ABSTRACT

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Snake envenomation has been recognized as a neglected tropical disease by the World Health Organization. Every year millions of snakebite cases around the world result in many cases of mortality and morbidity. India records the highest cases of snake envenomation annually; however, magnitude of the snake envenomation issue is far greater than documented in the published material. The Indian subcontinent is inhabited by more than 52 species of venomous snakes. Among them, the Indian cobra (*Naja naja*), Indian common krait (*Bungarus caeruleus*), Indian Russell's viper (*Daboia russelii russelii*), and Indian Saw-scaled viper (*Echis carinatus*), commonly known as "Big Four" venomous snakes of India, account for the majority of snakebite deaths and morbidity.

Across the globe, scorpion stings result in thousands of deaths yearly; therefore, scorpion sting envenomation is considered a neglected public health concern in tropical and sub-tropical countries. Among the scorpion species found throughout India, only the Indian red scorpion (*Mesobuthus tamulus*) belonging to the family Buthidae, and the Indian black scorpion (*Heterometrus swammerdami*) belonging to the Scorpionidae family pose a significant threat to humans; however, limited clinical reports suggest that *Mesobuthus tamulus* venom (MTV) is more toxic compared to any venomous scorpion in this subcontinent.

Management of snake envenomation is imperative to curb the deadly effects of snake venom on the bite victim. There are no detection tests or kits available for detection of snake venom in India and generally, the bitten snake species are identified through the description or photograph of the snake provided by the patient and/or their family or friends or witnesses, an examination of the bite site, local symptoms of envenomation, biochemical analysis of urine, and 20-min whole blood clotting test (20WBCT). However, there are cases where no good evidence exists to identify the culprit snake and sometimes healthcare providers struggle to identify snakes, leading to cases of misidentification. In case of scorpion envenomation diagnosis also there are no specific tests or methods available. Clinical practitioners and physicians examine the possible ramifications associated with scorpion envenomation such as, evaluating the potential for renal failure by measuring creatinine levels, assessing pancreatic enzyme levels in cases of scorpion sting-induced pancreatitis and ordering an electrocardiogram to detect electrocardiographic abnormalities.

Snakebite from a venomous snake may be either a 'Wet Bite' with mild local symptoms to severe systemic toxicity and ultimately death, or they may be a 'Dry Bite' without local or systemic signs of envenomation due to little or no venom injection. Antivenom administration, the only therapy for snakebite envenomation has several adverse and costly effects. Hence, using a method or kit to precisely determine whether a particular snakebite case is classified as a 'wet bite' or a 'dry bite' will significantly deter hospital authorities or physicians from delivering antivenom in every instance of snakebite. Similarly, in some parts of the world, efforts have been made to detect the venom of several scorpion species. However, no studies have been documented to create a method for detecting and quantifying MTV in India.

For easy understanding, this thesis is structured into following six chapters-

Chapter I: This chapter provides an introduction to the global burden of snakebite including the Indian scenario, and global burden of scorpion envenomation including the Indian scenario. This chapter discusses the classical or contemporary methods for snake and scorpion venom detection, and the key issues related to these methods of detection. This chapter also briefly discusses the proteome composition of the Indian snake venoms and the Indian red scorpion venom. This chapter further illustrates the use of peptide as antigens to raise antibodies for detection, gold nanoparticles as colorimetric sensor and colorimetric assays. The aims and objectives of the study are described in this chapter.

Chapter II: This chapter reviews the published literature on modern analytical tools and techniques for rapid detection of snake envenomation including the only currently available diagnostic kit for clinical diagnosis of snake envenomation in Australia. This chapter also reviews the immunodiagnostic tests for the detection of scorpion venom.

Chapter III: This chapter enlists the chemical and consumables used in the study and the methods and protocols employed for performing various experiments.

Chapter IV and V: These chapters include results and discussions, and the content of each chapter is briefly discussed below:

Chapter IV: This chapter describes the custom peptides (CPs) designed from major toxins of 'Big Four' venomous snakes. Further, this chapter depicts the immunoassays performed to study immune-recognition of the antibodies raised against the CPs and commercial anti-snake polyvalent antivenom towards Indian snake venoms under *in vitro* and *in vivo*

conditions. This chapter also illustrates the characterization of gold nanoparticles (AuNPs) synthesized and the AuNP-antibody conjugates obtained from conjugation of the antibodies raised against the CPs to the AuNPs, and their use for detection of Indian snake venoms in envenomed Wistar strain rats (*in vivo*) using digital image colorimetry.

Chapter V: This chapter describes the CPs designed from major toxins of MTV. This chapter depicts the immunoassays performed to study immune-recognition of the antibodies raised against the CPs and commercial anti-scorpion antivenom towards MTV under *in vitro* and *in vivo* conditions. This chapter also illustrates the use of acetonitrile precipitation method to augment the MTV detection sensitivity enriched the low molecular mass peptide toxins in envenomed rat plasma. This chapter also demonstrates the biophysical characterization of the synthesized AuNPs and the AuNP-antibody conjugates from the conjugation of the antibodies raised against the CPs with AuNPs. This chapter further describes the detection and quantitation of MTV in envenomed plasma by AuNP-antibody conjugates.

Chapter VI: This chapter presents the conclusion of this study and visualizes the prospects of the study's findings.

In the present study a method has been described for the diagnosis of medically significant ‘Big Four’ Indian snake venoms (*Naja naja, Bungarus caeruleus, Daboia russelii, Echis carinatus*) and *Naja kaouthia* venoms in the plasma of experimentally envenomed animals (envenomed under laboratory conditions). Rabbit polyclonal antibodies (PAbs) were raised against the five custom peptides designed using the antigenic sites of the main toxins found in the proteome of India's 'Big Four' venomous snakes identified by computational analysis. The individual PAbs and PAb formulations obtained by combining the PAbs in different combinations were studied for their immune-recognition of the ‘Big Four’ venomous snakes and *Naja kaouthia* venoms and it was observed that the PAb formulation (FPAb) prepared by mixing the five representative PAbs in the ratio of 1:1:1:1:1 (w/w/w/w/w) demonstrated synergistic immune recognition of the ‘Big Four’ snakes and *Naja kaouthia* venoms. The recognition for these venoms under *in vitro* and *in vivo* conditions by FPAb was significantly higher than the recognition by commercial polyvalent antivenom produced against native venom toxins. The FPAb demonstrated detection of the venoms in subcutaneously envenomed rat plasmas until 240 minutes post-injection. UV-Visible (UV-Vis) spectroscopy, Fourier-transform infrared

(FTIR) spectroscopy, zeta potential, transmission electron microscopy (TEM), and atomic force microscopy (AFM) characterised AuNP conjugated with FPAb. The FPAb-conjugated AuNP demonstrated aggregation upon interaction with venom toxins, changing the colour from red through burgundy to blue, monitored using a smartphone. From the digital image colorimetry analysis of the images, calibration curves for venoms were obtained, and each venom in the envenomed plasma at different time intervals was quantified using these curves.

This study further describes another novel, simple, and rapid method for detecting MTV in the plasma of envenomed animals using PAbs raised against four modified custom peptides representing the antigenic epitopes of K^+ (Tamapin) and Na^+ (α-neurotoxin) channel toxins, the two major MTV toxins identified by proteomic analysis. From the immune-recognition assays performed using individual PAbs, PAb formulations obtained by combining the PAbs in different combinations and ratios, and commercial anti-scorpion antivenom (developed against native toxins), it was observed that the PAb formulation (PAbF) containing PAb 1, 2, and 3 in proportion (1:1:1, w/w/w) acted synergistically, demonstrating significantly higher immunological recognition of MTV. The PAbF could detect MTV optimally in envenomed rat plasma (intravenous and subcutaneous routes) at 60 min post-injection. The acetonitrile precipitation method developed in this study to augment the MTV detection sensitivity enriched the low molecular mass peptide toxins in envenomed rat plasma, which was ascertained by mass spectrometry analysis. The AuNPs conjugated PAbF characterized by biophysical techniques such as UV-Vis spectroscopy, FTIR, zeta potential, TEM and AFM, demonstrated their interaction with low molecular mass MTV peptide toxins in envenomed rat plasma. This interaction results in the accumulation of the AuNPs, thus leading to signal change in absorbance spectra that can be discerned within 10 min. From a standard curve of MTV spiked plasma, the quantity of MTV in envenomed rat plasma could be determined by AuNP-PAbF conjugate.