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Laustsen, Andreas Hougaard; Lohse, Brian; Lomonte, Bruno; Engmark, Mikael; Maria Gutierrez, Jose

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Letter to the Editor

Selecting key toxins for focused development of elapid snake antivenoms and inhibitors guided by a Toxicity Score

[1]Andreas H. Laustsen, [1]Brian Lohse, [2]Bruno Lomonte, [3]Mikael Engmark, [2]José María Gutiérrez*

[1] Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark; [2] Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica; [3] Department of Systems Biology, Technical University of Denmark, Denmark

Abbreviated title: Toxicity score

*Corresponding author: José María Gutiérrez, Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica; pone: 506-2511-7888; fax: 506-22920485; e mail: jose.gutierrez@ucr.ac.cr

For more than 100 years, antivenoms have been produced by traditional methods of immunization of large mammals with mixtures of snake venoms (WHO, 2010; Gutiérrez et al., 2011). With the introduction of proteomic and transcriptomic tools in the molecular analysis of both venoms (venomics) (Calvete, 2014) and antivenoms (antivenomics) (Calvete, 2011, Calvete et al., 2014), in combination with the toxicological assessment of venoms, a more in-depth understanding of venom composition and antivenom efficacy is being built. As retrieved from current public databases on Elapidae, values for Median Lethal Dose (LD_{50}) are known for 203 toxins, belonging to seven protein sub-families, originating from 40 species (Fig. 1). Furthermore, the number of elapids for which venom-wide proteomics or transcriptomics studies have been reported has now reached 49 out of 355 described species (our unpublished data; http://www.reptile-database.org). Information is now available for a considerable number of species of high medical relevance.

Taken together, these insights, and new information being published, may provide the grounds for a knowledge-based design of future antivenoms, based on the identification of the toxicologically most relevant toxins in venoms. Scientific efforts have so far mainly been centered around molecular targeting of relevant snake toxins by using synthetic inhibitors (de Oliveira et al., 2003, Howes et al., 2007), nanobodies (Richard et al., 2013), or antibody fragments (Kulkeaw et al., 2009, Tamarozzi et al., 2006). However, a limitation for introducing novel antitoxins or inhibitors is that they usually only target one or few individual toxins, and not the whole venom, which typically contains many toxins belonging to various protein families (Calvete, 2011). For many snake venoms, this challenge could be overcome if a complete overview of the effect of each toxin in the venom were at hand; this would allow a distinction between toxins that are essential to neutralize in envenoming cases, and toxins/proteins that are not pathophysiologically important. This could in the future enable researchers to develop antitoxins against the medically relevant toxins and use mixtures of these antitoxins as a replacement, or reinforcement, of antivenoms (Roncolato et al., 2013), potentially providing better efficacy and safety, and lower cost of treatment. We argue that despite the wealth of efforts within characterization of snake venoms, a lack of a systematic approach for evaluating the importance of individual toxins within whole venom still exists. Furthermore, no simple tool has been presented for evaluating whether a given toxin in a snake venom is of sufficient medical relevance to justify an antitoxin discovery program against it. Without such a tool, modern

63 antivenom research based on molecular biology, medicinal chemistry, and biotechnology risks
64 becoming unfocused in the jungle of snake venom and antivenom data.

65 Primarily two properties of toxins are relevant when evaluating their potential medical
66 impact: 1) Intrinsic toxicity, and 2) abundance in venom. A systematic method for evaluating the
67 relevance for acute toxicity, i.e. lethality, of each toxin in whole venom, taking both toxicity and
68 abundance into account, was recently presented (Laustsen et al., 2015a). This method is based on a
69 Toxicity Score, which is calculated for either a toxin or whole venom itself by dividing the relative
70 toxin abundance in the venom (in percentage) by the LD_{50} value for the toxin in mice. If the
71 confidence intervals are known for both the abundance and LD_{50}, a confidence interval for the
72 Toxicity Score can be easily calculated. This parameter was initially proposed for ranking the medical
73 importance of toxins in the venom of the black mamba, *Dendroaspis polylepis* (Lautstsen et al.,
74 2015a). Further studies on *Naja kaouthia* and *Aipysurus laevis* venoms strongly suggest that Toxicity
75 Score is likely to be a better parameter to determine the medical relevance of a toxin than LD_{50}
76 estimation alone, and may be used for assessing the relative importance of toxins in whole venom
77 (Laustsen et al., 2015b, Laustsen et al., 2015c). The Toxicity Score can be used to quickly assess
78 whether, for example, a toxin of high abundance and moderate toxicity is of higher importance than a
79 very potent toxin of low abundance. This approach provides an easy, systematic method for
80 identifying the key toxins that antivenom development should focus on. Table 1 presents an example
81 of estimation of the Toxicity Score.

82 When the Toxicity Score is used to select the relevant toxins, which should be neutralized by
83 an antivenom or an inhibitor, a decision has to be made to define a threshold value for relevance. For
84 snakes that are able to inject large amounts of venom into their prey or predators, even toxins with a
85 low Toxicity Score may become important. Therefore, the minimum cut-off value for the Toxicity
86 Score has to be analyzed on a case-by-case basis and has to take into account the total amount of
87 venom that the specific snake is able to inject in a bite. Table 2 illustrates an example on how to
88 determine the relevant toxins in a venom on the basis of a cut-off value for the Toxicity Score of 5.
89 Using reported venom yields from milking of snakes (e.g. the values reported on
90 http://snakedatabase.org/pages/LD50.php) provides a basis for the worst-case upper limit of venom
91 that may be injected in a bite from different snake species. For example, from our previous work on

92 the venom of *Naja kaouthia* (Laustsen et al., 2015b), reported in this database to deliver up to 742 mg
93 of venom, a cut-off value of the Toxicity Score of 5 would select a group of 5 neurotoxins of the three-
94 finger toxin family and one PLA_2 among a total of 28 fractions as the most relevant targets to
95 neutralize. Fractions with a Toxicity Score below this value would have less than 37 LD_{50}s for an
96 envenoming with the maximum yield of 742 mg, and are thus likely not to play a significant role in
97 overall venom toxicity.

98 The issue of potential synergism between venom components has to be taken into
99 consideration when selecting key toxins to focus antivenom development efforts on. Using the
100 Toxicity Score allows for the identification of venoms where toxins display synergistic effects. As
101 shown in the cases of *D. polylepis* (Laustsen et al., 2015a) and *A. laevis* (Laustsen et al., 2015c)
102 venoms, the Toxicity Score of the whole venom is higher than the sum of Toxicity Scores of individual
103 toxins, thus indicating the presence of synergism. In these cases it is relevant to further investigate
104 the synergistic effects by assessing the toxicities of pairs of toxins in order to identify proteins of low
105 Toxicity Score, but which exert synergistic effects. An example of such an approach is given by
106 Strydom (1976) for toxins from *D. polylepis* venom. Despite their low intrinsic toxicity, synergistically
107 acting toxins would also be good candidates to include in an antivenom development program.

108 The Toxicity Score can be readily applied to the study of elapid snake venoms because: (a)
109 the main toxic activity of these venoms is neuromuscular paralysis leading to respiratory arrest and
110 death, thus making the assessment of lethality a clinically-relevant parameter; (b) the solvents used in
111 many separation methods (such as the commonly used reverse-phase HPLC) do not generally
112 denature the most relevant elapid venom toxins, such as three-finger toxins, dendrotoxins, and
113 phospholipases A_2 (PLA_2) (Fry et al., 2015); and (c) the mouse model used in the determination of LD_{50}
114 is generally relevant to the human situation, as the physiological mechanisms involved in
115 neuromuscular transmission in mice and humans are similar.

116 The use of this parameter in viperid snake venoms might be more complicated, for the
117 following reasons: (a) Although lethality is the most serious complication of viperid envenomings,
118 other aspects also have high medical relevance, such as local tissue damage, i.e. necrosis and
119 hemorrhage, and systemic effects such as coagulopathy and systemic hemorrhage (Warrell, 2010). (b)
120 The solvents used in reverse phase-HPLC, especially acetonitrile, denature relevant toxins in viperid

venoms, particularly zinc-dependent metalloproteinases (SVMPs), thus affecting their toxicity. It is likely, nevertheless, that these two hurdles might be overcome in the future, with the development of non-denaturing separation methods of high resolution. At present, using the Toxicity Score is therefore only feasible for focusing development efforts on elapid antivenoms, where the toxins do not loose activity in the purification process. An exception is the case of some Australian elapid venoms containing potent procoagulant serine proteinases playing a significant role in toxicity (Kini, 2005), which could be affected by chromatographic solvents; however, the key toxins in the vast majority of elapid venoms can withstand solvents used in reverse-phase HPLC.

The Toxicity Score has been employed for directing drug discovery efforts against α-cobratoxin, which was shown to have the highest relevance in *Naja kaouthia* venom (Laustsen et al., 2015b), using phage display screening (Laustsen et al., 2015d). In this example, the Toxicity Score predicts that blocking the action of α-cobratoxin will abrogate the overall toxicity of the venom. In conclusion, when working with elapid venoms, using the Toxicity Score may thus provide a clearer path for determining the medical importance of different toxins in whole venom for the development of toxin inhibitors and the improvement of antivenoms.

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Table 1: Example of how Toxicity Scores are calculated for three toxins and whole venom.

	Abundance (%)	LD_{50} (mg/kg)	Toxicity Score*
Toxin 1	10	0.1	100
Toxin 2	20	2	10
Toxin 3	50	1	50
Whole venom	100	0.25	400

* $Toxicity\ Score = \frac{Abundance\ (\%)}{LD_{50}}$

Table 2: Example of how Toxicity Scores may help distinguish between medically relevant and non-relevant toxins/proteins, when selecting targets for focused antivenom development.

	Abundance (%)	LD_{50} (mg/kg)	Toxicity Score	Medically relevant
Whole venom	100	0.25	400	
Toxin A	10	0.05	200	Yes
Toxin B	20	0.2	100	Yes
Toxin C	50	1	50	Yes
Toxin D	10	10	1	No
Toxin E	10	10	1	No

Figure legend

Figure 1: Overview of numbers of elapid snake venom toxins for which toxicity, as judged by estimation of Median Lethal Dose (LD_{50}), has been assessed. Snake genera are highlighted in **A** by color codes.

Figure 1
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