COMPARISONS OF THE AFRICAN COBRAS USING ELECTROPHORETICAL ANALYSIS OF VENOM AND THEIR MORPHOLOGY

Zuzana Andrejčáková[*1], Vladimír Petrilla[1], Drahomíra Sopková[1], Radoslava Vlčková[1], Samuel Bila[3], Lenka Krešáková[1], Monika Petrillová[2]

[1]*The Department of Anatomy, Histology and Physiology, Institute of Physiology, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, Košice 041 81, Slovak Republic*

[2]*The Department General Education Subject, Institute of Foreign Languages, University of Veterinary Medicine and Pharmacy in Košice, Komenského 73, Košice 041 81, Slovak Republic*

[3]*Faculty of Veterinary Medicine, Eduardo Mondlane University, Maputo, Mozambique*

ABSTRACT

The African spitting cobras involve the genus Hemachatus (Hemachatus haemachatus species) and some species of the genus Naja (Naja nigricollis, Naja aschei, Naja nigricincta, Naja mossambica, Naja katiensis, Naja nubiae, Naja pallida). Naja aschei was considered as an identical species of Naja nigricollis. After genetic analysis, the Naja aschei was described as new species of spitting cobra. The aim of our work was the characterization and comparison of venomous snakes, Naja aschei and Naja nigricollis (caught in Tanzania and Ghana) using electrophoretic analysis and morphological comparison of their skulls. Our results suggest a different structure of skull formation and composition of snake venom, suggesting generic difference between snakes Naja aschei and Naja nigricollis.

Key words: spitting cobras, venom, electrophoresis, protein, morphology

Introduction

In terms of general opinion, cobras belong to the best-known snakes in the world. Their natural behaviour and characteristic defensive position have caught the human

imagination for millennia, and made them cultural icons and objects of veneration in many parts of the world (Wüster and Broadley, 2003). Some species have the ability to spit their venom at an adversary (Wüster and Thorpe, 1992). Spitting cobras (genus *Naja*) from Africa, Asia and South Africa (*Hemachatus haemachatus*) have perfectly adapted venomous apparatus, which allows them to squirt venom from the fang for a distance of several metres to defend themselves (Bogert, 1943).

According to phylogeographic studies, the African spitting cobras significantly introduce an ideal model organism in open areas of sub-Saharan Africa. They are extended in the drier parts of Africa, occurring from southern Egypt in the north to the Western Cape and KwaZulu-Natal in South Africa, and from Senegal in the west to Somalia in the east. Over the past 40 years, the six species of the genus had been recognized the six species that originally belonged to species *Naja nigricollis* (Broadley, 1968 and 1974; Wüster and Broadley, 2003 and 2007; Wüster et al., 2007).

Snakes' venom is a viscous liquid containing mixtures of biologically active components, protein toxins and enzymes, polypeptide and peptide toxins, polysaccharides, low molecular weight substances and ions. Toxins are peptides, polypeptides and proteins without enzymatic activities with an approximate molecular mass of 3–30 kDa (Valenta, 2008).

Spitting cobras are important causes of snakebite accidents in sub-Saharan Africa (Warrell et al., 1976; Warrell and Ormerod, 1976; Tilbury, 1982), and their research of venom composition is essential both for academic and applied purposes (Fry et al., 2003).

Thanks to new research methods, the snake systematics is still refreshing. Studies on the molecular level based on analysis of mitochondrial DNA (mtDNA) sequences identified a new type of cobra, *N. nubiae* from the north-east Africa which was described and previously considered as identical species of *N. pallida*. The new species differs from *N. pallida* especially in the fact that has more than one dark stripe on the neck (Wüster and Broadley, 2003). Another new kind of a giant cobra, *N. ashei* from East and North-East of Africa studied in our experiment, was also described and previously considered as colourful variation of *N. nigricollis*. According to the mtDNA sequences, the research indicates that it is more closely related to *N. mossambica* than *N. nigricollis* (Wüster and Broadley, 2007). The new

species *N. nigricincta* was until recently considered as identical species of *N. nigricollis*, that underwent the examination by mtDNA sequences. The conclusions confirmed that *N. nigricincta* as a separate species, is more closely related to *N. ashei* and *N. mossambica*, than *N. nigricollis* (Wüster et al., 2007).

The aim of our study was to investigate the spectra from venom proteins in snakes (*N. nigricollis* caught in Ghana and Tanzania, and *N. ashei* caught in Kenya) by zone electrophoresis on alkaline-buffered agarose gels. Simultaneously, we focused on morphometric analysis of their skulls.

Materials and methods

For morphological study we compared the presence, size and shape of the bone structures of selected bones of the skull of *Naja ashei* and *Naja nigricollis* caught in Ghana and Tanzania. We used a scalpel, scissors and tweezers at the preparation. For the final purification of the preparation we used boiled water and the 15% hydrogen peroxide. For the final joining of parts of the skulls we used superglue Super Bond, needles, washer and wire. The observation was performed using a magnifying glass table and documentary material was made by camera Canon EOS 1100 D with a camera lens Canon EFS 18-135 mm f / 3.5-5.6 IS. Length of venom tooth was measured using a sliding scale (200 Digital) and common twine.

The samples of venom were taken from the African cobras: *N. nigricollis* (first caught in Ghana and second in Tanzania) and *N. ashei* (caught in Kenya). The snakes' venom was selected after the snake's bite into the measuring vessel with a plastic film and stored in the transport cryogenic microtubes under liquid nitrogen (-195 ˚C) and stored in deep freezer (-70 ˚C). Due to the fact that the venom extracts contained some dead cells, the samples were centrifuged (Centrifuge EBA 21, HETTICH GmbH & Co. KG, Tuttlingen, Germany) for 5 min at 600 x g until other determination was done.

The concentration of the total protein was assessed using commercial diagnostic kits (RANDOX, Crumlin, United Kingdom) with automatic biochemical analyser ALIZÉ (LISABIO, Pouilly-en-Auxois, France). The absorbance used was measured at 540 nm.

For electrophoretic study, 10 µl of venom extract was used for each separation. HYDRASYS device (SEBIA, Lisses, France) was used for the determination of protein

spectrum in the snakes' venom. The samples were separated using electrophoresis (HYDRAGEL 7 PROTEIN) on alkaline buffered (pH 9.2) agarose gels. The separated proteins were visualized using 0.4 g/dl Amido Black. The dried gels were prepared for visual examination and densitometry to obtain accurate relative quantification of individual zones. Then photographs of the gels were taken. Qualitative evaluations of the gels were done directly from the electrophoretograms and the densitometric curves of the separations were created by means of EPSON PERFECTION V 700 PHOTO densitometer scanning at 570 nm and evaluated using PHORESIS software (Version 5.50, SEBIA, Lisses, France).

Results

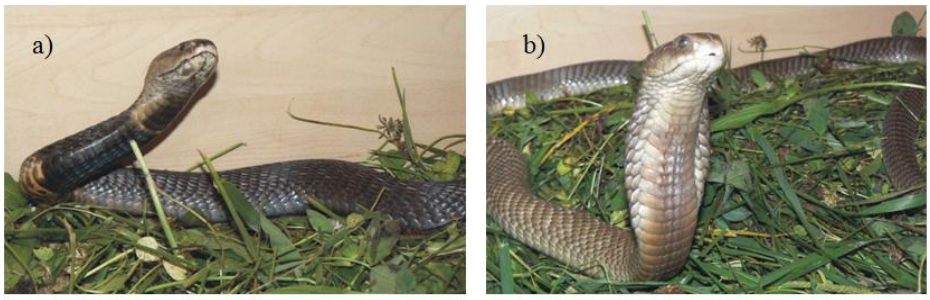

Figure 1 Photo of *N. nigricollis* caught in Tanzania (a) and *N. ashei* caught in Kenya (b).

Our work was focused on the comparison of African spitting cobras' venom and their morphology, especially *N. nigricollis* (caught in Tanzania and second in Ghana) and *N. aschei* (caught in Kenya) as shown in Figure 1. The snakes were similarly sized and they were the same female specimens.

Morphological comparison of the skull bone formations of *N. nigricollis* (caught in Tanzania) and *N. ashei* (caught in Kenya) is shown in Figure 2. In the caudal portion of the upper jaw (*os maxillare*), the number of non-venomous teeth were 2 in *N. nigricollis*, and in *N. ashei* we found 3 non-venomous teeth. On the palatine bone (*os palatinum*), we found 6 non-venomous teeth and on the wing bone (*os pterigoideum*) was found 17 non-venomous teeth in all species. We found two prominent ventral protrusions in the cranial part of the rear parietal bone (*os basioccipitale*). Biometric studies revealed differences among observed species. Total body length of *N. ashei* was 1600 mm, in *N. nigricollis* (caught in Ghana) it was 2100 mm, and in *N. nigricollis* (caught in Tanzania) it was 2200 mm in length. The

length of fang in observed species was: *N. ashei* – 8 mm, *N. nigricollis* (caught in Ghana) – 9 mm, and *N. nigricollis* (caught in Tanzania) – 9.1 mm.

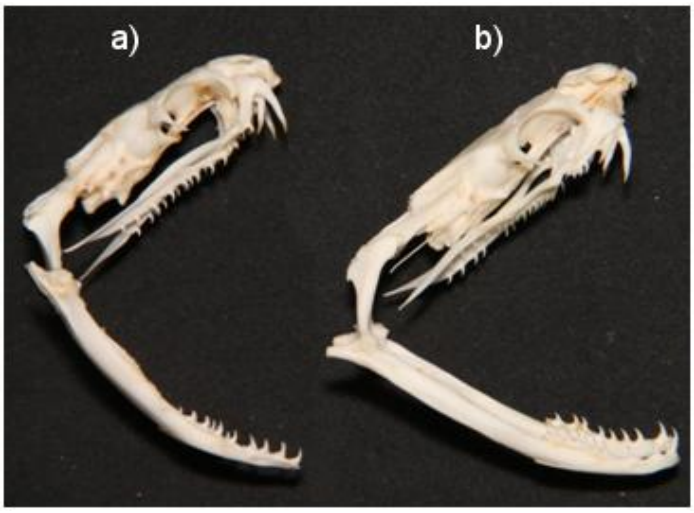

Figure 2 Morphological comparison of the skull bone formations of a) *N. nigricollis* (caught in Tanzania) and b) *N. ashei* (caught in Kenya).

The gel electrophoretograms of the venom protein samples are shown in Figure 3. The measured results indicated the significant differences between protein fractions in observed cobras.

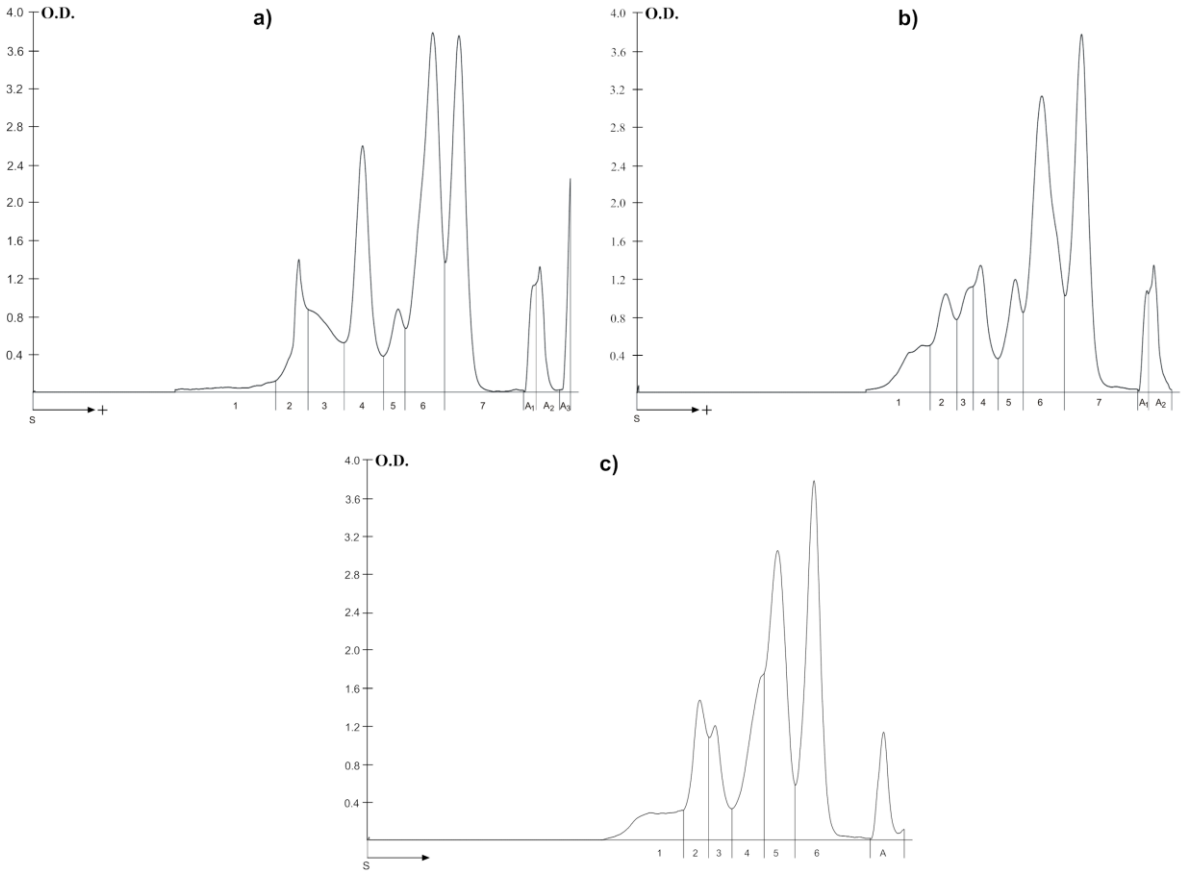

Figure 3 Gel photograph shows the electrophoretic separation of the venom protein sample obtained from: a) *N. ashei*, b) *N. nigricollis* (caught in Tanzania), and c) *N. nigricollis* (caught in Ghana), S: Start (junction between the stacking and separation gels), O.D.: Optical Density. The X-axis represents the scanned area of gel strip, 1–7: Labeling of globulin-like fractions, A1–A3: Labeling of albumin-like fractions.

The electrophoretic patterns of the venom protein samples showed quite a qualitative difference in both albumin-like and globulin-like regions, which suggest that *N. nigricollis* and *N. aschei* are clearly distinct at the sub-specific level. The number of clear globulin-like fractions or fraction groups in *N. aschei* and *N. nigricollis* (caught in Tanzania) samples was 7; while in *N. nigricollis* (caught in Ghana) were 6 globulin-like fractions observed. There were many differences in albumin-like fractions: *N. ashei* – 3; *N. nigricollis* (caught in Tanzania) – 2; *N. nigricollis* (caught in Ghana) – 1 albumin-like fraction. There was one albumin-like fraction in *N. ashei*, which was beyond the capabilities of sharing sequence on agarose gel electrophoresis. Pictures of electrophoretic strips of the venom protein samples obtained from mambas showed clear differences in *N. ashei* contrary to both species of *N.*

nigricollis (Fig. 4). The quantification (in percentage and g/l) of each fraction is shown in Table 1. Our results showed different structures of the *N. ashei* and *N. nigricollis* (caught in Tanzania and caught in Ghana) venoms.

Table 1 Protein characteristic of *N. ashei* (caught in Kenya), *N. nigricollis* (caught in Tanzania) and *N. nigricollis* (caught in Ghana) venoms.

		The proportion of proteins					
		N. ashei		*N. nigricollis* (Tanzania)		*N. nigricollis* (Ghana)	
Name of fraction		%	g/l	%	g/l	%	g/l
Total protein		100	273.4	100	258.3	100	304.4
Globulin-like fraction	**1**	1.7	4.6	6,1	15.8	6.8	20.7
	2	7	19.1	8,2	21.2	10.7	32.6
	3	8.5	23.2	6	15.5	7.5	22.8
	4	16.4	44.8	7.9	20.4	14.2	43.2
	5	4.9	13.4	7.7	19.9	26.5	80.7
	6	29.3	80.1	30.9	79.8	28.4	86.4
	7	22.3	61	25.9	66.9	-	-
Albumin-like fraction	**A1**	2.8	7.1	2.6	6.7	5.9	18
	A2	3.9	10.8	4.7	12.1	-	-
	A3	3.2	8.7	-	-	-	-

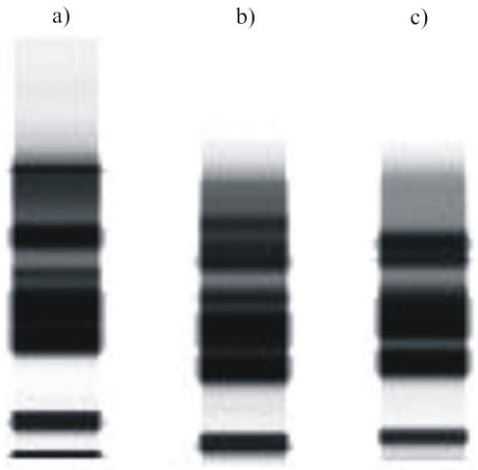

Figure 4 Pictures of electrophoretic strips of the venom protein sample obtained from: *N. ashei* (a), *N. nigricollis* caught in Tanzania (b) and *N. nigricollis* caught in Ghana (c).

Discussion

Morphological comparison of selected species of snakes is relevant in scientific as well as in breeding aspects. We noticed differences in total length of fangs of *N. ashei* and *N. nigricollis* (caught in Tanzania and caught in Ghana), and the numbers of non-venomous teeth in observed cobras. We demonstrated the presence of ventral protrusion on the cranial part of the rear parietal bone (*os basioccipitale*), which could be responsible for spitting function alone, similar to study of Freyvogel and Honneger (1965) or Wüster and Thorpe (1992).

It is known that the complex composition of venom is responsible for the diversity of pathophysiological effects noticed after snake bite (Chérafi et al., 2010). We used electrophoretic method for the determination of venom's protein. Protein electrophoresis is a specific method intended to characterize sample composition, protein interactions, molecular weight, isoelectric point, and to purify little weight of protein for further analysis (Westermeeier and Naven, 2002; Ostapchenko et al., 2011).

The electrophoretic method was used in study by Göçmen et al. (2006) who compared Levantine vipers and Macrovipera lebetina (Linnaeus) from Cyprus and southern Anatolia. They investigated differences in their electrophoretic types of venom proteins, as well as differences in hemipenial morphology among individuals above. Therefore they made conclusions that the southern Anatolian population should not be nominated as the exclusive subspecies M. l. lebetina (Linnaeus) that lives in Cyprus. Arikan et al. (2014) also studied venom proteins of *Vipera kaznakovi* and *Vipera ammodytes* using similar electrophoresis and they noticed the differences between them. The differences in venom from *N. ashei* and *N. nigricollis* were observed in the study by Petrilla et al. (2014), who found that toxins of the above mentioned species are not identical but show great differences by using the atomic force microscopy and fluorescence spectra methods, which are shown as a distinct characteristic fluorescence fingerprints.

Our work was focused on the morphological study (biometry) and biochemical comparison of *N. ashei* and *N. nigricollis* venom by zone electrophoresis on alkaline-buffered agarose gels. We found the differences between the snakes that were previously considered as a phylogenetically identical species. Anti-snake venom is a protein product that was made from venom of *N. nigricollis* in the past. Based on recent studies of mtDNA and other

methods (including electrophoresis), comparing *N. ashei* and *N. Nigricollis*, we could expect that there is a need for production of new antivenom from *N. ashei*. The factors such as different geographic location, altitude, temperature, type of food, food of potential prey, length of hibernating, waste of venom and frequent application of venom in the defence against prey should not affect the efficacy of antivenom. Knowledge and characteristics of snake venom composition may also result in the production of new drugs (e.g. against cancer) in the future.

Acknowledgements

We would like to show our gratitude to Viperafarm Biely Kostol from Slovakia team and the team Tropical world Trakovice from Slovakia for professional help during taking of the venom from snakes and also for taking photos. Finally, we want to thank professional herpetologist Thea Litschka Koen from Swaziland (Africa) who has given us the antivenom and to Mária Kozáčková from the Department of Physiology, University of Veterinary Medicine and Pharmacy in Košice for technical support during electrophoretic analysis. The study was supported by the grant VEGA No 1/0579/12 and VEGA No 1/013/613.

References

Arikan, H., Göçmen, B., İğcı, N. and Akman, B., 2014. Age-dependent variations in the venom proteins of Vipera kaznakovi (Nikolsky, 1909) and Vipera ammodytes (Linnaeus, 1758) (Ophidia: Viperidae). Turk. J. Zool. 2014, 38: 216-221.

Bogert, C. M., 1943. Dentitional phenomena in cobras and other elapids, with notes on adaptive modification of fangs. B. Am. Mus. Nat. Hist., 81: 285-360.

Broadley, D. G., 1968. A review of the African cobras of the genus *Naja* (Serpentes: Elapinae). Arnoldia, 3: 1-14.

Broadley, D. G., 1974. A review of the cobras of the *Naja nigricollis* complex in south-western Africa. Cimbebasia (A), 2: 155-162.

Chérifi, F., Rousselle, J. C., Namane, A., and Laraba-Djebari, F., 2010. CCSV-MPase, a novel procoagulant metalloproteinase from *Cerastes cerastes* venom: purification, biochemical characterization and protein identification. Protein J., 29: 466-474.

Freyvogel, T. A., and Honneger, C. G., 1965. Der "Speiakt" von Naja nigricollis. Acta Trop., 22: 289-302.

Fry, B. G., Winkel, K. D., Wickramaratna, J. C., Hodgson, W. C., and Wüster W., 2003. Effectiveness of snake antivenom: species and regional venom variation and its clinical impact. J. Toxicol—Toxin Rev., 22: 23-34.

Göçmen, B., Arikan, H., Marmer, A., Langerwerf, B., and Bahar, H., 2006. Morphological, Hemipenial and Venom Electrophoresis Comparisons of the Levantine Viper, *Macrovipera lebetina* (Linnaeus, 1758), from Cyprus and Southern Anatolia. Turk. J. Zool., 30: 225-234.

Ostapchenko, L., Savchuk, O., and Burlova-Vasilieva, N., 2011. Enzyme electrophoresis method in analysis of active components of haemostasis system. ABB, 2: 20-26.

Petrilla, V., Tomečková, V., Komanický, V., Lichardusová, L., Šutorová, M., Petrillová, M., and Sopková D., 2014. Fluorescent profiling of venom-selected cobra species. Spectroscopy letters: An international journal for rapid communication, 47: 1-5.

Tilbury, C. R., 1982. Observations on the bite of the Mozambique spitting cobra (Naja mossambica mossambica). S. Afr. Med. J., 61: 308-313.

Valenta, J., 2008. Jedovatí hadi: Intoxikace, terapie; 1st ed. Press: Galén, Praha, Czech Republic.

Warrell, D. A., Greenwood, B. M., Davidson, N. Mc. D., Ormerod, L. D., and Prentice, C. R. M., 1976. Necrosis, haemorrhage and complement depletion following bites by the spitting cobra (Naja nigricollis). Quart. J. Med., 45: 1-22.

Warrell, D. A., and Ormerod, L. D., 1976. Snake venom ophthalmia and blindness caused by the spitting cobra (*Naja nigricollis*) in Nigeria. Amer. J. Trop. Med. Hyg., 25: 525-529.

Westermeeier, R., and Naven T., 2002. Proteomics in practice: A laboratory manual of proteome analysis, Wiley-VCH Verlag GmbH.

Wüster, W., and Broadley, D. G., 2003. A new species of spitting cobra (*Naja*) from north-eastern Africa (Serpentes: Elapidae). J. Zool. Lond., 259: 345-359.

Wüster, W., and Broadley, D. G., 2007. Get an eyeful of this: a new species of gianty spitting cobra from eastern and north-eastern Africa (Squamata: Serpentes: Elapidae: Naja). Zootaxa, 1532: 51-68.

Wüster, W., Crookes, S., Ineich, I., Mane, Y., Pook, C. E., Trape, J. F., and Broadley, D. G., 2007. The phylogeny of cobras inferred from mitochondrial DNA sequences: evolution of venom spitting and the phylogeography of the African spitting cobras (Serpentes: Elapidae: Naja nigricollis complex). Mol. Phylogenet. Evol., 45: 437-453.

Wüster, W., and Thorpe, R. S., 1992. Dentitional phenomena in cobras revisited: fang structure and spitting in the Asiatic species of *Naja* (Serpentes: Elapidae). Herpetologica, 48: 424-434.