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# Determination of Lethal Dose ($LD_{50}$) of Venom of four Different Poisonous Snakes found in Pakistan

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## Abstract

Pakistan is the highly fertile region for the envenoming and deadly snakes. The envenoming fatality and snake bite cases are increasing day by day not only in Pakistan but also in World. In Pakistan, almost 40,000 snake bite cases and 8000 fatal cases has been identified. This high-risk issue needs to be addressed with an easy accessible and affordable treatment by producing anti snake venom serum. $LD_{50}$ (50% Lethal Dose) of venom is the prime parameter to determine the toxicity and lethality of venom extracted from the four poisonous snakes present in the Pakistan. The main objective of this study waste produces highly potent and cost-effective anti-snake venom serum by the determination of $LD_{50}$. The venom was extracted from four different species of snakes i.e., *Echis carinatus*, *Vipera russelli*, *Bungares caeruleus* and *Naja naja* (Cobra) present in Biological Production Division of NIH, Pakistan. The four to five serial dilutions were injected intravenously into the mice tail and observations were recorded to calculate the $LD_{50}$ of each species by Reed and Munch method in Bacteriology section of Quality Control Laboratory, National Institute of Health, Islamabad. Then 3 to 5 fold $LD_{50}$ is the neutralization dose of Anti-snake venom serum used for the calculation of $ED_{50}$ of each batch/lot of anti-snake venom serum(as per WHO). The results of the study shows that $LD_{50}$ of *Naja Naja* (Cobra) lies approximate between 6 to 7 µg/dose, *Echis carinatus* (*Saw Scaled Viper*) 11 to 12 µg/dose, *Vipera russelli* (*Russel viper*) 5 to 6 µg/dose and *Bungares caeruleus* (*Krait)* 4 to 5 µg/dose in intravenous injection of dilution.

**Keywords:** Venom; $LD_{50}$; Anti snake venom serum; $ED_{50}$

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## Introduction

There are almost 5.4 million venomous snake bites, about 2.5 million envenoming and over 125,000 deaths annually. As per global burden of snake bite incidents, (morbidity and mortality), South Asia is the most affected region. India has the highest number of deaths with 35,000–50,000 per year [1,2]. In Pakistan, 40,000 snake bites and 8000fatal cases per year [3] in Nepal, 1,000 deaths per year [4] by snake bite, in Sri Lanka, around 33,000 snake bite victims are reported annually [5]. The fatality rate is almost 20% [6]. Snakes are cold-blooded vertebrates and provoke a high number of human deaths due to envenoming characteristic [7].

In Pakistan, *Echis carinatus* (*saw Scaled viper*) is found in Thar and Cholistan desert, and Astola island of Makran in Baluchistan. It is almost 0.4 to 0.6-meter-long with flattened body and short tapered tail. It is active and can move rapidly in aside winding motion. *Vipera russelli* is found in Pakistan from India-Pakistan border to Indus Valley in Provinces of Sindh and Punjab at moist and cool places. Viper snakes distributed north to the Indus valley of Pakistan and Kashmir, regarded as one of Pakistan's deadliest snakes. *Bungares caeruleus Sindanus (Krait)* is about 1.0 to 1.8-meter-long with glossy appearance, flattened body and jerky movement. It is very active at night. *Naja naja (Cobra)* has two subspecies of almost 1.9 to 2.4-meter long. The hood appearance varies greatly and it is present in eastern Pakistan and Karachi at sea level [8].

The almost 3000 species of snake are found in all over the world

 

and near about 600 among them are found venomous. These snakes inject modified saliva (venom) containing toxins into the body of their prey through their fangs cause bleeding, muscle paralysis, and tissue destruction (necrosis) around the bite site [9].

The assessment of snake venom median lethal doses ($LD_{50}$) is an important step for an accurate evaluation of the toxic activity of specific venom, and is also regularly used to select the relevant anti-venom batch, as well as to establish the neutralizing capacity of each vial. According to the WHO, venom lethality is expressed as median lethal dose ($LD_{50}$). The $LD_{50}$ value is defined as the amount of a substance (or venom) causing death of 50% injected mice [10]. The $LD_{50}$ for snake venom was first determined in mice by Meier and Theakston in 1986 [11]. According to their study the approximate $LD_{50}$ for *Naja naja* snake was determined in mice to be equal to 0.05 µg/g body weight.

The potency of anti-snake venom serum is expressed by its action of neutralizing the $LD_{50}$ of snake venom. The toxic effect of venom is expressed in term of its $LD_{50}$. This $LD_{50}$ also helped in the establishment of the effective titer of the anti-snake venom serum as median effective dose ($ED_{50}$). The median effective dose is the minimum amount of anti-snake venom serum to neutralize and protects 50% population of the mice injected [12].

The current study is based upon the calculation of $LD_{50}$ of venom of different poisonous snakes present in Pakistan and its evaluation in terms of $LD_{50}$. The findings from this investigation will help in the production of highly potent and cost effective anti-snake venom serum in Pakistan.

## Procedure

The $LD_{50}$ was calculated by Reed & Munch method according to WHO guidelines. The calculation is carried by observing and calculating cumulative survival/death, proportionate of difference in dilution factor and mortality rate. The standardized $LD_{50}$ of venom is essential for the neutralization of antivenom i.e., for potency determination of each manufactured batch.

The study has been performed on following four venomous snakes found in Pakistan.

*Echis carinatus (Saw Scaled Viper)*

*Bungares caeruleus (Sung Choor, Krait)*

*Vipera russelli (Russell's viper, Dobia russuii)*

*Naja naja (Cobra or Sheesh Nag or Kala Nag)*

## Results

Each dilution was injected in group of eight mice both male and female 18 to 20 g weight. The dose of venom was inoculated intravenous in tail. The observations were noted after 24 hours of inoculation and $LD_{50}$ were calculated by Reed and Munch

**Table 1** shows the mortality/survival rate of the mice after injecting specified amount of venom intravenously in mice.

**Table 1** Determination of LD50 values through reed and munch method.

| Venom of the Snake | No. of doses | Dose in µg | No. of Mice injected | Weight of mice (g) | Route of inoculation/dose in ml | No. of Survival after 24Hrs. |
|---|---|---|---|---|---|---|
| *Echis carinatus (Saw scaled viper)* | 1 | 32 | 8 | 18-20 | Intravenous in Tail | 0 |
| | 2 | 16 | 8 | | | 3 |
| | 3 | 8 | 8 | | | 5 |
| | 4 | 4 | 8 | | | 8 |
| *Vipera russelli* (Russell's viper) | 1 | 20 | 8 | 18-20 | Intravenous in Tail | 0 |
| | 2 | 10 | 8 | | | 1 |
| | 3 | 5 | 8 | | | 6 |
| | 4 | 2.5 | 8 | | | 8 |
| *Bungares caeruleus Sindanus* (Krait) | 1 | 16 | 8 | 18-20 | Intravenous in Tail | 0 |
| | 2 | 8 | 8 | | | 3 |
| | 3 | 4 | 8 | | | 5 |
| | 4 | 2 | 8 | | | 8 |
| *Naja Naja* (Cobra) | 1 | 16 | 8 | 18-20 | Intravenous in Tail | 0 |
| | 2 | 8 | 8 | | | 3 |
| | 3 | 4 | 8 | | | 5 |
| | 4 | 2 | 8 | | | 8 |

**Table 2** LD50 values of different snake venom through intravenous mode of injection.

| Venom of the Snake | Dose in µg as dilution | | | | Weight of Mice (g) | Route of inoculation/ dose in ml | No. of Survival after 24Hrs. | | | | $LD_{50}$ µg/dose | $LD_{50}$ µg/g |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Echis carinatus* | 32 | 16 | 8 | 4 | 18 – 20 | Intravenous in Tail | 0 | 3 | 5 | 8 | 11.311 | 0.5655 |
| Russell viper | 20 | 10 | 5 | 2.5 | 18 – 20 | Intravenous in Tail | 0 | 1 | 6 | 8 | 6.643 | 0.3321 |
| Krait | 16 | 8 | 4 | 2 | 18 – 20 | Intravenous in Tail | 0 | 3 | 5 | 8 | 5.656 | 0.2828 |
| Cobra | 16 | 8 | 4 | 2 | 18 – 20 | Intravenous in Tail | 0 | 3 | 5 | 8 | 5.656 | 0.2828 |

**Table 2** shows that the approximate lethal dose ($LD_{50}$) of each species of the snakes in microgram per dose as well as in µg/g of body weight. The $LD_{50}$ of venom of *Echis carinatus* is 11.311 µg/dose(approx.0.5655 µg/g), Russell Viper is 6.643 µg/dose (approx.0.3321 µg/g), Krait is 5.656 µg/dose (approx.0.2828 µg/g), and Cobra 5.656 µg/dose (approx.0.2828 µg/g).

## Discussion

This study is carried out to estimate approximate lethal dose ($LD_{50}$) of the four snakes venom i,e., *Echis carinatus,* Russell Viper, Krait and Cobra which are 11.311 µg/dose (0.5655 µg/gm), 6.643 µg/dose (0.3321 µg/gm), 5.656 µg/dose (0.2828 µg/gm), 5.656 µg/dose (0.2828 µg/gm).

In this study $LD_{50}$ value obtained for venom of *Echis carinatus* was 0.5655 µg/g which shows that its lethality is lesser as compared to Cobra, Krait and Russell viper. Now specific studies in Pakistan has been carried out to standardize the values of $LD_{50}$ for Ekis Carinatus venom throughout the country however results obtained by international researchers depicts Different value of $LD_{50}$ for instant a study reported by Christensen in 1979 demonstrate the $LD_{50}$ of *Echis carinatus* 22 µg/18-20 g of mice or 1.2 µg/gm through intravenous route [13,14] while according to Australian online Biodata of Snake Venom, the $LD_{50}$ of *Echis carinatus* multisquamatus (found at Iran) is 3.26 µg/g [15]. It is strongly felt there is dire need to do more work in this field because most of $LD_{50}$ value varies from study to study.

In the case of Russell Viper, the $LD_{50}$ obtained through I.V injection is 0.3321 µg/g, previously no specific study had been carried out to evaluate the lethality of local species of viper snake in Pakistan. Kankokar and Rao et al. characterize the venom of Indian Krait on the basis of its lethality and composition and its $LD_{50}$ was demonstrated to be 0.31 µg/g in mice through I.V route [16]. In another study performed by Meier and Theakston revealed that the lethality of venom of Russell viper varies with change in route of injection as their results predicts the $LD_{50}$ of 0.4 µg/g through Intraparietal (I.P) route, 0.75 µg/subcutaneous (S.C) route and 0.3 µg/g through intravenous(I.V) route [7,14]. This fact of high lethality through I.V route could be attributed to the size of venom molecules in Russell viper, such as anti-coagulants, hemorrhagic compounds, edematous proteases and molecules with amidolytic and caseinolytic properties.

*Bungarus caeruleus* (Krait) is found in Peninsular India spreaded from Sindh (Pakistan), to the West Bengal plains [17]. In Pakistan, no specific work is done on local species of *Bungarus caeruleus* for the determination of $LD_{50}$. In our study the $LD_{50}$ value of its venom in mice model is 0.2828 µg/g which is contradictory to previous reports demonstrated Engelmann and Obst et al. their obtained $LD_{50}$ value is 0.169 µg/g through I.V mode of injection [18] Other findings obtained by Mirajkar and More et al. demonstrated that when crude venom administered intravenously cause lethality in mice with usual neurotoxic symptoms and resultant $LD_{50}$ value was 160 µg/kg or 0.16 µg/g [19]. According to geographical conditions and other factors, venom composition varies widely as demonstrated in values. In another study $LD_{50}$ of Krait species is reported to be 0.30 µg/g [14]. Further research and online data of $LD_{50}$ of Krait's venom obtained from Australian Venom and Toxin database depicts the $LD_{50}$ value 0.169 µg/g which is also inconsistent to our results [15]. *Bungarus candidus* venom show high lethal activity with an I.V. $LD_{50}$ of 0.2828 µg/g while previous values collected from Sean Thomas biodata depicts the value as 0.169 µg/g. Although the value is inacceptable range in comparison to our results, the small difference is due to geographical variation and other factors. Another study carried out at Malaysia for the determination of $LD_{50}$ of crude venom of *Bungarus candidus* depicts the variation in the lethality of venom with an I.V. $LD_{50}$ of 0.11 µg/g [20].

Interestingly, the venom of *Naja naja* (cobra snake) is far more poisonous than viper venoms, in agreement with previously described values in literature. [10]. These results **(Table 2)** indicate small molecular weight of cobra venom (with molecular weights < 15 kDa). The venom toxicity of the potent *Naja naja* is resultant of low molecular weight (usually <30 kDa) toxins, such small molecules have a shorter residence time at the site of inoculation and quick diffusion causing an instant bioavailability in the blood.[21] Riaz and Zaman et al. also determined Lethal dose 50 ($LD_{50}$) of *Naja naja* in Pakistan by Reed and Munch method through intramuscular route (I.M) and the value obtained were 1.2 mg/kg or 1.2 µg/g which shows that lethality of snake venom is decreased through I.M route [22]. It is demonstrated that the route of injection of venom could affect the $LD_{50}$ values. Another study reveal this impact in which the $LD_{50}$ value of *Naja naja* venom has increased about four times when mode of injection was changed from I.V to I.M [10].

Our results of $LD_{50}$values for Pakistani *Naja naja* are in concordance with the recently reported study carried out at University of Malaya by Wong, Tan et al. They determined the subcutaneous and intravenous LD50 values for Pakistani *Naja naja* in mice model. The $LD_{50}$value obtained through intravenous mode of injection was 0.22 µg/g which also favors our results. [23] Diverse immunological properties of cobras from different geographical regions have fascinated researchers specially protein chemists to work for developing new antidotes.

All these findings show that variation in lethality and composition of snake venom is a ubiquitous phenomenon both interspecific and intraspecific. Venom variation can lead to severe consequences for snakebite victims by rendering the particular antibodies present in antivenom unproductive against heterologous toxins in venoms. [24].

The $LD_{50}$ reflects the venom of the snakes which are found in Pakistan is of high quality and very much potent in terms of its toxicity and lethality [25] This high-quality venom [26,27] provokes the production of highest quality of anti-snake venom serum.

## Conclusion

In the present work, we determined experimentally the $LD_{50}$ values of reference snake venoms in mice, and evaluated the venom potency with relevant to previous literature. [28-30] The

$LD_{50}$ values obtained from this study can effectively be utilized to develop potent anti-venom serum in Pakistan. The determination of $LD_{50}$ of each venom is important not only to produce potent antivenom serum but also for determination of neutralization capacity of each produced lot of antivenom serum before release for consumption. The results show that most of the venom obtained from same species with geographical barrier has different $LD_{50}$ values depicting the effect of geographical regions, route of injection and several other factors so it is the need of time for in-depth research in this area, to develop antivenom serum for specific species based upon their exact lethality. In addition, the $LD_{50}$ value of these assays must be noticed with caution since these are obtained using a mouse model.

## Future Perspectives

The results of current study could persuade scientific community to explore further in-depth knowledge to identify the myotonic components and characterization of venom of deadliest snakes found in Pakistan, which may be of huge biodiscovery potential for the development of antivenom. Moreover, these findings indicate that there is dire need for effective antivenom in Pakistan based upon the lethality of local species. Sera Processing Lab. NIH, Pakistan is capable enough to produce polyvalent antivenom against the venom of local snakes and will fulfill the country demand of antivenom in near future by the expansion of its manufacturing capacity.

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