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Forensic Analysis of Snakebite Venom and its Effects on the Living Organism

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ABSTRACT

Snakebite has been a health problem throughout the world including the sub-continent where it has caused hundreds of thousands of causalities in the last half-century and so on. The four species of snake i.e., *Elapidae, Viperidae, Colubridae*, and *hydrophobia* are further divided into their sub-families which are highly venomous. First of all, blood samples of affected people were collected, and serum was separated which was then analyzed using UV-Vis. spectroscopy and HPLC. The RBCs, WBCs, and HGB, of the blood, were recorded to investigate the effect of venom on the patient. All the patients showed a decrease in RBCs, WBCs, clotting of HGB, and the deficiency of all the blood components examined. Heartbeat increased, but in rare cases decreased. The venoms of different snakes were analyzed by using RP-HPLC and a densitometer. The data was collected w.r.t time, age, gender, occupation, season, and body parts and analyzed. The serum of different venomous is analyzed. So, it was concluded the cases of snakebite patients in Pakistan are low, but the mortality rate is high due unavailability of the vaccine.

Keywords: Snake Venom; Naja *Kaouthia; Elapidae; Viperidae; Colubridae*; Russel's Viper; Peptide Molecules

Abbreviations: HGB: Hemoglobin; WBS: White Blood Cells; RBC: Blood Cells; SDS: Sodium Dodecyl Sulfate; PAG: Polyacrylamide Gel

Introduction

In tropical and sub-tropical areas, snakebite is a major health problem in the whole world. It is estimated that more than 6 million people are affected by snakes in tropical areas of the world [1]. The poisoned snake is too dangerous. According to the survey, approximately 2.11×10^5 deaths occur among 3.1×10^6 by poisonous snake bites [2,3]. The rural areas are much more affected by snake bites whereas the city areas are less affected. Although the effects of snake bites are much greater, the whole estimation is done generally. The labor causes snake bites in the marshy fields while working on rice crops [4,5]. Snakes bite people with their vicious venom which is produced naturally. The venom is produced in *Elapidae* and some groups of the *Colubridae* snake in venom glands called salivary glands [6,7]. During the biting or spitting the snake sprays his venom on the pray with the help of tabular fangs. The venom constitution consists of toxic and nontoxic enzymes. The molecular enzyme components are found between 1.3×10^4 to 1.5×10^4 Da and the polypeptides like mycotoxin, neurotoxin, platelet inhibitors (disintegrins), and cytotoxin (cardiotoxin) are found in the presynaptic and postsynaptic form. As well as these are called potassium binder channels.

The molecular weight of these polypeptides lies in the range of 5.1×10^4 to 1.1×10^5 Da. The components of venom such as organic ac-

ids, carbohydrates, lipids, nucleotides citrate salts, and carbohydrates contain molecular weight below 1.5 kDa [8-10]. From experiments, it was observed venom with smaller molecular weight constituents was less active. If we want to count the total number of humans affected by snake venom it would be hard to estimate. But people thousands are bitten by a snake and the majority die due to the unavailability of the anti-venomous vaccine [11,12]. The death ratio of patients by venoms can be decreased by applying some preventive measures. Through awareness to people, on-door delivery of anti-venomous vaccines, and education of the people, the death rate can be decreased [13,14]. Snake venom is the oldest known poison for human beings and many myths are related to it. Some herbs and many folks are associated with them, especially in Hinduism. Many scientists have studied peptide toxins and protein venom. The major issue in the synthesis of the anti-venom vaccine is that the mixture is very complicated, and drugs are required with surprising activity [14-17].

Material and Methods

Sample Collections

The samples of different patients affected by snake biting were collected from different hospitals like Mayo Hospitals, Sheikh Zaid Hospital, and the Services Hospitals Lahore. Samples were centrifuged and analyzed. The snake venom was separated from the blood. By using a densitometer and Gel electrophoresis the blood sample was analyzed. From Table 1, it can be understood that people ages 21-40 are more affected whereas children are less because they visit land and agriculture form, so they are affected Table 2 shows if we study gender-wise then males are more affected than females due to their routine work. If we study the aspect of occupation, then people who belong to agriculture occupation are more affected as compared to any other as shown in Table 3. Data related to the time of the incident reveals interesting facts. Table 4 indicates that more than half of cases occur between 1 pm to 5 pm, as compared to the morning. The data clearly shows snake bitness is due to the invasion of humans into their habitat means snake bitness is defensive, not offensive. The data in Table 5 shows that snake bites are enormous in the rainy season as compared to others. The reason behind that is during the rainy season the snakes come out of their habitats because water enters their habitat due to shallowness.

Table 1: Percentage of patients with snake bit relative to the age groups.

Serial Number	Age of Patients (years)	Number of Patients	Percentage (%)
1	5-12	2	4
2	12-22	12	23
3	22-32	25	46
4	32-42	19	39
5	42-52	21	40
6	52-72	7	11

Table 2: Gender-specific number of patients.

Gender	No. of Patients	Percentage (%)
Male	62	71
Female	38	29

Table 3: Occupation of patients.

Field of Work	Number of Patients	Percentage (%)
Agriculture	64	90
Housewives	4	7
Students	1	2
Unemployed/Visitors	3	6

Table 4: Data related to the incident of time of snake bite.

Time of Accident	Number of Patients	Percentage (%)
7:00 am –10:00 am	9	23
10:00 am –2:00 pm	22	41
2:00 pm –6:00 pm	34	52
6:00 pm – 11:00 pm	7	18
11:00 pm – 7:00 am	2	6

Table 5: Season of snakebite and affected people.

Biting Season	Months	Number of Patients	Percentage (%)
Winter	Start of Oct – End of Feb	3	7
Autumn	End of Feb – Mid of March	9	23
Spring	End of Mar – Mid of Apr	28	39
Summer	May – End of Sep	72	89

Table 6 shows about ninety percent of snake bite cases are on hands, feet, or legs because they are easily approachable and visible. Table 7 represents snake bite serum. The normal values of different analyses and values with serum are shown. There is a major difference observed between normal values and observed values. The deficiency of hemoglobin (HGB), white blood cells (WBS), and red blood cells (RBC) was observed in snake venomously affected patients. It causes a cardiac attack and damage to the kidney's slow nervous system. The venom of the snake belonging to the *Elapidae* and Cobra family is neurotoxic and that of the *Viperidae* family is cytotoxic and myotoxic [18,19].

Table 6: Percentage of snake bites concerning the part of the body affected.

Site of bite	No. of Patients	Percentage
Shoulder	1	2
Hands	43	79
Feet	11	23
Belly	1	2
Forearms	5	11

Legs	39	58
Ears	3	7
Scrotum	2	5

Table 7: Complete blood count of the snake-bitted patient.

SAMPLE	OBSERVED	NORMAL
WBC	590	10^3 /UL
RBC	4300	10^6 /UL
HGB	11.4	21 g/dl
MCV	37.6	91Fl
PLT	562	10^3 /UL
LY	4.2	46.4 %
MO	0.5	5.5 %
GR	4.3	48.1 %

Sample Preparation Methodology for UV-Visible Analysis

After collection of the sample, centrifuged for 4-7 minutes at 3500 RPS. The serum was separated into vials carefully and analysis was performed through UV-Vis. spectrophotometer immediately. With the help of the SDS-PAGE method, proteins were separated. For protein estimation, a serum named Bovine Albumin was used.

For Crude Venoms Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate (SDS) an anionic detergent was implied for the negative charge on proteins. By increasing the concentration of SDS more binding happened with proteins, so large molecules were restrained from migrating fast compared to small molecules having acrylamide concentration in gel above critical density. All the samples treated against lamellar sample buffer contain the following characteristics, 6.8pH, 20% glycerol, 62.50 mM, Tris-HCl, sodium dodecyl sulfate SDS 2%, bromophenol blue 0.01% while the temperature was 10°C. By using a 5-min micropipette, 30 µL of each poured sample that was toxic into the well which consisted of polyacrylamide gel (PAG) of about 12%. At the temperature of (25°C), an electrophoresis process was performed. at the SALMONE pharmaceutical industry, Manga Mandi Lahore. By using Coomassie Brilliant Blue R-250 gel was stained 40mA and then scanned with a densitometer named GS-710. The molecular weight of proteins was calculated by using precise proteins. A mixture of 9 recombinant proteins was collected from the Salmone Pharmaceutical industry, 36 km, Multan Road, near Manga Mandi, and these proteins were through the two outer wells that contain similar gel. These proteins are 250, 200, 150, 100, 75, 50, 37, 25, 15, and 10 kDa.

Reverse Phase High Performances Liquid Chromatography (RP-HPLC) of Venoms Protein

Depending on hydrophobicity, the protein molecules were separated by using reversed-phased chromatography. The hydrophobic groups like hydrocarbon chains ranging from C-4 to C-8 were immobilized in the RP-HPLC stationary phase. Trifluoroacetic acid was in C-18 columns for reversed-phase chromatography for peptide molecules. Trifluoroacetic acid and peptide molecules provide adequate hydrophobic behavior that is retained through the stationary phase. The chromatographic column is developed in such a way by acetonitrile nitrile as the solvent that nonpolar components will be eluted last while polar components will elute early. In the first solution, dissolved the venom of the crude snake was in 0.08%, trifluoroacetic acid and then quickly injected into chromatography's reverse phase C-18 with10µm, ST 3.9/260 mm column equilibrated with 0.1% tetra fluoro acetic acid. By increasing concentration (80% ACN in 0.1% trifluoroacetic acid) all proteins were eluted in solution 2 at a flow rate of 2mL/min. the AKTA explore system was used for the elution monitoring range is 215-280 nm.

Sodium Dodecyl Sulfate Page

Venom SDP-Page profiles are shown in the figure. The dissolved venom is in a homogenous mixture of SDS-PAGE while the conditions are nonreducing. The molecular weight of all four venom samples is different ranging from low to medium, and high. Revealed proteins in Coomassie blue-stained gel, those proteins whose molecular weight is in the range of 10^{-20} kDa. The protein contains two *Viperidae* snakes (*C. rhodostoma* and *T. albolabris*) and their molecular weight are high and low respectively. On the other hand, the low molecular weight was observed in proteins that contain *Elapidae* snakes (*O. Hannah* and *N. kaouthia*).

Reverse Phase- High-Performance Liquid Chromatography Profiles (RP-HPLC)

All four samples of venom were analyzed by using the PR-HPLC system. A small amount of unbounded venom was detected in samples. The unbounded proteins were eluted in solutions ranging from 80% to 25 % concentration [20]. The sodium dodecyl sulfate-polyacrylamide gel having venom was scanned through a GS-710 densitometer. The molecular weight marks were taken by lane of MW markers having samples in the sequence (W) *T. albolabris* venom, while (X) *C. rhodostoma* venom, (Y) *N. kaouthia* venom, and (Z) *O. Hannah* venom respectively (Figures 1-4).

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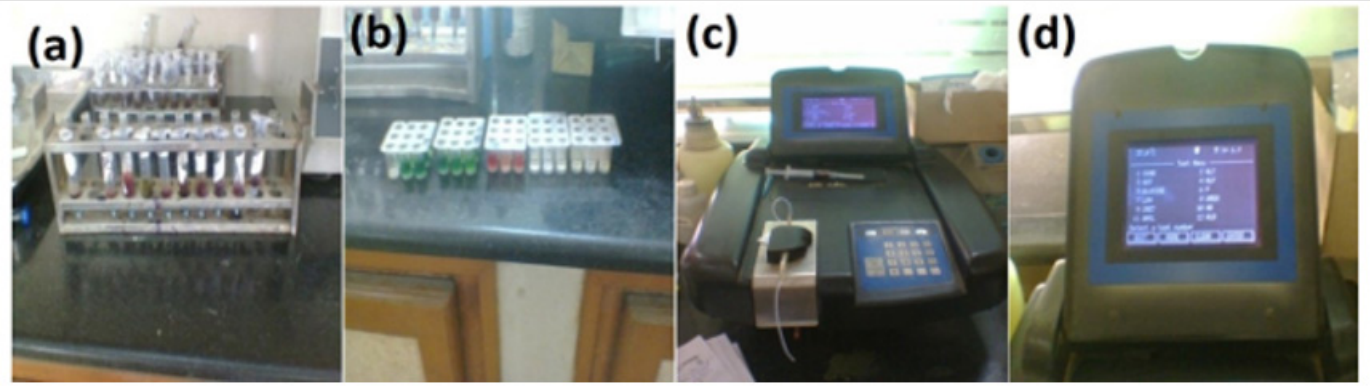

Figure 1:
a. Blood samples of snake bite patients.
b. Serum samples after centrifugation and prepared samples for analysis.
c. Samples injected in UV-Vis Spectrophotometer.
d. Analysis displayed on the screen of a spectrophotometer.

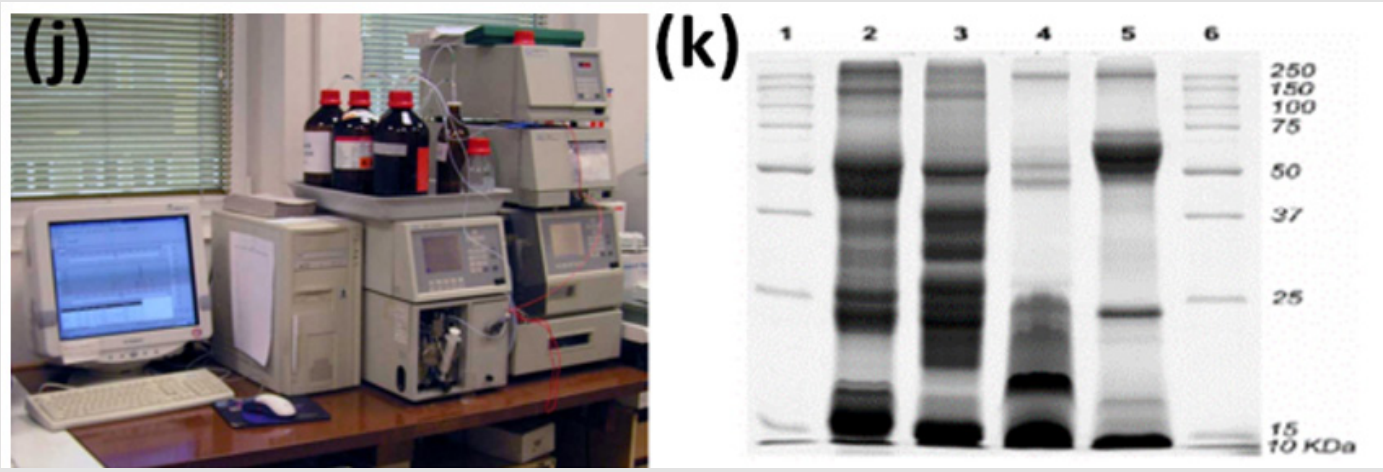

Figure 2: SDS–PAGE profile of snake venom, HPLC that was used for the analysis of the analysis of SDS PAG was performed through polyacrylamide gel (PAG) in non-reducing conditions.

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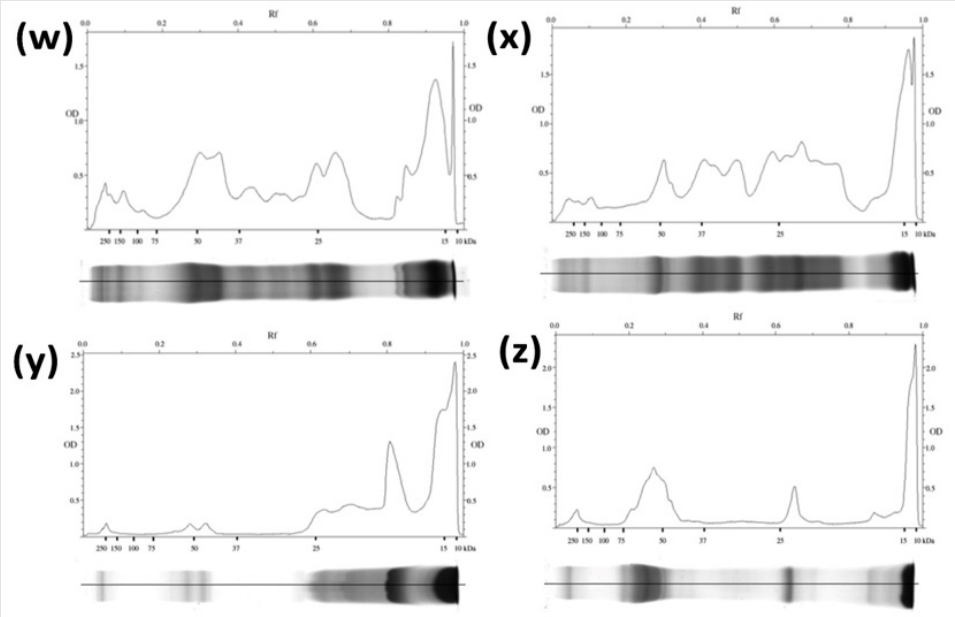

Figure 3: Represents densitometer (w, x, y, and z) tracing curves measured by Quantity One software of (SDS-PAGE).

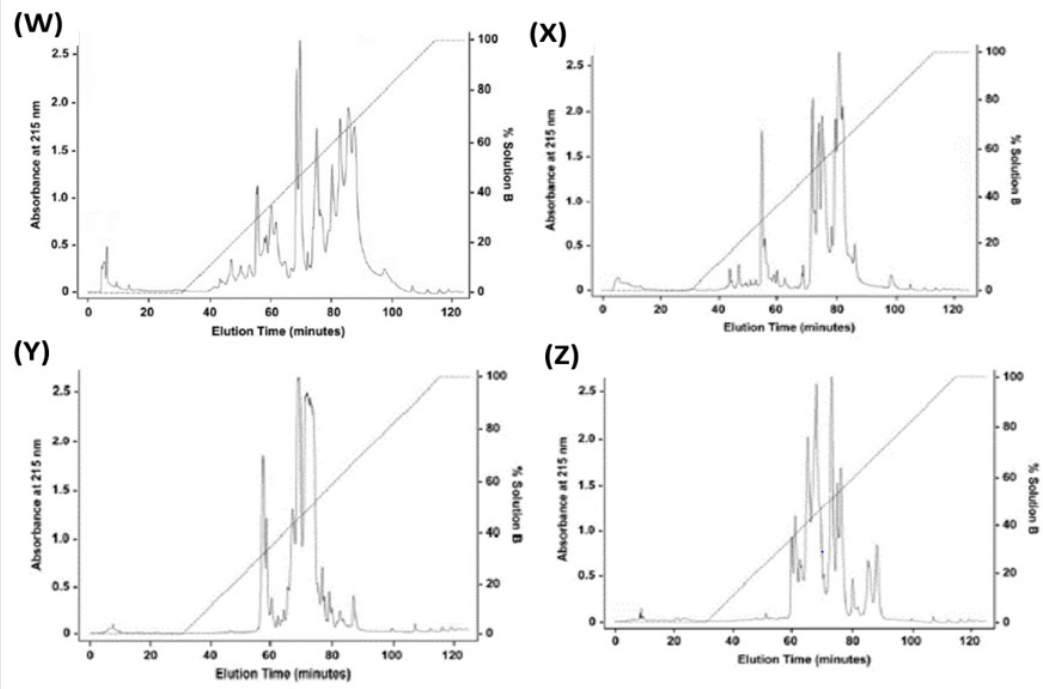

Figure 4: HPLC instrument, (W, X) RP-HPLC profiles of venom of 4 most common venomous snakes of PAKISTAN on C-18, 10µm ST 4.6/250-millimeter column (W) *T. albolabris* venom and (X) *C.rhodostoma* venom. The (Y, Z) RP-HPLC profiles of venom of 4 most common venomous snakes of PAKISTAN on C-18, 10µm ST 4.6/250-millimeter column.

Results and Discussion

It was found that snake venom severity is influenced by various factors like season, diet, climate altitude, and age of the snake. The biological activity of snakes is affected by processing methods such as storage, sample collection, and drying. The venom of *Naja kaouthia* and *Ophiphagus Hannah* was more toxic than *Viperidae* species like C and T [21] albolabris. The snakes of the same species are less toxic than in other countries, but the death rate is high due to the unavailability of anti-venom vaccines. The results that were obtained from experiments give us information about further research. The venom of the elapid snake accumulated shows some major bands in the range of 200-250 kDa. Whereas a wide range of protein bands mainly from 15 to 200 is observed in vampire snakes' venom. The venom proteins of *Elapidae* snake having molecular less than 15 kDa show high density [22]. The bands that show very high density represent high neurotoxin. These neurotoxins are a sign of the high toxicity of cobra venom. According to SDS-PAGE analysis, the venom of the *Viperidae* snake contains small components of normal size proteins. Mostly elution of about 50-80% of *Viperidae* snake venom components takes place in solution 2. On the other hand, 30 to 60% elution of Elapsidae snake venom takes place in solution 2. So, this varying range of elution shows the hydrophobic nature of the venom of *Viperidae* snake is more as compared to Elapsodae snake venom.

Conclusion

Venom is a complex mixture of proteins, peptides, and small molecules secreted by an animal to cause harm to another. It is mainly used in predation or self-defense. Among the components, peptides, in particular, have been studied extensively after the discovery of their diverse pharmacological properties. Moreover, there are a large number of bioactive venom peptides that have been isolated, and these represent a huge and undiscovered source of new therapeutic leads. One of the reasons may be because of the problems of low bioavailability and stability associated with peptides. The development of peptidomimetics, using organic compounds with a lower molecular weight or shorter peptides to mimic the action of peptides, has become an approach to overcome these problems. Tirofiban and eptifibatide are typical examples of using peptidomimetics in drug development. The snake toxins exhibit various therapeutic activities *in vitro* or *in vivo* models as well as in clinical studies. As there is an argument about the cytotoxic effect of toxins, some new technologies have been used to modify them into detoxified versions. Besides, the conjugation of toxins with polymeric materials, such as liposomes, microspheres, hydrogels, and nanoparticles for targeted drug delivery usually reduces toxicity, protects from protease degradation, and enhances therapeutic activities. This specific delivery system can deliver the toxins directly to their primary site of action and reduce the side effects encountered in systemic administration. The serum of different samples was analyzed, and it determined that cases of snakebite patients in Pakistan are low, but the mortality rate is high due inaccessibility of the vaccine.

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