rain forest up to 1200 m in the north of South America east of the Andes. Moreover, we have purified major key venom proteins within different toxin families (SVMPs, SVSPs, L-AAOs and PLA_2s) which have been selected for their efficiency to subdue prey and tested cross-neutralization by the two α-BS. The data provided a pattern of extensive cross-neutralization of toxic activities of these venoms by both bothropic antivenoms. Therefore, this report may be relevant to our understanding of how to better treat the important neglected snake bite-induced tropical disease in several regions of the South American countries.

2. Materials and methods

2.1. Venoms and isolated toxins

Venoms were pooled from many wild *Bothrops atrox* specimens of both sexes, adults and juveniles captured in the rain forest region