



Artemia salina as an animal model for the preliminary evaluation of snake venom-induced toxicity

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ABSTRACT

Lethality and cytotoxicity assays of snake venoms and their neutralization by antivenom require many mice for the experiments. Recent developments have prompted researchers to seek alternative strategies that minimize the use of mice in line with Russel and Burch's 3Rs philosophy (Replacement, Reduction, and Refinement). *Artemia salina* is an animal model widely used for toxicity screening. However, its use in snake venom toxicology is limited by a lack of data. The present study compared the toxicity of venoms from *Bitis arietans*, *Naja ashei*, and *Naja subfulva* using mice and *Artemia salina*. In the *Artemia salina* test at 24 h and the dermonecrotic test in mice, the toxicity of the venoms was in the order *Naja ashei* ~ *Naja subfulva* > *Bitis arietans*. In the lethality test in mice, the toxicity of the venoms was in the order *Naja subfulva* > *Naja ashei* > *Bitis arietans*. These findings suggest that the toxicity of the venoms in *Artemia salina* and the dermonecrotic bioassay in mice have a similar trend but differ from the lethality test in mice. Therefore, it may be relevant to further explore the *Artemia salina* bioassay as a potential surrogate test of dermonecrosis in mice. Studies with more venoms may be needed to establish the correlation between the *Artemia salina* bioassay and the dermonecrotic assay in mice.

1. Introduction

Antivenom efficacy is primarily evaluated in mice using the neutralization of lethality assay (WHO, 2016). This is a preclinical test that is routinely carried out by antivenom manufacturers in fulfillment of regulatory requirements (Gutiérrez et al., 2013, 2021). Investigating the capacity of antivenoms to neutralize other toxic effects of venom e.g. dermonecrosis may also be important (Gutiérrez et al., 2021; 2013; WHO, 2016). Towards this end, the World Health Organization (WHO) recommends the *in vivo* minimum necrotizing dose (MND) assay in mice (WHO, 2016).

Assays on the toxicities of venom e.g. lethality and dermonecrosis and their neutralization by antivenom require many mice for the experiments, which are costly and labor-intensive. Criticism by animal

welfare groups is also rife (da Silva et al., 2015; Kakanj et al., 2015; Kerkkamp et al., 2018; Stransky et al., 2018). Therefore, researchers are motivated to seek alternative techniques which may significantly minimize the use of or eliminate the need to use mice (Calvete et al., 2016; Barbosa et al., 1995; Chacón et al., 2015; De souza et al., 2015; Khaing et al., 2018; Oguiura et al., 2014; Pornmuttakun and Ratanabangskoon, 2014; Rial et al., 2006; Rungsiwongse and Ratanabangskoon, 1991; Segura et al., 2010; Theakston and Reid, 1979).

The lethality assay in brine shrimp (*Artemia salina*) is rapid and requires minimal resources (Hamidi et al., 2014). It has many applications in toxicology (Barahona and Sanchez-Fortun, 1999; Gadir, 2012; Hernández-Matehuala et al., 2015; Kerster and Schaeffer, 1983; Mirzaei and Mirzaei, 2013; Mwangi et al., 2015; Okumu et al., 2020; Hamidi et al., 2014; Rajabi et al., 2015, 2012). However, its suitability as a

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replacement or surrogate test to the more technically and ethically challenging lethality or necrotizing assays is limited by a lack of data. This study compared the *Artemia salina* lethality assay with the lethality and necrotizing assays in mice using venom from some snakes of medical importance in Sub-Saharan Africa namely *Bitis arietans* (Puff adder), *Naja ashei* (large brown spitting cobra), and *Naja subfulva* (Eastern Forest Cobra).

2. Materials and methods

2.1. Ethical considerations

Ethical approval was obtained from the institutional Biosafety, Animal Care, and Use Committee REF BAUEC/2019/220.

2.2. Snake venom

Venom was collected from snakes (*Bitis arietans*, *Naja ashei*, and *Naja subfulva*) which are maintained in Kenya (Broken Snake Farm, Malindi). Freshly collected venom from each snake species was pooled, snap frozen, lyophilized, and stored at -20°C . Freeze-dried venom samples were reconstituted in phosphate-buffered saline at the time of use.

2.3. *Artemia salina* (brine shrimp) lethality assay

Artemia salina (brine shrimp) eggs were commercially sourced (Batch number; X001M8M5IZ) and hatched in a trough using marine salt solution (MSS; 38.5% w/v) over 48 h. *Artemia* nauplii/larvae were transferred from the hatching trough to 5 mL sample vials. Venom aliquots (5, 50, and 500 μL) were pipetted from venom stock solutions (5 mg/mL) into the vials which contained brine shrimp. The contents were made up to the mark with MSS resulting in 10, 100, and 1000 $\mu\text{g/mL}$ venom concentrations (Meyer et al., 1982). The nauplii that died were counted after 24 and 72 h. Experiments were carried out in quintuples of 10 nauplii per vial. Mortality was analyzed by probit analysis and the results were expressed as LC_{50} : Median lethal concentration (Bliss, 1935; Finney, 1952). In cases where there were deaths in the control group (phosphate-buffered saline), the data were corrected using the formula by Abbot (Abbott, 1925).

$$\% \text{Death} = \frac{\text{Test} - \text{Control}}{100 - \text{Control}} \times 100$$

2.4. The median lethal dose (LD_{50}) assay in mice

Eighty mice weighing 18–20 g were randomly assigned to 16 groups of 5 mice each. Graded doses of *Bitis arietans*, *Naja ashei*, and *Naja subfulva* venoms were prepared in phosphate-buffered saline and administered to 75 mice (WHO, 2016). Five mice served as control and received phosphate-buffered saline only. Mortality was recorded after 24 h and the least dose of *Bitis arietans*, *Naja ashei*, and *Naja subfulva* venom which was responsible for 50% mortality was determined by Probit analysis (Finney, 1952).

2.5. The minimum necrotizing dose (MND) assay in mice

Eighty mice weighing 18–20 g were shaved (Theakston and Reid, 1983; WHO, 2016). Solutions (50 μL) containing graded doses of *Bitis arietans*, *Naja ashei*, and *Naja subfulva* venoms were injected intradermally in the shaved skin of mice (Theakston and Reid, 1983; WHO, 2016). After 3 days, the mice were humanely sacrificed and the skin was carefully removed. The diameter of snake venom-induced necrotic lesions on the inner side of the skin was measured using a digital Vernier caliper (Theakston and Reid, 1983; WHO, 2016). A plot of the mean lesion diameter against the venom doses was used to estimate the minimum necrotizing dose (MND) which was defined as the dose of

venom which corresponded to a 5-mm necrotic lesion (Theakston and Reid, 1983; WHO, 2016). A negative control group received phosphate-buffered saline only.

2.6. Statistical analysis

The *Artemia salina* LC_{50} values of the venoms were determined by probit analysis (Bliss, 1935; Gaddum, 1948). The minimum necrotizing dose was defined as the least amount of venom which when injected intradermally produced a necrotic lesion of 5 mm diameter. It was estimated by plotting mean lesion diameter against venom dose and reading off the dose which corresponded to a 5-mm diameter (Theakston and Reid, 1983; WHO, 2016).

3. Results

Information on the gender, size, location of capture, and reference number of the snakes used in venom extraction is shown in Table S1. Adult, male and female snakes were collected from Watamu, Kizingo, the Arabuko Sokoke Forest, Kilifi, Kakamega, Busia, and Nandi. Table S1.

The mortalities of *Artemia salina* larvae treated with phosphate-buffered saline only (negative control) are summarized in Table S2. Some mortality was observed in controls at 72 h. *Bitis arietans*, *Naja ashei*, and *Naja subfulva* snake venom-induced mortality in *Artemia salina* is summarized in Tables S3, S4, and S5 respectively. Generally, there were dose and time-dependent increases in the venom-induced mortalities of *Artemia salina*. See Tables S3, S4, and S5.

According to the data of Table 1, the toxicity of snake venoms in *Artemia salina* after 24 h was in the order *Naja ashei* ~ *Naja subfulva* > *Bitis arietans*. Table 1. The toxicity (lethality) of the venoms in mice after 24 h was in the order *Naja subfulva* > *Naja ashei* > *Bitis arietans*. Table 1. The necrotizing activity of the venoms in mice after 72 h of exposure was in the order *Naja subfulva* ~ *Naja ashei* > *Bitis arietans*. Table 1.

4. Discussion

The search for alternatives to the mouse lethality/toxicity assays in snake venom research has been a subject of fascination for many researchers past and present. As far back as 1907, Albert Calmette described a relationship between toxicity in mice and *in vitro* proteolysis and hemotoxicity (Calmette, 1907). Russel and Burch's 3Rs philosophy (Reduction, Replacement, and Refinement) limits the number of mice used in experimental assays and is widely celebrated in the snake venom toxinology community (Russel and Burch 1959). In line with this philosophy, the present study presents baseline data on the applicability of an alternative animal model for the preliminary evaluation of snake venom-induced toxicity.

Lethality is an acute response to venom while dermonecrosis takes some time to unravel (i.e. up to 72 h) (WHO, 2016). In the present study, murine LD_{50} and *Artemia salina* bioassays were compared at different times i.e. the toxicity in *Artemia salina* (24 and 72 h) and the toxicity in mice (24 and 72 h). A good relationship was observed between the 24 h *Artemia salina* bioassay and the 72-h dermonecrosis bioassay. In other words, the *Artemia salina* test does not necessarily have to be done at 72 h. However, a major problem of running the *Artemia salina* test for an extended period (72 h) was the high number of deaths in the negative control (phosphate-buffered saline) samples. Therefore, based on these results, the best time to judge the lethality of *Artemia salina* would be after 24 h.

The mortality trends of the venom in *Artemia salina* were inconsistent with mortality in mice. On the strength of these findings, the *Artemia salina* model does not appear to be suitable for evaluating neurotoxic venoms. It could be argued that the neurotoxins of elapid venom (*Naja subfulva*), most of which act on nicotinic acetylcholine receptors of muscle cells (Kukhtina et al., 2000), do not act on the synapsis of *Artemia*

Table 1Comparison of the toxicity of *Bitis arietans*, *Naja ashei* and *Naja subfulva* snake venoms in *Artemia salina* and in mice.

Snake species	Toxicity in <i>Artemia salina</i> ^a		Toxicity in mice	
	LC ₅₀ (24 h)	LC ₅₀ (72 h)	LD ₅₀ (24 h) ^b	MND (72 h) ^c
<i>Bitis arietans</i>	410.47 (228.21–729.14)	57.36 (26.50–125.47)	9.24 (7.50–11.38)	112.92
<i>Naja ashei</i>	41.28	5.40	3.02 (2.45–3.72)	36.12
<i>Naja subfulva</i>	47.54	12.06	0.55 (0.49–0.63)	31.48

^a Expressed as Median Lethal Concentration (LC₅₀) in µg/mL, with 95% confidence limits in parentheses.^b Expressed as Median Lethal Dose (LD₅₀) in µg/g, with 95% confidence limits in parentheses.^c Expressed as Minimum Necrotizing Dose (MND) in µg.

salina. However, the observation that high doses of the venoms were able to kill the *Artemia salina* suggests that there may be some neurotoxic effect realized. Perhaps these neurotoxins cannot readily penetrate the tissues of *Artemia salina*.

It was interesting to note that the cytotoxic venom (*Naja ashei*) was more toxic to *Artemia salina* at 72 h than the neurotoxic *Naja subfulva* venom. This could imply that the cytotoxins (either 3FTxs or PLA₂s) in *Naja ashei* venom (Hus et al., 2018) have the capacity to damage membranes of the *Artemia salina*. The findings of this study when taken together with the advantages of the *Artemia salina* assay (affordability, rapidity, simplicity, convenience, and robustness) and its utility in antivenom efficacy testing (Okumu et al., 2020) make a strong case for its use as an alternative to the more technically challenging, and expensive minimum necrotizing dose assay in mice. However, despite the many positive aspects of the assay, there are some drawbacks associated with the use of *Artemia salina*. First, it does not provide adequate information on the mechanism of cytotoxicity of the test substance (Hamidi et al., 2014). Secondly, the hatching of the *Artemia* nauplii requires a container that should be large enough to hold an air pump for equal distribution of oxygen (Vanhaecke et al., 1981). Moreover, 48-h old nauplii (2nd to 3rd instar stage) are more sensitive to test compounds (Vanhaecke et al., 1981). Thus many of the nauplii which hatch beyond the 48-h incubation period may not be suitable for experimental assays. Variations in pH and temperature may also affect the hatching process of the nauplii (Sorgeloos et al., 1978).

5. Conclusion

These findings suggest that the toxicity of the venoms in *Artemia salina* and the dermonecrotic bioassay in mice have a similar trend. Therefore, it may be relevant to further explore the *Artemia salina* bioassay along the lines of the 3Rs concept aimed at reducing the use of mice in snake venom research. Studies with a larger number of venoms are warranted to determine the correlation between the *Artemia salina* bioassay and the dermonecrotic assay in mice.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.toxcx.2021.100082>.

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Author contributions

Conceptualization: MO, FO, and JG; data curation: all authors; formal analysis: MO; investigation: all authors; methodology: MO, FO, and MJ.; project administration: MO, JG, and FO, resources: all authors; software: MO; supervision: JM, JG, PM, and VM; validation: FO, JM, JG, PM, VM, and MJ; visualization: MO, and MJ; writing original draft: MO and FO; writing review and editing: all authors. All authors read and approved the final manuscript.

Ethical considerations

This study was approved by the Biosafety, Animal Care, and Use Committee of the Faculty of Veterinary Medicine, University of Nairobi. REF BAUEC/2019/220 which was issued on April 24, 2019. All the used cell lines were disposed of per the Kenya Medical Research Institute (KEMRI)-University of Nairobi (UoN) protocols.

Consent for publication

Not applicable.

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Abbreviations

MND Minimum necrotizing dose; WHO: World Health Organization; BAUEC: Biosafety Animal Use and Ethics Committee; KEMRI: Kenya Medical Research Institute; UoN: University of Nairobi; LC₅₀: the lethal concentration responsible for 50% mortality in *Artemia salina*; LD₅₀: the median lethal dose responsible for 50% mortality in mice.

References

- Abbott, W.S., 1925. A method of computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18, 265–267.
- Barahona, M.V., Sanchez-Fortun, S., 1999. Toxicity of carbamates to the brine shrimp *Artemia salina* and the effect of atropine, BW284c51, iso-OMPA, and 2-PAM on carbaryl toxicity. *Environ. Pollut.* 104, 469–476.
- Barbosa, C.F., Rodrigues, R.J., Olortegui, C.C., Sanchez, E.F., Heneine, L.G., 1995. Determination of the neutralizing potency of horse antivenom against bothropic and crotalic venoms by indirect enzyme immunoassay. *Brazilian Journal of Medical and Biological Research. Revista Brasileira de Pesquisas Medicas e Biologicas* 28, 1077–1080.
- Bliss, C.I., 1935. The calculation of the dosage-mortality curve. *Ann. Appl. Biol.* 22, 134–167. <https://doi.org/10.1111/j.1744-7348.1935.tb07713.x>.
- Calmette, A., 1907. Les venins: les animaux venimeux et la serotherapie antiveneuse. Masson.
- Calvete, J.J., Arias, A.S., Rodríguez, Y., Quesada-Bernat, S., Sánchez, L.V., Chippaux, J. P., Pla, D., Gutiérrez, J.M., 2016. Preclinical evaluation of three polyspecific antivenoms against the venom of *Echis ocellatus*: neutralization of toxic activities and antivenomics. *Toxicol.* 119, 280–288. <https://doi.org/10.1016/j.toxcx.2016.06.022>.

- Chacón, F., Oviedo, A., Escalante, T., Solano, G., Rucavado, A., Gutiérrez, J.M., 2015. The lethality test used for estimating the potency of antivenoms against *Bothrops asper* snake venom: pathophysiological mechanisms, prophylactic analgesia, and a surrogate in vivo assay. *Toxicon* 93, 41–50. <https://doi.org/10.1016/J.TOXICON.2014.11.223>.
- da Silva, C.P., Costa, T.R., Paiva, R.M.A., Cintra, A.C.O., Menaldo, D.L., Antunes, L.M.G., Sampaio, S.V., 2015. Antitumor potential of the myotoxin BthTX-I from *Bothrops jararacussu* snake venom: evaluation of cell cycle alterations and death mechanisms induced in tumor cell lines. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 21, 1–8.
- De souza, L.L., Stransky, S., Guerra-duarte, C., Flor-sá, A., Schneider, F.S., Kalopothakis, E., Chávez-olortegui, C., 2015. Determination of toxic activities in *Bothrops* spp. snake venoms using animal-free approaches: correlation between in vitro versus in vivo assays. *Toxicol. Sci.* 147, 458–465. <https://doi.org/10.1093/toxsci/kfv140>.
- Finney, D.J., 1952. *Probit Analysis: Statistical Treatment of the Sigmoid Response Curve*. Cambridge university press, Cambridge.
- Gaddum, J.H., 1948. Probit analysis. *Nature* 161, 417–418. <https://doi.org/10.1038/161417a0>.
- Gadir, S.A., 2012. Assessment of bioactivity of some Sudanese medicinal plants using brine shrimp (*Artemia salina*) lethality assay. *J. Chem. Pharmaceut. Res.* 4, 5145–5148.
- Gutiérrez, J.M., Solano, G., Pla, D., Herrera, M., Segura, Á., Villalta, M., Vargas, M., Sanz, L., Lomonte, B., Calvete, J.J., León, G., 2013. Assessing the preclinical efficacy of antivenoms: from the lethality neutralization assay to antivenomics. *Toxicon* 69, 168–179. <https://doi.org/10.1016/J.TOXICON.2012.11.016>.
- Gutiérrez, J.M., Vargas, M., Segura, A., Herrera, M., Villalta, M., Solano, G., Sánchez, A., Herrera, C., León, G., 2021. In vitro tests for assessing the neutralizing ability of snake antivenoms: toward the 3Rs principles. *Front. Immunol.* 3394. <https://doi.org/10.3389/FIMMU.2020.617429>, 0.
- Hamidi, M., Jovanova, B., Kadifkova Panovska, T., 2014. Toxicological evaluation of the plant products using Brine Shrimp (*Artemia salina* L.) model. *Macedonian Pharmaceutical Bulletin* 60, 9–18. <https://doi.org/10.33320/maced.pharm.bull.2014.60.01.002>.
- Hernández-Matehuala, R., Rojas-Molina, A., Vuelvas-Solórzano, A.A., García-Arredondo, A., Alvarado, C.I., Olguín-López, N., Aguilar, M., 2015. Cytolytic and systemic toxic effects induced by the aqueous extract of the fire coral *Millepora alcornis* collected in the Mexican Caribbean and detection of two types of cytotoxins. *J. Venom. Anim. Toxins Incl. Trop. Dis.* 21, 1–13. <https://doi.org/10.1186/s40409-015-0035-6>.
- Hus, K.K., Buczkowicz, J., Petrillóvá, M., Łyskowski, A., Legáth, J., Bocian, A., 2018. First look at the venom of *Naja ashei*. *Molecules* 23, 609. <https://doi.org/10.3390/molecules23030609>.
- Kakanj, M., Ghazi-Khansari, M., Mirakabadi, A.Z., Daraei, B., Vatanpour, H., 2015. Cytotoxic effect of Iranian *Vipera lebetina* snake venom on HUVEC cells. *Iran. J. Pharm. Res. (IJPR): IJPR* 14, 109.
- Kerkkamp, H., Bagowski, C., Kool, J., Soelingen, B. Van, Vonk, F.J., Vlecken, D., 2018. *Toxicon* Whole snake venoms: cytotoxic, anti-metastatic and antiangiogenic properties. *Toxicon* 150, 39–49. <https://doi.org/10.1016/j.toxicon.2018.05.004>.
- Kerster, H.W., Schaeffer, D.J., 1983. Brine shrimp (*Artemia salina*) nauplii as a teratogen test system. *Ecotoxicol. Environ. Saf.* 7, 342–349.
- Khaing, E.M., Hurtado, P.R., Hurtado, E., Zaw, A., White, J., Warrell, D.A., Alfred, S., Mahmood, M.A., Peh, C.A., 2018. Development of an ELISA assay to determine the neutralising capacity of horse serum following immunization with *Daboia siamensis* venom in Myanmar. *Toxicon* 151, 163–168. <https://doi.org/10.1016/J.TOXICON.2018.07.012>.
- Kukhtina, V.V., Weise, C., Muranova, T.A., Starkov, V.G., Franke, P., Hucho, F., Wnendt, S., Gillen, C., Tsetlin, V.I., Utkin, Y.N., 2000. Muscarinic toxin-like proteins from cobra venom. *Eur. J. Biochem.* 267, 6784–6789. <https://doi.org/10.1046/j.1432-1327.2000.01775.x>.
- Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E. j., McLaughlin, J. L., 1982. Brine shrimp: a convenient general bioassay for active plant constituents. *Planta Med.* 45, 31–34.
- Mirzaei, M., Mirzaei, A., 2013. Comparison of the *Artemia salina* and *Artemia uramiana* bioassays for toxicity of 4 Iranian medicinal plants. *Int. Res. J. Biol. Sci.* 2, 49–54.
- Mwangi, G.G., Wagacha, J.M., Nguta, J.M., Mbaria, J.M., 2015. Brine shrimp cytotoxicity and antimalarial activity of plants traditionally used in the treatment of malaria in Msambweni district. *Pharmaceut. Biol.* 53, 588–593. <https://doi.org/10.3109/13880209.2014.935861>.
- Oguiura, N., Kapronezai, J., Ribeiro, T., Rocha, M.M.T., Medeiros, C.R., Marcelino, J.R., Prezoto, B.C., 2014. An alternative micro method to access the procoagulant activity of *Bothrops jararaca* venom and the efficacy of antivenom. *Toxicon* 90, 148–154. <https://doi.org/10.1016/J.TOXICON.2014.08.004>.
- Okumu, M.O., Mbaria, J.M., Gikunju, J.K., Mbuthia, P.G., Madadi, V.O., Ochola, F.O., 2020. Enzymatic activity and brine shrimp lethality of venom from the large brown spitting cobra (*Naja ashei*) and its neutralization by antivenom. *BMC Res. Notes* 13, 1–7. <https://doi.org/10.1186/s13104-020-05167-2>.
- Pornmuttakun, D., Ratanabanangkoon, K., 2014. Development of an in vitro potency assay for antivenom against Malayan pit viper (*Calloselasma rhodostoma*). *Toxicon* 77, 1–5. <https://doi.org/10.1016/J.TOXICON.2013.10.021>.
- Rajabi, S., Ramazani, A., Hamidi, M., Naji, T., 2012. Biochemical tolerance during low dose propylene glycol exposure in neonates: a formulation-controlled evaluation. <https://doi.org/10.1186/s40199-015-0105-x>.
- Rajabi, S., Ramazani, A., Hamidi, M., Naji, T., 2015. *Artemia salina* as a model organism in toxicity assessment of nanoparticles 1–6. <https://doi.org/10.1186/s40199-015-0105-x>.
- Rial, A., Morais, V., Rossi, S., Massaldi, H., 2006. A new ELISA for determination of potency in snake antivenoms. *Toxicon* 48, 462–466. <https://doi.org/10.1016/J.TOXICON.2006.07.004>.
- Rungsiwongse, J., Ratanabanangkoon, K., 1991. Development of an ELISA to assess the potency of horse therapeutic antivenom against Thai cobra venom. *J. Immunol. Methods* 136, 37–43. [https://doi.org/10.1016/0022-1759\(91\)90247-D](https://doi.org/10.1016/0022-1759(91)90247-D).
- Russel, W.M.S., Burch, R.L., 1959. *The Principles of Humane Experimental Technique*. Methuen & Co, London. Available from: <http://117.239.25.194:7000/jspui/bitstream/123456789/1342/1/PRILIMINARY%20%20AND%20%20CONTENTS.pdf>.
- Segura, A., Castillo, M.C., Núñez, V., Yarlequé, A., Gonçalves, L.R. de C., Villalta, M., Bonilla, C., Herrera, M., Vargas, M., Fernández, M., 2010. Preclinical assessment of the neutralizing capacity of antivenoms produced in six Latin American countries against medically relevant *Bothrops* snake venoms. *Toxicon* 56, 980–989.
- Sorgeloos, P., Remiche-Van Der Wielen, C., Persoone, G., 1978. The use of *Artemia* nauplii for toxicity tests—a critical analysis. *Ecotoxicol. Environ. Saf.* 2, 249–255.
- Stransky, S., Costal-Oliveira, F., Lopes-de-Souza, L., Guerra-Duarte, C., Chávez-Olortegui, C., Braga, V.M.M., 2018. In vitro assessment of cytotoxic activities of *Lachesis muta muta* snake venom. *PLoS Neglected Trop. Dis.* 12, e0006427.
- Theakston, R.D.G., Reid, H.A., 1979. Enzyme-linked Immunosorbent Assay (ELISA) in assessing antivenom potency. *Toxicon* 17, 511–515. [https://doi.org/10.1016/0041-0101\(79\)90284-8](https://doi.org/10.1016/0041-0101(79)90284-8).
- Theakston, R.D.G., Reid, H.A., 1983. Development of simple standard assay procedures for the characterization of snake venoms. *Bull. World Health Organ.* 61, 949.
- Vanhaecke, P., Persoone, G., Claus, C., Sorgeloos, P., 1981. Proposal for a short-term toxicity test with *Artemia* nauplii. *Ecotoxicol. Environ. Saf.* 5, 382–387.
- WHO, 2016. Post-ECBS version expert committee on biological standardization geneva, 17 to 21 october 2016. WHO guidelines for the production, control, and regulation of snake antivenom immunoglobulins. World Health Organization 1–138.