Toxicon 55 (2010) 612–618

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Venom analysis of long-term captive Pakistan cobra (*Naja naja*) populations

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ARTICLE INFO

Article history:
Received 11 June 2009
Received in revised form 11 October 2009
Accepted 14 October 2009
Available online 21 October 2009

Keywords:
Captive species
Intraspecific variation
Naja naja
Isoforms
Snake venom proteome

ABSTRACT

Venom production facilities keep established colonies of captive snakes to obtain venom for research and antiserum production. Due to strict regulations of importation, some of these colonies are formed with only a small number of initial animals and consist of closely related individuals (sometimes siblings). To understand the effect of long-term captivity on the venom composition and its impact on antiserum production, we analyzed 15 long-term captive *Naja naja* (Pakistan) originating from two separate venom production colonies using liquid chromatography-mass spectrometry and electrophoresis. The chromatogram produced from each individual cobra venom was found to be different. When the protein molecular masses of the peaks were identified, it was found that all the venoms consisted of the same protein composition, but the concentration of the proteins were different. Although three-finger toxins and phospholipase A_2 enzymes are the major toxic components present in these venoms, there was a clear difference in the amounts of each individual isoform. Such variation may affect the ability of antivenoms in neutralizing the toxic components of the wild type venom.

1. Introduction

Venom production facilities try to captive breed snakes as a matter of business efficiency, to produce better adapted and healthy snakes and to reduce the need to rely on wild populations. Captive-bred individuals also have the potential to produce better quality venoms and higher yields, perhaps due to better hygienic conditions, hydration, and consistent food supply throughout their lifetimes (Panizzutti et al., 2001). These snakes rarely use their venom to capture their prey as they are fed with processed foods hence the yield of the venom is higher as compared to the wild species which constantly use venoms to capture their prey (Panizzutti et al., 2001). Although declining, venom production facilities in several countries still operate on a 'catch-milk-replace when it dies' basis.

Venom variation is most commonly studied in wild or short-term captive venomous snakes. Venom is variable in composition and protein concentration (Willemse et al., 1979). Not only are there clear distinctions between the venom from snakes of separate genera, but also within the same species (Chippaux et al., 1991). Intraspecific venom variation has been documented to be the result of many factors, including age (Minton and Weinstein, 1986; Daltry et al., 1996a; Saravia et al., 2002; Alape-Giron et al., 2008), gender (Menezes et al., 2006), locality (Minton and Weinstein, 1986; Jayanthi and Gowda, 1988; Mukherjee and Maity, 1998; Saravia et al., 2002; Creer et al., 2003; Wei et al., 2003; Ferquel et al., 2007; Alape-Giron et al., 2008), diet (Daltry et al., 1996b; Li et al., 2005; Sanz et al., 2006; Pahari et al., 2007; Gibbs and Mackessy, 2009), genetic

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doi:10.1016/j.toxicon.2009.10.018

relationships (Menezes et al., 2006), season (Gubensek et al., 1974; Williams and White, 1992), frequency of extractions (Willemse et al., 1979) and even processing (Willemse and Hattingh, 1979). In fact, the venom from the same individual can be subject to ontogenetic changes (Daltry et al., 1996a), seasonal variation (Gubensek et al., 1974), and differences in secretions from right and left venom glands (Johnson et al., 1987). Venom variability may have an impact on both primary venom research and management of snakebite, including a selection of antivenoms and a selection of specimens for antivenom production (Chippaux et al., 1991). Venom variability from long-term captive snakes raised at venom production facilities has not been well studied and the factors resulting in venom variation are still poorly understood. Studies of venoms of captive-bred snakes indicate the intraspecific variation which is genetically inherited unlike the environmentally induced variations (Daltry et al., 1996a). The data from such studies may have practical applications in future venom research and antiserum production as most of the venoms are being collected from established captive colonies. If the venom composition is being affected by the reduction in genetic diversity, it could produce antiserum that might not be as effective against snakebite from wild specimens and also lead to inconsistent results in venom research.

It is not unusual for captive colonies to consist of closely related individuals, since sometimes it is only from a few initial animals that these colonies are constructed. This is the case for the black Pakistan cobras, *Naja naja,* used in this study. Convention on International Trade in Endangered Species (CITES of Wild Fauna and Flora) regulates the exportation and importation of animals and can make certain species of snakes difficult to acquire in captivity without captive breeding. *N. naja* is listed as a CITES II animal.

In this study, the venom from a total of 15 *N. naja* housed at two different venom production facilities were investigated (Supplementary Table 1). Nine of these cobras originated from the bloodlines maintained at the Kentucky Reptile Zoo in the United States. The other six cobras were produced from the bloodlines located at Latoxan in France. All cobras were second generation captive animals and all with the exception of one, were siblings of either the same or different litters. The difficulties in acquiring unrelated individuals due to CITES may be one of the reasons for such closely related colonies of siblings. Here we have attempted to analyze venom variation patterns within long-term captive *N. naja* colonies.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used were of analytical grade and were purchased from either Sigma or Merck (KGaA Darmstadt, Germany), unless mentioned otherwise.

2.2. N. naja *venom samples and animals*

N. naja venoms were obtained from two sources and each venom sample was collected from a single cobra (no pooled samples were used). Nine individual *N. naja* housed at the Kentucky Reptile Zoo (Slade, Kentucky) were extracted from August through September 2008 and their venoms were lyophilized (10–15 mg dry weight). Of the nine individuals, #1 was the mother of two clutches, including individuals #6–9 (first clutch, approximately 2.5 years old) and #2–5 (second clutch, approximately 1.5 years old). Gender, age (if known) and snout-vent length (SVL) was recorded for each individual (Supplementary Table 1). During captivity the diet for these animals consisted of rats/mice depending upon snake size and occasional chicks.

Six individual *N. naja* housed at Latoxan were extracted on July 15th, 2008, and the collected venoms were lyophilized (10–18 mg dry weight). Of the six individuals, all are siblings, but from two separate clutches. *Naja* A, B and D are from the same clutch born 01 September 2001 and *Naja* C, E, and F are from the same clutch born on 1st November 2002. The diet for these animals also consisted of rats/mice depending upon snake size.

2.3. Reverse phase liquid chromatography-mass spectrometry (LC/MS)

Venom samples were dissolved in MilliQ water (1 mg/ml) and filtered to remove undissolved debris. All samples were stored at −20 °C until further use. A total of 150 μl of venom sample was subjected to reverse phase liquid chromatography on a RP-Jupiter C18 (10 μ, 300 Å, 10 mm × 250 mm) column equilibrated with buffer A [0.1% trifluoroacetic acid (TFA)] attached to a Perkin–Elmer Sciex Triple-quad API-300 LC/MS system mass spectrometer (Thornton, Canada). The crude mixture was eluted using a linear gradient of buffer B [0.1% TFA in 80% (v/v) acetonitrile] at a flow rate of 40 μl/min for a total of ∼3 h and detected with at 214 nm. The elution gradient was 20–55% in 105 min. Electrospray ionization mass spectrum was acquired in positive ion mode with an orifice potential of 80 V. Nitrogen was used as a curtain gas with a flow rate of 0.6 l/min and a pressure setting of 100 psi. Full scan data were acquired over the ion range from 500 to 2500 mass/charge (m/z) with step size of 1 Da. Data processing was performed with the aid of BioMultiview/Analyte software (Perkin Elmer Sciex, Thornton, Canada). Protein masses for each peak in the chromatogram were determined with this software. Elution of proteins was also monitored at 280 nm.

2.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Pre-cast SDS-PAGE Tris–HCl gradient gel (4–20%) and Precision plus prestained protein dual-color SDS-PAGE standards were obtained from Bio-Rad Laboratories (Hercules, CA). Venom proteins (20 μg in 20 μl) along with 7 μl of protein marker were loaded into the gel. Gel was run at 120 V for ∼1.5 h and stained with Coomassie brilliant blue and destained in acetic acid:methanol:water (10:40:50, v/v) and protein bands were recorded using a gel documentation system.