Development of the First Specific Antivenom for Sindh Krait: Addressing a Neglected Health Threat in South Asia

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Abstract- Sindh Krait (*Bungarus sindanus*) is a highly venomous snake found in South Aisa that have critical threat due to unavailability of specific antivenom and low efficacy of commercially available antivenom against this deadly snake. This study reports the first holistic development of specific antivenom against Sindh Krait addressing the treatment gap for the envenomation of this snake. The venom was collected from Sindh Krait from the Sindh Province of Pakistan and traditional approach was used to develop antivenom in horses. Hyperimmune horse plasma was processed using caprylic acid and ammonium sulphate precipitation methods and the resulting antivenom was tested for its safety and efficacy. The results showed that the newly developed antivenom is highly effective against venom of Sindh Krait and hence provides a promising solution for improving the treatment of envenomation of Sindh Krait.

Index Terms- Antivenom, Envenomation, Neglected Tropical Disease, Sindh Krait, Snake, Snakebite, South Asia, Venom

1. INTRODUCTION

Snakebite is a serious health concern in many parts of the world. it is often described as the 'disease of the poor' along with the other neglected tropical diseases as is mostly effect the poor people like farmers, shepherds, women and children and those living in poor rural areas[1]. WHO has declared the snakebite as a priority neglected tropical disease in 2017 showcasing the severity of the problem.[2, 3]. Snake bite envenoming effects millions of people worldwide each year resulting in thousands of deaths and amputations. Africa and Asia are the most effected from this disease with around 2 million envenomation cases in Asia and up to 0.6 million cases in Africa. The total number of snakebite case are estimated to be 4.5 to 5.5 million globally [4]. The snakebite burden is highest in the low- and middle-income countries with poor infrastructure and weak healthcare system [1].

Although effective treatment against snakebite envenomation is available in shape of antivenom but its limited availability and low specificity leads to disparity in treatment of snakebite around the world. Moreover, limited data is available regarding the snakebite cases and management which adds to the problem of estimation of real snakebite burden [5, 6]. Another significant factor is the complex geography of snake distribution and representation of regional snake venoms is respective antivenom. WHO has identified all the venomous snakes and categorized them based on their medical importance, but representation of medically important snakes is still limited in antivenom like Pakistan has *Bungarus caeruleus*, *Bungarus Sindanus*, *Naja naja*, *Naja oxiana* from *Elapidae* family in category 1 medically important snakes but it is manufacturing antivenom against *Bungarus caeruleus* and *Naja naja* only [7]. Same goes for many other countries where antivenom does not include representation of all the medically important snakes that makes the antivenom less effective against un-represented species resulting in increase in morbidities and mortalities. This problem persists throughout the world where interspecies and intraspecies variations in venom composition, complex and diverse geographical distribution of snakes, limited availability of quality venoms leads to low quality and less effective antivenoms [8]. Also, weaker health systems in regions of higher snakebite prevalence often lack proper infrastructure and data collection and reporting system that contributes to underreporting of snakebite cases and ultimately a poorer understating to severity of the problem which further complicates the efforts to address the problem [9, 10].

Although, there have been efforts in the past to build the antivenom against Sindh krait but those were often limited studies covering a specific aspect of antivenom production [11]. This is the first antivenoms of its kind against the Sindh krait that

provides a potential solution to the long existing treatment gap of the Sindh Krait. In this study, we have explored the implications of this groundbreaking development, examined not only the lethality of the venom but also raised a safe and effective antivenom against this deadly snake. This study highlights the critical need for the development of targeted antivenoms against the medically important yet ignored snake species specially Sindh krait. It also paves the way for the development of commercial antivenom against this deadly snake to contribute in combating global snakebite crisis and saving precious human lives.

2. MATERIALS AND METHODS

2.1. Snake collection and identification

The Sindh krait was collected from Tharparker, Umerkot, Sanghar and Khairpur districts of Sindh province of Pakistan (Fig. 1). The Sindh krait was identified from its characteristics 17 dorsal scale rows with jet black color as compared to the 15 rows of common krait with greyish black color. Its body features broad white bands contrasting with the common krait's broader black bands with lighter white bands. Sindh kraits display white spots covering approximately one-third of their bodies, which gradually develop into bands [12]. The snakes were housed at the serpentarium of National Institute of Health Islamabad Pakistan.

Fig. 1. Sampling districts of Sindh province, Pakistan for snake collection

2.2. Venom collection and LD_{50} determination

The venom was collected from snakes after two months of quarantine period. The venom was collected through standard procedure as recommended by WHO [7], after which it was freeze dried and stored for further use.

The LD_{50} of the venom was determined using the probit analysis. Mice having weight of 18-20g were selected and injected with two-fold decreasing dose of venom intravenously through caudal route as 10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 µg/dose. The deaths were recorded after 24 hours and probit analysis was performed and LD_{50} was calculated using the using the R software (version 4.3.3, 'dose.p()' function from the 'MASS' package).

2.3. Horse immunization and hyperimmunized plasma collection

Two large inbred horses having at least three years of age was used for immunization. Horses were given multisite injections of 10ml sterile venom solutions with two weeks interval according to the schedule given in table 1.

Table 1. Immunization schedule for horse

Day	**1st**	**14th**	**28th**	**42th**	**56th**
Dose	5µg/0.005mg	10µg/0.01mg	17µg/0.017mg	30µg/0.03mg	50µg/0.05mg
Day	**70th**	**84th**	**98th**	**112th**	**126th**
Dose	100µg/0.1mg	200µg/0.2mg	500µg/0.5mg	1mg	2mg
Day	**140th**	**154th**	**168th**	**182th**	**-**
Dose	4mg	8mg	12mg	15mg	-

Antibody titer was checked at regular intervals from the serum collected from horses against $2LD_{50}$ of venom challenge dose, and horses were bled after 14^{th} dose. A total of 10L blood was collected from horse in two episodes of 48 hours interval in a sterile container, using 4% potassium oxalate solution as anti-coagulant. The blood was centrifuged at 5000rpm for 10 minutes to separate plasma from blood.

2.4. Plasma processing and purification

Plasma was processed with the caprylic acid fractionation method as described by [13]. In this method the pH of the plasma was lowered to 5.8 by using 1.75N acetic acid (Merck cat# 100063) and then 5% caprylic acid (Merck cat. no. 800192) was added slowly with constant stirring. The mixture was then subjected to vigorous stirring for one hour and then filtered. Saturated solution of ammonium sulphate (Merck cat. no. 101217) having specific gravity of 1.245 was then added to this mixture at 45% concentration and solution was gently stirred for 10 minute and the solution was then filtered. The filtrate was discarded and precipitate containing pure immunoglobulins were dialyzed using Spectra Pro® 2 dialysis membrane (MWCO 12,000-14,000 Da) for 14 days against distilled water to remove residual ammonium sulphate [14]. The residual ammonium sulphate was detected using 4% barium chloride solution. Total proteins and albumin levels was determined using the Spectrum Total Protein (Single Reagent) kit (cat. no. 310 001) and Spectrum Albumin - BCG (Acetate Buffer) kit (cat. no. 210 001) respectively. Based on its results the solution was diluted to four times to get optimal protein concentration in final product. Phenol was then added to the final solution as preservative to a concentration of 0.15g/dL and sterile filtration was performed using Sartobran® P (cat. no. 5235307H8), Pore size: 0.45, 0.2 µm (CA double layer) filter.

2.5. Antivenom safety and efficacy testing

Turbidity analysis was done using at 600nm at using Spectronic 20D+ spectrophotometer. Sterility test was performed using Tryptic Soy Broth (TSB) media by Merck/Sigma (catalog number 22092-500G) for aerobic growth and for anaerobic growth, Fluid Thioglycollate Medium (FTM) by Merck/Sigma (cat no. STBMFTM12) was utilized. The abnormal toxicity was performed using five mice (18-20g each) and two guinea pig (900-1000g each). Each guinea pigs received 2.5ml and each mice received 0.5ml of antivenom. Control was also injected with same amount of normal saline. Readings were recorded for seven days at alternative days for any signs of toxicity, weight loss and paralysis. Pyrogen test was performed through LAL test using Pyrostar™ ES-F 80 test kit (cat. no. WPEK4-20025).

To calculate ED_{50} antivenom in dilution ratio of 1:0 (pure), 1:1, 1:2, 1:3 and 1:4 was injected into mice along with $6LD_{50}$ challenge dose intravenously through tail route. Each group was containing 8 mice of 18-20g. The survival rate was calculated and dose response data was analyzed through probit analysis using the dose.p function from the MASS package in R version 4.3.3. The neutralization potency of antivenom was calculated using the ED_{50} with the help of formula.

3. RESULTS

3.1. Snake Identification

Sindh krait was primarily identified from jet black color with 17 mid body dorsal scale rows as compared to the 15 mid body dorsal sales of the common krait with greyish black color. Common krait has broader black bands and narrower white bands while Sindh krait has wider white bands and narrower black bands. The average length of adults of Sindh krait was about 4-6ft. Fig. 2 shows the scale pattern of Common krait, fig. 3 shows the scale pattern of Sindh krait and fig. 4 shows the Sindh krait along with common krait.

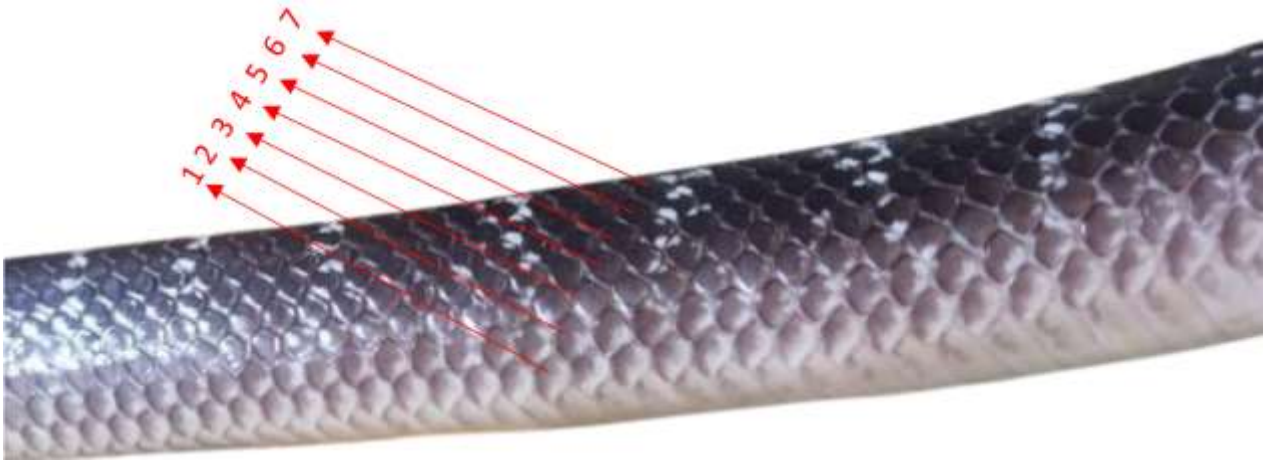

Fig. 2. Scale pattern of Common krait

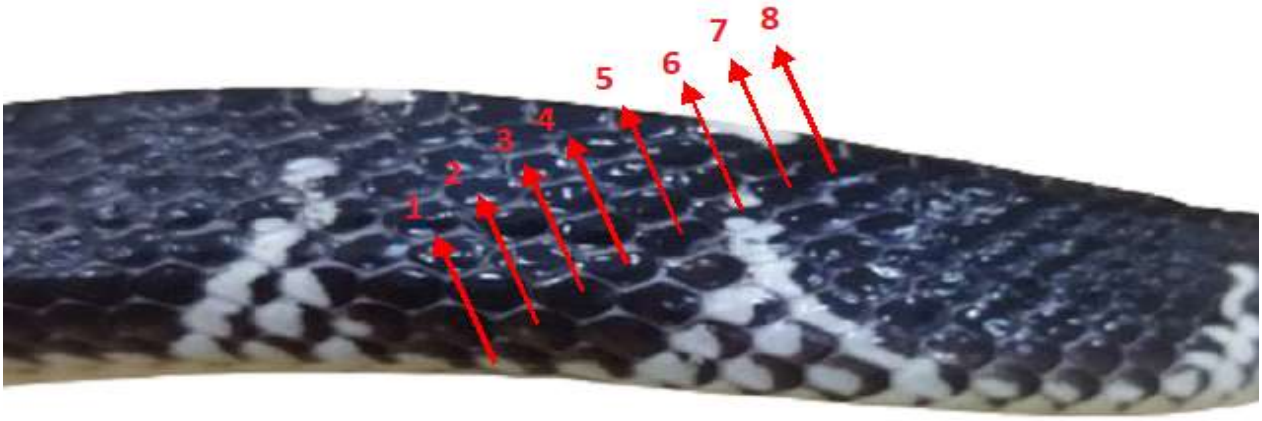

Fig. 3. Scale pattern of Sindh Krait

Fig. 4. Sindh Krait along with Common Krait

3.2. LD_{50} of Sindh krait

LD_{50} of Sindh krait was found to be 1.1283µg/dose or 0.0564µg/g. Fig. 5 shows the dose response curve showing the LD_{50} values of Sindh krait.

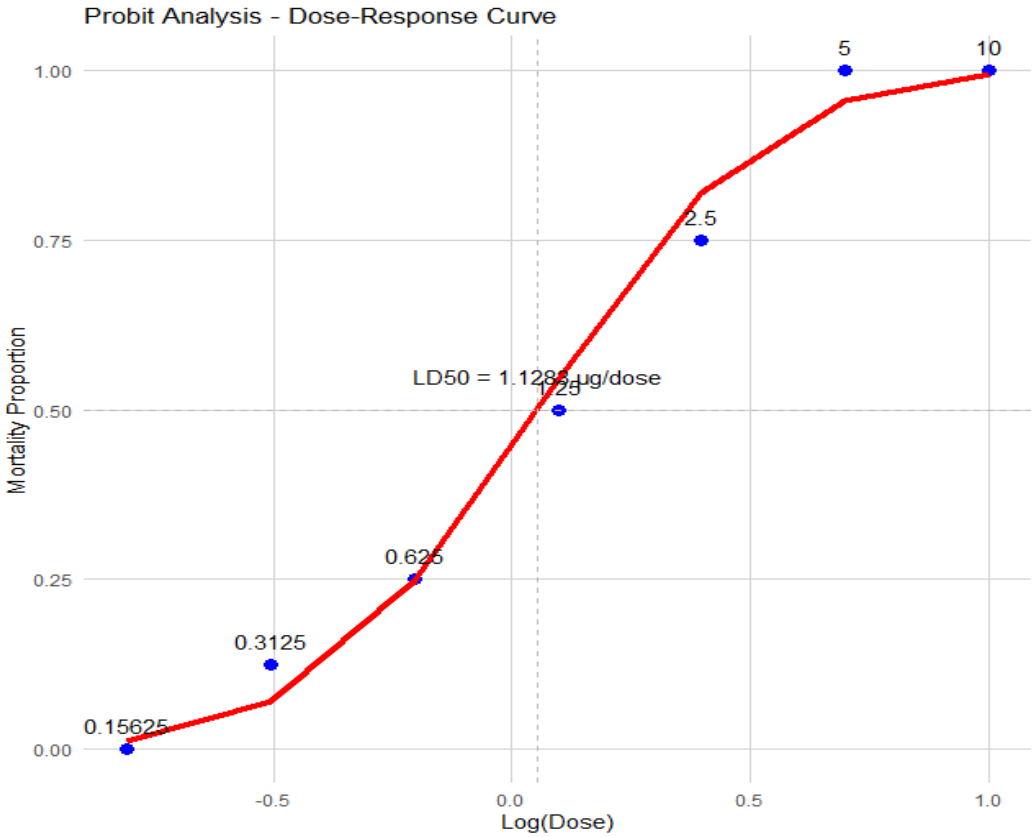

Fig. 5. Dose response curve and LD_{50} of venom of Sindh Krait

3.3. Immunization and Hyperimmunized Plasma Collection

Horses were immunized after 14 venom doses. The antibody titer was checked in vivo against $2LD_{50}$ challenge dose. The horse plasma showed no apparent potency after first three doses, but increased steadily after 4^{th} dose until the 12^{th} dose. After that horse plasma showed little increase in titer and hence bled thereafter. Fig. 6. shows the in vivo neutralization ability of horse plasma and antibody titer buildup after each venom dose. A total of 12.4L of plasma was obtained from 20L of blood which was stored at 2-8^{o}C for further processing.

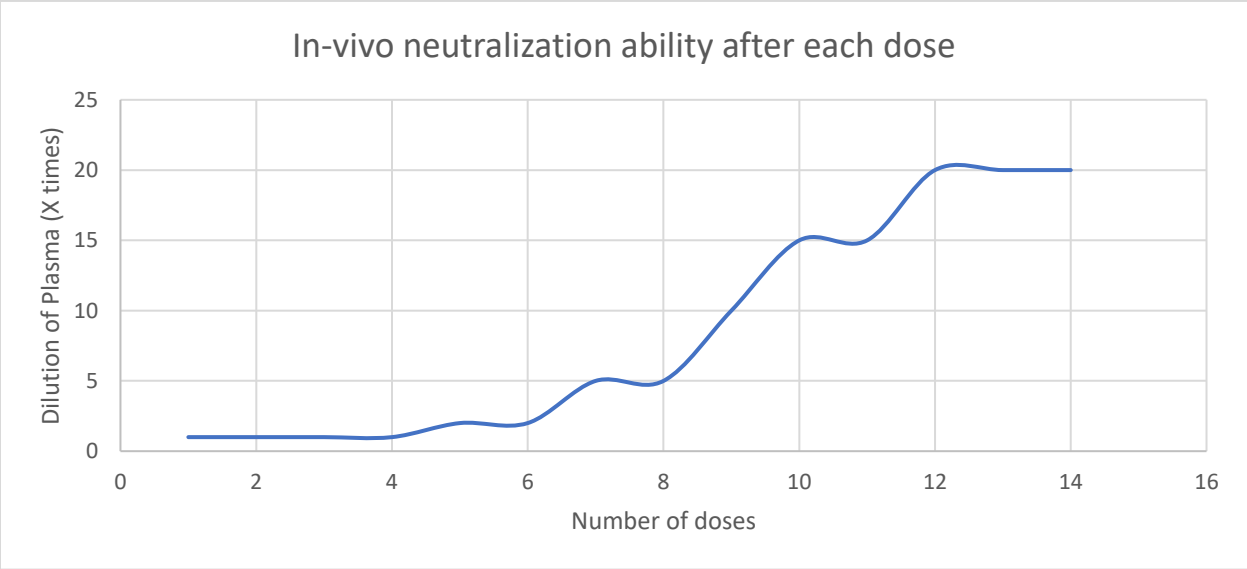

Fig. 6. In vivo neutralization potential of horse serum after each dose of venom

3.4. Plasma Processing

The plasma was processed with caprylic acid process. This process precipitates all the non-specific proteins in the plasma which can be easily filtered and immunoglobulins are obtained in filtrate. This solution can be further purified through tangential flow filtration or ammonium sulphate precipitation method. We used ammonium sulphate precipitation method for purification of immunoglobulins from the solution. Table 2. shows the different parameters that were measured after caprylic acid and ammonium sulphate precipitation. The processed plasma was diluted 4 times to obtain the optimal concentration of proteins in the final product.

Table 2. Parameters measured for caprylic acid and ammonium sulphate precipitation

Concentration	Total Protein concentration g/dl	Abnormal toxicity	Albumin g/dl	Total solids g/dl	Turbidity A(600nm)
Caprylic acid concentration (5% V/V)	7.88	Not detected	0.37	8.3	0.071
Ammonium concentration	28.4	Not tested	1.48	30.1	0.194

3.5 Safety and Efficacy parameters

Different safety parameters like pyrogen, abnormal toxicity and sterility were determined and antivenom was found safe for use. The values for these parameters are given in the table 3.

The effective dose 50% (ED_{50}) was calculated using probit analysis. The ED_{50} value at 1:2 (33%) dilution was found to be 31.59 µl. It means that 31.59 µl of antivenom at this specific dilution is required to achieve 50% survival rate in mice with

$6LD_{50}$ challenge dose. Similarly for pure antivenom, ED_{50} is 10.42 µl of undiluted antivenom. Fig. 7 represents the dose response curve that shows the relationship between different doses of antivenom and survival rate.

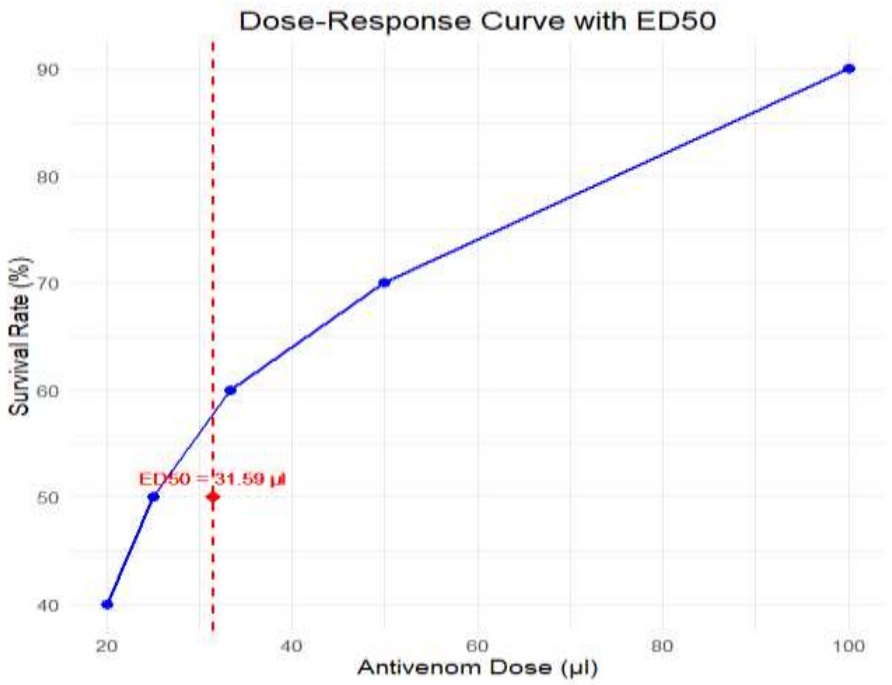

Fig. 7. Dose response curve and ED_{50} of antivenom

Subsequently, the neutralization potency of antivenom is calculated from this ED_{50}. For the pure antivenom with ED_{50} 10.42 µl and challenge dose of 6.7698 µg the neutralization potency is 649.69µg/ml or 0.649mg/ml, and for antivenom dilution of 1:2 (33%) the potency was 214 µg/ml. Such high neutralization potency i.e. ~0.65mg/ml shows that the antivenom has very good neutralization potential against the venom of Sindh Krait

Table3. Prominent Safety and quality parameters of antivenom

Parameter	Testing method	Result	Regulatory requirement
Sterility	Filtration and TSB media for aerobic growth Filtration and FTM for anaerobic growth	No microbial growth	Absence of microbial growth
Abnormal Toxicity	Guinea pigs and mice	No significant signs of toxicity i.e. weight loss, paralysis or behavioral change	Compliance with pharmacopeia
Pyrogen	LAL test	1.62 EU/ml	Compliance with pharmacopeia
Appearance	Visual inspection	Golden yellowish liquid	Compliance with marketing requirements
Turbidity	Observation with Spectrophotometer at 600nm	0.059	No cloudiness

4. DISCUSSION

Sindh krait has not been studied much over the time. It was first reported in 1897 by renowned zoologist G. A. Boulenger [15]. Although reported for a long time, this snake has got very little attention due to its close similarity with common krait

that has much greater prevalence and geographical distribution. Due to this reason, although it is categorized among category 1 medically important snake [7], its antivenom is still not available and its bites are often misdiagnosed with that of common krait. This was first explained by Pillai in 2012, when envenomation cases with similar signs to common krait was recorded in Maharashtra, India and it the Sindh krait was reported with its characteristics 17 dorsal scales as the reason [16]. Similar envenomation cases were also reported from other parts of India that showcases the greater geographical distribution of this snake.

Sindh krait has a significant presence in Pakistan and a wide geographical distribution as well [17], but only few studies have been done on this. Also, there is lack of proper identification and reporting system of snakes and snakes and snakebite that further widens the gap of recognition of this snake [10]

The Sindh krait is far more dangerous and venomous than its more commonly known relative i.e. common krait having a much lower LD_{50} than the common krait [18]. Compounding the problem is the fact that the commercially available antivenoms have been proven to be the least effective against Sindh krait that results in critical gap in the treatment of envenomation cases of this snake. Sindh krait most commonly occurs in the Sindh province of Pakistan, from where it has also got its name, this snake shares far more wide geographical distribution that this mere locality [16]. Its geographical presence extends into India, Iran, Bangladesh, and Nepal but it is yet to gain its representation in the antivenoms of the entire South Asian region [18, 19]. Moreover, the krait bite usually occurs at night and due to the short fangs of krait, its bite often goes unnoticed and the victim dies due to envenomation before its realization [20]. The venom was extracted from snakes that purely belongs to the Sindh region where the Sindh krait originally got its name. The LD_{50} 0.0564µg/g was comparable to the previous studies i.e. 0.04 µg/g [21] and 0.02 µg/g [18] which showed that the venom was of high quality.

The antivenom was produced through the combination of two popular methods of antivenom production methods i.e. caprylic acid fractionation method and ammonium sulphate fractionation method. Both methods have been widely used around the world and recommended by WHO a well. Although, caprylic acid method gives high process efficiency, its product of often diluted that needs to be further purified from ultrafiltration and diafiltration by using TFF machine. Ammonium sulfate precipitation method provides an easy and cheap alternative to this where one can easily fractionate the diluted product of caprylic and get a concentrated antivenom that can be easily diluted to the requirement [13].

The antivenom produced through this method was very good quality by fulfilling the recommended regulatory requirements and also showed the good neutralization potency against the venom of Sindh krait. There have been studies for the commercially available antivenom for its efficacy against the Sindh Krait venom but all showed a lower neutralization potential because the venom proteome of the Sindh krait differs from common krait [18, 21]. The antivenom produced in this study is not only specific for the Sindh krait but it also showed a very good neutralization potential against the venom of Sindh krait that is comparable to the neutralization potential of commercially available antivenoms against common krait i.e. 0.6mg/ml [21]. In this way this study opens the ways for the commercial producers of antivenom to actively include the representation of Sindh krait in their antivenom. This approach will not only increase the recognition of this snake but will also save the precious human lives.

5. CONCLUSION

The development of antivenom specific antivenom against Sindh Krait is a significant step in addressing the critical gap of snakebite treatment in South Asian region. This study describes the method to produce a safe and highly effective antivenom against venom of Sindh Krait. This will not only contribute to reduction of snakebite related deaths but also highlights the importance of representation of medically important yet neglected snake species. Future efforts should focus on commercial production of antivenom against Sindh Krait or at least its inclusion in currently available antivenoms to save precious human lives and improve overall public health outcomes.

Conflict of Interest

The authors declare no conflict of interest.

Authors Contributions

HSH and NH conceived the idea, TH and AN helped to design the experiment and provision of supplies and support for the study. HSH performed the experiment and TH helped in data analysis. HSH prepared the original draft of this work. NH, AN and TH contributed to the revision and proofreading of this manuscript.

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REFERENCES

[1] R. A. Harrison, A. Hargreaves, S. C. Wagstaff, B. Faragher, and D. G. Lalloo, "Snake envenoming: a disease of poverty," *PLoS Negl. Trop. Dis.,* vol. 3, no. 12, p. e569, 2009.
[2] T. Lancet, "Snake-bite envenoming: a priority neglected tropical disease," vol. 390, ed, 2017, p. 2.
[3] S. Bhaumik, A. B. Zwi, R. Norton, and J. Jagnoor, "How and why snakebite became a global health priority: a policy analysis," *BMJ Global Health,* vol. 8, no. 8, p. e011923, 2023.
[4] WHO. "Snakebite envenoming: Fact Sheet." https://www.who.int/health-topics/snakebite#tab=tab_1 (accessed 23 September, 2023).
[5] A. Kasturiratne *et al.*, "The global burden of snakebite: a literature analysis and modelling based on regional estimates of envenoming and deaths," *PLoS Med.,* vol. 5, no. 11, p. e218, 2008.
[6] C. Bravo-Vega, C. Renjifo-Ibañez, M. Santos-Vega, L. J. León Nuñez, T. Angarita-Sierra, and J. M. Cordovez, "A generalized framework for estimating snakebite underreporting using statistical models: A study in Colombia," *PLoS Negl. Trop. Dis.,* vol. 17, no. 2, p. e0011117, 2023.
[7] WHO. "Guidelines for the production, control and regulation of snake antivenom immunoglobulins, Annex 5, TRS No 1004." World Health Organization. https://www.who.int/publications/m/item/snake-antivenom-immunoglobulins-annex-5-trs-no-1004 (accessed 16 September, 2023).
[8] B. G. Fry, K. D. Winkel, J. C. Wickramaratna, W. C. Hodgson, and W. Wüster, "Effectiveness of snake antivenom: species and regional venom variation and its clinical impact," *Journal of Toxicology: Toxin Reviews,* vol. 22, no. 1, pp. 23-34, 2003.
[9] A. F. Pintor *et al.*, "Addressing the global snakebite crisis with geo-spatial analyses–Recent advances and future direction," *Toxicon: X,* vol. 11, p. 100076, 2021.
[10] R. Memon, T. B. Erickson, and C. E. Goldfine, "Challenges in care of snake envenomation in rural Pakistan: a narrative review," *Toxicology Communications,* vol. 7, no. 1, p. 2223049, 2023.
[11] S. Yaseen and A. A. Khan, "Antivenom production in chicken against Sind krait (Bungarus sindanus) venom and its efficacy assessment using different immunoassays," *Ciência Rural,* vol. 54, p. e20220639, 2023.
[12] Wildlifeofpakistan. "Snakes of Pakistan: Common Krait, Sindhi Krait, Northern Punjab Krait." www.wildlifeofpakistan.com (accessed 24 September, 2023).
[13] G. Rojas, J. Jiménez, and J. Gutiérrez, "Caprylic acid fractionation of hyperimmune horse plasma: description of a simple procedure for antivenom production," *Toxicon,* vol. 32, no. 3, pp. 351-363, 1994.
[14] H. Hassan, A. Najam, G. Parveen, H. Ali, R. Channa, and S. Adeel, "An optimized method of IgG's precipitation with ammonium sulphate from hyper immune horse plasma for snake anti venom production," *International Journal of Molecular Biology: Open Access,* vol. 5, no. 4, pp. 166-169, 2020.
[15] G. Boulenger, "A new krait from Sind (Bungarus sindanus)," *J. Bombay Nat. Hist. Soc.,* vol. 11, pp. 73-4+ Pl, 1897.
[16] L. V. Pillai, D. Ambike, S. Husainy, A. Khaire, A. Captain, and U. Kuch, "Severe neurotoxic envenoming and cardiac complications after the bite of a 'Sind Krait'(Bungarus cf. sindanus) in Maharashtra, India," *Trop. Med. Health,* 2012.
[17] M. S. Khan, *A Guide to the Snakes of Pakistan*. Edition Chimaira Frankfurt am Main, 2002.
[18] K. Sunagar *et al.*, "A wolf in another wolf's clothing: Post-genomic regulation dictates venom profiles of medically-important cryptic kraits in India," *Toxins (Basel),* vol. 13, no. 1, p. 69, 2021.
[19] S. Kumar, H. S. Banyal, and I. Sharma, "First record of Sind Krait, Bungarus sindanus Boulenger, 1897 (Reptilia: Squamata: Elapidae) from Narayan Sarovar Wildlife Sanctuary, Kachchh, Gujarat," *Records of the Zoological Survey of India,* vol. 121, no. 2, pp. 215–218, 2021.
[20] H. S. Bawaskar, P. H. Bawaskar, and P. H. Bawaskar, "Snake bite in India: a neglected disease of poverty," *The Lancet,* vol. 390, no. 10106, pp. 1947-1948, 2017.
[21] A. M. F. Oh, C. H. Tan, K. Y. Tan, N. H. Quraishi, and N. H. Tan, "Venom proteome of Bungarus sindanus (Sind krait) from Pakistan and in vivo cross-neutralization of toxicity using an Indian polyvalent antivenom," *J. Proteomics,* vol. 193, pp. 243-254, 2019.

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