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Next Generation Antivenoms and Their Neutralizing Efficacy against Snakebite Envenoming

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Abstract:**Snake envenomation is a major neglected tropical disease and remains a serious threat in many countries at the present day. The impoverished rural populations are vulnerable to snakebite envenoming which eventually strengthens the cycle of poverty. The highly diversified components of snake venoms are primarily responsible for severe clinical manifestations in the victim. The only available treatment is the use of animal-derived broad-spectrum polyvalent antivenoms having a very low level of case-specific antibodies. A significant number of drawbacks of this antivenom are possessing a key challenge in snakebite treatments. The recent advancements into the toxin-specific monoclonal antibodies proved to be promising for future envenoming treatments, although its use is still in a naïve stage since it also holds some limitations in neutralizing the complex snake venoms. In the emerging fields of molecular biology along with transcriptomic and proteomic analysis protocols, the snake venom constituents have been well characterized and this holds another promising approach to using DNA immunization for next-generation therapeutics. This strategy is more compatible with the human immune system and exerts the least adverse effects. In this review, the comparative analysis of present polyvalent antivenom and future therapeutic protocols has been discussed.**

Index Terms:**Antivenom, Envenomation, Recombinant, Therapeutics, Toxicity.**

I. INTRODUCTION

Envenomation caused by snakes is one of the major problems in tropical countries and is considered a 'neglected tropical disease'. An estimated extent of snakebites occurring per year in the world is within the range of 1.8 to 2.7 million. India ranks first in the number of deaths occurring due to snakebites, with an average of 50,000 per year ((Chippaux, 2017; Mohapatra et al., 2011). The increasing human-snake conflict in tropical countries and lack of efficiency in the proper diagnosis of snakebite is causing an elevation in the number of victims globally.The detrimental consequences of such cases include economic degradation in the families of snakebite victims, mental health implications in the survivors with an increased risk of depressive and post-traumatic stress disorders (Williams et al., 2019). Lack of proper knowledge about snakes and snakebite imparts one of the major inhibitors of improving snakebite victims.Neglecting or postponement of the initiation of treatment can lead to several health issues such as tissue necrosis, amputation, and compartmental syndromedepending on the type of snake and its venom composition. Thus, snakebite is not only a public health issue but also one of the major reasons for socioeconomic degradation. The significant numbers of mortality along with other public health concerns are due to the action of snake venoms which are cocktails of toxic and non-toxic constituents. The toxic components contain both enzymatic and non-enzymatic molecules affecting different organ systems of the victim leading to its death (Alangode et al., 2020).

Upon incorporation of snakebite as a 'neglected tropical disease' by WHO, it has been a focus to look at with strategic improvements in its defensefrom both educational and medical facilities. Currently, 22 countries from the 5 continents are involved in the resolution of fighting against this so-called 'disease of the poor', and it has been taken into action at the 71st World Health Assembly.Data from a global survey from 2020 showed the presence of 22 antivenom manufacturers for 65 distinct products, but the availability and gross yearly production are unclear (Potet et al., 2021). The annual bite cases in India alone are quite high, and when this number is combined with Sub-Saharan countries the supply of antivenom vials is insignificant rendering high mortality in the rural areas. It was earlier reported that the antivenom vials intended to be used in India in a year can reach up to 2 million (Whitaker andWhitaker, 2012). But it is still not determined how many vials are effective to neutralize the actions of snake venom in Indiadue to variations in efficacy of the polyvalent antivenom currently in use. These represent a serious need for producing more antivenom vials annually or changing the approach to antivenom production.

The conventional medical treatment to neutralize snakebite toxicity is the use of heterologous antivenoms, a century-old nonspecifictreatment protocol that is still giving lives to thousands of snakebite victims. This type of antivenom is obtained from horse immunization with sub-lethal doses of respective snake venoms (Laustsen et al., 2018). The conventional heterologous antivenom is the only effective procedure implemented against snakebite envenomation, but it contains several unwanted health problems due to the toxins that may vary between taxa, geographic range, age, and sex of the snakes. One of the major concerns in the neutralization of the toxic effects is the batch variation of snake venoms. Apart from this, the negative reactions elicited by the polyvalent antivenom such as pyrogenic reactions, serum sickness, allergic reactions, etc. are imposing the bigger limitations in its use and induces the urge to innovate and implement new and effective antivenom strategies.

To reduce or eliminate the drawbacks caused by the current antivenom, there is an urgent requirement to innovate another therapeutic approach for the effective treatment of snakebite envenomation. Different alternative strategies have been proposed including the use of monoclonal antibodies, DNA immunization, synthetic epitope strings, etc.Introduction of monoclonal antibodies against snake venom toxins may be of great advantage in the future by using antibody library based on phage display technique as it can overcome the existing limitations of polyvalent antivenom. But low half-life stability, high production cost, and low immunogenicity of monoclonal antivenom are the hindrances in pushing it into clinical trials. Although broadly neutralizing monoclonal antibodies can be an answer to this (Fernandes et al., 2010).

Another recent advancement in the field of antivenoms is the use of recombinant antibodies and antibody fragments specific to the toxic components within a venom (Laustsen et al., 2018). These proposed antivenom strategies are well-investigated and do not exert any undesirable reaction in the bodyof model organisms such as mice. The recombinant antibodies of human origin are compatible with the human immune system and thus represent better safety profiles. Broadly neutralizing monoclonal antibodies only target the therapeutically active toxins not only of snake venoms, but also of the scorpion, spider, and bee venoms. But the use of monoclonal antivenom possesses the limitation of not being cost-effective. Moreover, the selection of specific toxins and the production of antibodies against them require extensive steps, and more investigations are required to identify different isoforms of very complex snake venoms (Ahmadi et al., 2020). There are certain proposed low molecular weight formats of recombinant antivenoms such as single-chain variable fragments (scFvs), Fab, diabodies, bivalent formulations, etc. that have lower chances to induce adverse immune reactions in the body. Pharmacokinetic considerations are also needed to be balanced to implement such therapeutic approaches (Laustsen et al., 2018).

Recently with the emergence of advanced biotechnological protocols, the study of DNA immunization with transcriptomic analysis has also expanded the horizons in the field of snakebite envenomation. In this elegant and robust process, specific DNA coding sequences of toxic antigens is directly injected into the cells andexpressed in an immunized animal. This technique is comparatively much easier to develop specific antibodies without the involvement of recombinant technologies and purification of proteins from heterologous organisms such as *Escherichia coli* (Suntravat et al., 2013). This approach can be beneficial to the proposed antivenom strategies for its cost-effective production and least harmful effects on the victim. Not only the aforementioned advantages are there, but the DNA immunization can be highly specific for other venomous animals also (Liu et al., 2021).

In this present review, the neutralizing efficacy of the present Indian polyvalent antivenom is compared to the recombinant antivenoms with a directional view on the DNA immunization against the snakebite envenomation.

II. DIVERSITY OF SNAKE VENOM COMPONENTS

Treatment of snakebites is highly difficult due to the intense variability of snake venoms which is again dependent on the taxonomic and geographical diversity of venomous snakes worldwide (Shashidharamurthy and Kemparaju, 2007). The venomous species along with their subspecies add further diversity in the composition of venoms due to the differences at the genetic level. There are many more unexplored species than the world currently deals with, and it is again another cause of additional venom diversity in the snakes. Even after such disparity, the venoms contain many similarities too. They all are complex mixtures consisting of different hydrolytic enzymes, non-enzymatic proteins, and several other peptide components (Williams et al., 2019; Gutierrez et al., 2017). The enzymatic components exert principal toxic effects in the snakebite victims through several diverse mechanisms which are listed in the table-1. Although enzymatic components are the main players in toxicological aspects of snakebite envenomation, numerous non-enzymatic constituents may have similar pathophysiological consequences. Not all of them are toxic to the body, and therefore need not to be neutralized. Some of the main non-enzymatic components are listed here in the table-2.

Table I. Enzymatic components of snake venom

Phospholipase A_2 (PLA_2)	Group I PLA_2	Present in the venoms of elapid and colubrid snakes. They are basically β-neurotoxins affecting voltage-gated ion channels to block pre-synaptic transmission of nerve impulses and sometimes can cause permanent neurotoxicity by hydrolysing phospholipids at the nerve terminals (Williams et al., 2018).
	Group II PLA_2	Present in the members of Viperidae, primarily acts as cytotoxic components causing myonecrosis (Dixon and Harris, 1996).
Snake Venom Metalloproteases (SVMPs)	P-I/ Group I	Present in all groups and comprise just a metalloprotease domain. They hydrolyse the components of the endothelial cell basement around blood capillaries (Mackessy, 2010).
	P-II/ Group II	Contain an additional disintegrin domain. Function in the integrin shedding and have myotoxic effects (Siigur and Siigur, 1991).

	P-III/ Group III	Cysteine-rich domains and disintegrin domains are present. Inhibit collagen-induced platelet aggregation and induce inflammation (Chatrath et al., 2011).
	P-IV/Group IV	Two C-type lectin-like domains along with Cysteine-rich and disintegrin domains. They activate platelets by activating tyrosine kinase-dependent receptors (Ferreira et al., 2018).
Snake Venom Serine Proteases (SVSPs)		They degrade blood components proteolytically and thus affect the haemostasis of the body (Serrano and Maroun, 2005). The major effect of these components is cleaving fibrinogen to promote coagulation; but can act as anticoagulants in some cases (Sanchez et al., 2000). They are also found to dilate the blood vessels, activate prothrombin, and alter blood pressure to cause blood clotting (Kisiel et al., 1987).
L-Amino Acid Oxidases (LAAOs)		They are found in the venoms of elapids and vipers (Tasoulis and Isbister, 2017). They function in the oxidative deamination of amino acids and produce ammonia and hydrogen peroxide as side products that have several cytotoxic effects and may induce oedema, apoptosis, and may act as anticoagulants (Meléndez-Martínez et al., 2017; Sharma et al., 2015).
Acetylcholinesterase		Hydrolyses the neurotransmitter acetylcholine synaptically and blocks effective neuronal transmission (Schetinger et al., 2009).
Hyaluronidases		They hydrolyse the hyaluronic acid in the interstitial space and facilitate the diffusion of other toxins across the body (Isoyama et al., 2006).

Table II. Non-enzymatic components of snake venom

Three-finger Toxins	Predominant in elapid venoms, and some viper and colubrid venoms (Aird et al., 2013). They are generally neurotoxic or cytotoxic having a variety of biological effects such as they block neuromuscular transmission by binding to the post-synaptic nicotinic receptors in the skeletal muscle, form ion pores and cause cell lysis, block calcium channels and inhibit platelet aggregation (Heyborne and Mackessy, 2013; Kini and Doley, 2010).
Cysteine-rich Secretory Proteins	Found in all the venomous families. These single-chain polypeptides exert their effect by blocking calcium and potassium ion channels and thereby affecting signal transmission and smooth muscle contractions (Fox and Serrano, 2005).
Kunitz-type Proteinase Inhibitors	Present in vipers and elapids (Yuan et al., 2008), and functions to inhibit serine proteases and numerous ion channels. One of the notable groups of Kunitz peptides is the dendrotoxins found in the mambas, they interact with voltage-gated ion channels and cause involuntary muscle contractions (Laustsen et al., 2015).
Disintegrins	Present in viper venoms inhibits the collagen-induced activation of platelets (Williams et al., 2019).
Natriuretic Peptides	Found in elapids and vipers, promote natriuresis affecting myocardial actions as well as causing hypotension (Collins et al., 2000).
Snake C-type Lectin-like Proteins (Snaclecs)	Both activate and inhibit platelets through various types of receptors (Clemetson et al., 2005).
Bradykinin-potentiating Peptides (BPPs)	Inhibit the angiotensin-converting enzyme and cleave bradykinin resulting in potent hypotensive actions (Sciani and Pimenta, 2017).
Nerve Growth Factor (NGF)	Helps in the prey incapacitation. Cause mast cells to release chemical mediators and increase vascular permeability to facilitate dispersal of other venom components (Sunagar et al., 2013).

III. TOXIC EFFECTS OF SNAKE ENVENOMING

Venomous snakes contain a specialized biting mechanism to deliver their venoms into the body of the prey. The biting apparatus contains a pair of fangs that may be located anterior to the maxillary bones in the cases of viperids, elapids, or posteriorly in the colubrids known as non-front-fanged snakes. The size of the fangs determines whether the venom is injected subcutaneously or intramuscularly. Once inside the body, the venom is translocated with the help of the blood and lymphatic system to several organs inside the body and exert their pathological effects. There are significant differences in the toxicological effects between diverse groups of venomous snakes. But they exert some similar pathophysiological effects in the victimized prey such as local tissue damage, inflammation, haemostatic alterations(Gutierrez et al., 2017).

A. Local Tissue Damage

Almost all of the venomous snakes induce local tissue injuries, but the most potent effects are exerted by the vipers. SVMPs induce hydrolysis of blood vessel walls that causes haemorrhage of the local tissue and the venom spreads quickly in the body. The spread of venom is also promoted by the hyaluronidases(Escalante et al., 2011). PLA_2 induce blistering and oedema at the injured location(Dixon and Harris, 1996). These components can also induce necrosis in the softer tissues. SVMPs degrade the dermal-epidermal interface causing blisters. Hyaluronidases along with SVMPs hydrolyse extracellular matrix components, including collagen, proteoglycans, hyaluronic acid that alter the structure and function of tissue

components inducing local tissue damage (Gutierrez et al., 2016). The local intra-muscular nerves and vasculature also get damaged due to the actions of the toxin components. The envenomed tissue develops an extensive inflammatory response following the synthesis and release of several inflammatory mediators inducing pain in the tissue (Rucavado et al., 2016).

B. Haemotoxicity

Snake venom components destroy basement membrane components resulting in the reduced mechanical stability of the blood vessels. SVSPs and SVMPs modulate the factors involved in coagulation and can prevent or promote blood clots. Many other components including the three-finger toxins, snaclecs, disintegrins, cysteine-rich secretory proteins dysregulate the platelet aggregation (Kini and Koh, 2016). The SVMP-induced vascular damage causes disruption of the endothelial cell-to-cell adhesions (Escalante et al., 2011).They enhance haemorrhaging and may induce thrombocytopenia. Natriuretic peptides increase vasodilation. High concentration of PLA_2 causes haemolysis in elapid bites. Bleeding is also associated with snakebite envenomation. Intracranial bleeding causes ischaemia, stroke and several neurological symptoms (Del Brutto and Del Brutto, 2011). Hypovolaemia can result from increased vascular permeability caused by SVSPs. The viperid venoms include bradykinin-potentiating peptides that inhibit the angiotensin-converting enzyme and may also contribute to haemostatic alterations(Hayashi and Camargo, 2005).

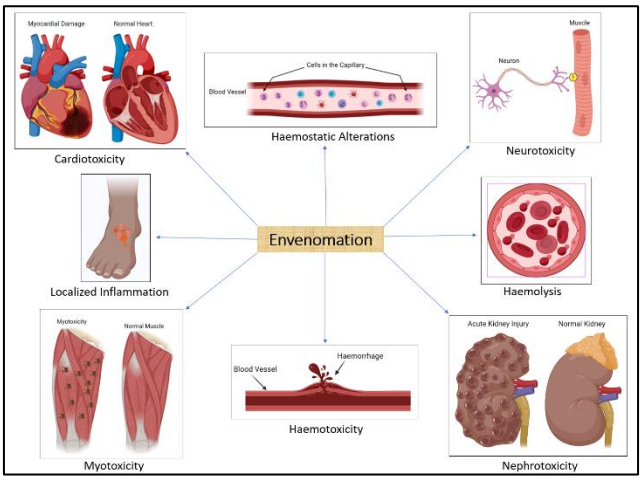

Fig.1. Toxicological effects of snake venoms

C. Myotoxicity

Myotoxic effects are majorly due to the actions of PLA_2 and three-finger toxins that can disrupt the integrity of the myofibrils via pore formations. They also cause hydrolysis of the phospholipids in the cell membrane. Following disturbances in the membrane structures, a rapid influx of calcium ions dysregulates the normal contractile mechanism of the myofilaments and causes other degenerative muscular events leading to irreversible muscle damage. SVMPs cause myotoxicity by cleaving collagen and other basement membrane components. The myotoxic effects are also caused by ischaemiaand localized oedema that alters tissue vasculature. Moreover, as a consequence of vascular and nervous damage of the tissues, muscular regeneration is impaired, often leading to permanent sequelae (Gutierrez et al., 2017; Ferreira et al., 2018).

D. Neurotoxicity

The nervous system is more affected in elapid envenomation and viperids generally do not severely act on the neurons, although crotoxin of some viper species such as *Daboia russelii* shows significant levels of neurotoxicity. The elapid venoms contain notable neurotoxins that cause neuromuscular paralysis resulting in respiratory failure and blockade of swallowing. Alpha-neurotoxins belonging to the three-finger toxin family acts post-synaptically and bind with the cholinergic receptors at the neuromuscular junction. Thus, they block the interactions of acetylcholine and induce flaccid paralysis (Barber et al., 2013). Beta-neurotoxins are predominantly PLA_2s that act pre-synaptically. These neurotoxins upon binding to their appropriate receptors cause enzymatic degradation of the membrane phospholipids causing neurotoxicity. Moreover, with the generation of lysophospholipids and fatty acids, the alterations in the cell cause immature fusion and release of synaptic vesicles (Rossetto and Montecucco, 2008). With an increased membrane permeability to ions followed by calcium influx and depolarization of the nerve membrane, the synaptic reserve vesicles are depleted due to exocytosis. These events ultimately lead to degenerative intracellular mechanisms resulting in degradation of the nerve terminals (Harris et al., 2000). These are the underlying causes for prolonged paralysis in snakebite victims. Some PLA_2s can act by blocking voltage-gated ion channels in the neuronal membrane (Pungerccar and Krizaj, 2007). Other snake venom components may also contribute to severe neurotoxicity such as acetylcholinesterase hydrolyses the acetylcholine and thus blocks neuromuscular signal transduction, cysteine-rich secretory proteins block specific ion channels (Ca^{2+} and K^{+}) to dysregulate the membrane potential (Harvey and Robertson, 2004).

E. Nephrotoxicity

Acute kidney injury is also connected with snake-bite envenomation. Haemodynamic disturbances caused by systemic bleeding and vascular damage often result in decreased renal blood flow that can cause kidney damage. SVMPs often hydrolyse the basement membrane components in the glomerular capillaries in the kidneys. Thrombotic microangiopathy may also develop due to the deposition of microthrombi. Some cytotoxic PLA_2s exert their effect directly by damaging tubular cells in the kidney. In some cases, cytotoxic and proteolytic activity can cause accumulation of myoglobin in the renal tubules resulting in severe nephrotoxicity (Sitprija and Sitprija, 2012).

F. Cardiotoxicity

Cardiotoxic effects are mainly caused by natriuretic peptides which mediate their effects by causing hyponatremia. The haemodynamic alterations also influence cardiological toxicity. In some elapid venoms, cardiotoxins directly cause myocardial damage. SVMPs often lead to cardiovascular shock from the haemodynamic changes due to venom-induced bleeding in the body (Hayashi and Camargo, 2005; Hojer et al., 2010).

G. Cytotoxicity

The major cytotoxic components are the PLA_2s and three-finger toxins. These constituents induce localized necrosis due to their cytotoxic effects. Three-finger toxins reduce the stability of the plasma membrane in different cell types through non-enzymatic mechanisms. Group-II PLA_2s can directly induce cytotoxic effects causing myogenic damage (Rivel et al., 2016; Duboyskii and Utkin, 2014).

IV. Conventional polyclonal antivenom

The anti-snake venom serum (ASVS) currently in use is a polyvalent antivenom extracted from horse serum after the animal is injected with respective snake venoms. The antivenom, also called antivenin, is F(ab')-based equine polyspecific antibodies developed using the venoms of four Indian venomous snakes which are Russel's viper (*Daboiarusselii*), Spectacled cobra (*Najanaja*), Common krait (*Bungarus caeruleus*), and Saw-scaled viper (*Echiscarinatus*) (Kini et al., 2018).

This conventional antivenom is produced through a multi-step process. First of all, the snakes are milked for obtaining the required venom from their venom glands and the venom is injected into a larger mammal, mostly horse or in some cases sheep to immunize the animal against snake venom toxins. After successful immunization of the animal, blood is collected from the body in a sufficient amount, and erythrocytes are separated from the plasma. Different precipitation techniques are employed to isolate manufactured IgG antibodies from the plasma. The concentration and formulations are tested before marketing as vials and administered into the snakebite victims (Kini et al., 2018).

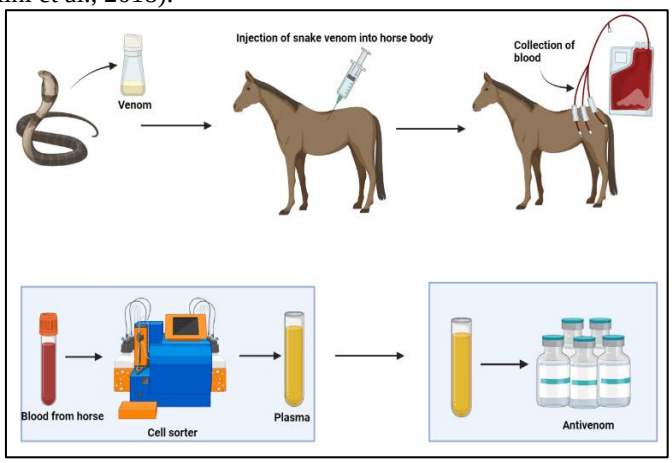

Fig.2. Manufacturing the conventional polyclonal antivenom

A. Limitations

1) Complexity

The process of manufacturing polyclonal antivenoms is largely dependent on the availability of associated snake venom and the immune system of the individual horse. The production system is also very much invasive and requires multiple tedious steps to produce a sufficient number of polyclonal antibodies.

2) Serum sickness

Serum sickness is a type of delayed-type hypersensitive reaction that occurs in several victims administered with the antivenom. The polyvalent antivenom contains only 5-36% antibodies able to bind to the snake venom toxins, and this is the major reason for administering multiple vials of antivenom (Segura et al., 2013). The high dosage of such poor neutralizing components increases the risk of serum sickness in the snakebite victims (Lavonas et al., 2011).

3) Inability to abolish local tissue damage

The local tissue damaged by snake venom components cannot be reverted back to its normal condition upon antivenom administration. The antibodies present in the administered antivenom have the limited pharmacokinetic properties to reach the deep layers of tissues and neutralize the catastrophic pathophysiology induced by multiple toxins in the snake venom (Gutierrez et al., 2017). This antivenom may not be effective to treat venom-induced consumption coagulopathy (VICC) which is one of the major outcomes of envenomation by elapid and viper species (Gulati et al., 2013). VICC being irreversible poses a great challenge to be neutralized by the polyvalent antibodies, and it has been shown to be less effective against *Echis*envenomation (Rogalski et al., 2017).

4) Allergic reactions

The horse-derived antivenoms are foreign to the human body and may act as antigens rather than acting as antibodies against snake venom toxins. These can lead to acute anaphylactic shock followed by severe allergic reactions in the victims (Lalloo and Theakston, 2003).

5) Unable to neutralize venoms from different regions

The chemical composition varies with geographical distribution in the venomous snakes. In certain cases, the antivenom fails to neutralize snake venoms in the victims from different locations and is the most potent failure of the currently available polyvalent antivenom (Goncalves-Machado, 2018). The neutralizing efficacy and the venom yield per bite are depicted in the given chart.

6) Lack of neutralizing ability against venoms of certain medically important snakes other than the 'big four'

The present antivenom cannot properly neutralize the toxins from the venomous species all over the country that are less known yet medically significant and also cause a good proportion of envenomation. There are about 29 unique venom components between *Najanaja*and *Najakaouthia*, and it exerts the resistance in neutralizing ability of the antivenom against the venom from *N. kaouthia* at the same level as that of *N.*

naja(Parvatam, 2018; Mukherjee, 2020). It is unable to reverse the myotoxic effects induced by the venom of Sri Lankan *Najanaja* once it has already begun damaging the tissue (Madhushani et al., 2021).

7) Expensive

Antivenom production takes several steps and diverse biological systems and thus it is much more expensive in clinical uses (Kini et al., 2018).

8) Pharmacodynamic consideration

Pharmacodynamics is the ability of therapeutic components to act *in vivo* to neutralize the toxins of venom and it is one of the key determinants of antivenom efficacy. The polyclonal antivenom has several limitations that make the measurements complicated. A single epitope in a toxin may be recognized by several antibodies with different affinities. Again, a particular antibody component in polyclonal antivenom may bind to homologous toxins with different specificities. The concentrations of individual antibody component that binds with a single epitope cannot be measured in this antivenom (Vauquelin and Charlton, 2013).

V. Requirements of Alternate Strategies

There are numerous reasons to find out effective intervention methodologies to treat snakebite envenomation. Currently available therapeutic strategies may be viable to some extent but possess several side effects and also have high mortality even after the application of the therapeutics. In snakebite envenomation, there is no incubation period for the toxins, although there may be some delay in starting the pathogenesis of systemic toxins that need to be transported through the blood from the site of biting. This is the reason to start treatment of envenomation as early as possible with effective measurements (Laustsen, 2019).

Larger snakes can inject a high dose of toxins into the body that can exert their effects immediately. This leads to the need for high amounts of antivenom that should be used against the venom (Harrison and Gutierrez, 2016). The higher dosage of antivenom influences the economic side of antivenom production. Effective and sound treatment protocol cannot be employed in the rural areas of India where the snake bite mortality is higher if the cost of antivenom is not within the limit (Laustsen and Dorrestin, 2018). Moreover, snake venoms are complex mixtures of different enzymes and chemicals considered as toxins. Therefore, it needs to be used a mixture of antivenom components. But it should be monitored which toxins are needed to be neutralized as some venom constituents may not be medically significant for the body. This will reduce the complexities in therapeutic methodologies (Laustsen et al., 2015; Laustsen, 2016).

The envenomation is not transmissible into a population and production of a suitable antivenom is sufficient as it will not require the development of herd immunity. Therefore, the development of a vaccine against snake venoms will be of limited value. There is a very low probability of developing resistance against the antivenoms, because of very slow mutation rates in reptiles, and the medically important toxins will barely change their composition in long periods of time (Laustsen, 2019). The polyvalent antivenoms, directed against the venom components after immunization of a host animal, contain several antibodies against those antigens that the host has encountered during its entire life. As a result, the antivenom carries a huge portion of antibodies that are not relevant for snakebite victims (Segura et al., 2013).

VI. Monoclonal Antivenom

Monoclonal antibodies (mAbs) have been reported to be effective therapeutic tools in several pathological states. The production of toxin-specific mAbs is one of the finest advancements in recombinant technology and it theoretically promises to overcome the limitation of ensuring that each of the IgGs in therapy is efficient to neutralize a toxin. There are other recombinant antibodies besides the mAb such as the single-chain variable fragments (scFvs), nanobodies, and antigen-binding fragments (Fab and $F(ab')_2$s) that have been studied to neutralize several toxins of different animal venoms (Richard et al., 2013; Boyer et al., 2013).

The first fully human-originated oligoclonal IgGs against animal toxins were reported recently. Carefully selecting the oligoclonal mixtures of human monoclonal IgGs can abolish the neurotoxicity caused by the dendrotoxins of the infamous black mamba (*Dendroaspispolylepis*) (Laustsen, 2018).

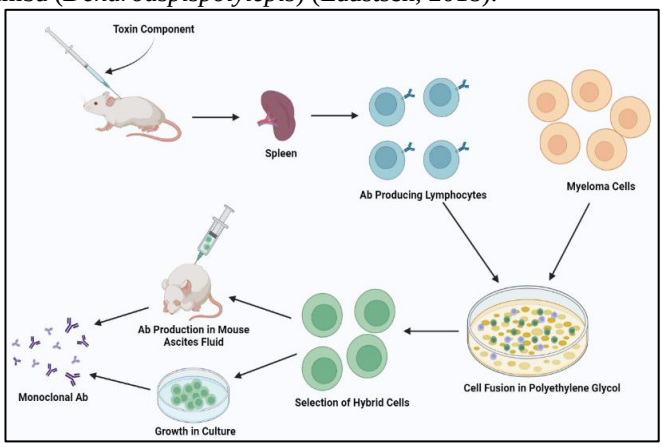

Fig.3. Schematic representation of producing monoclonal antibodies

The snake venom-mediatedhaemotoxicity can be addressed by using specific mAbs for species-specific toxins (Tanjoni et al., 2003). Successful evaluation of neutralizing efficacy for mAbs has been done against West African carpet viper (*Echis* sp.) (Idddon et al., 1988), Western diamondback rattlesnake (*Crotalus atrox*) (Perez et al., 1984), and *Bothrops*snakes (Fernandes et al., 2010). These approaches include mAb-induced antivenom responses only for very similar epitopes of a particular toxin of the whole venoms. They showed significant inefficacy against other venoms.

MAbs against the factor X activators present in the venom of Russel's viper is also investigated and proved to be effective in neutralizing the toxic component (Pukrittayakamee et al., 1983). A successful investigation has been done to find out the neutralization effects of myotoxic components of *Bothrops asper* snake venom (Lomonte et al., 1992). There are several studies regarding the neutralization efficacy of the mAbs raised against neurotoxic substances of snake venom as well. The specific

mAb against toxin alpha of *Najanigricolis* neutralizes its effects both *in vivo* and *in vitro* (Boulain et al., 1982). There are instances when mAbs neutralized long-chain neurotoxic curaremimetic toxins of snake venoms. Alpha-cobratoxin is also a type of these long-chain neurotoxins found in *Najakaouthia* snake venom (Pillet, 1992).

Human-originatedscFvs have also been proved to neutralize several toxins from distinct animal venoms, mostly scorpions (Riano-Umbarila et al., 2019; Rodriguez-Rodriguez et al., 2016).The injections of LD_{50} of whole venoms of certain scorpion species such as *Centruroides elegans, C. tecomanus, C. limpidus*, etc. caused death in all of the mice except in those administered with scFvs. It is more effective than using against the snake venoms, perhaps because the scorpion venoms are not so complex and even the molecular and pathological considerations are well distinguished for them (Riano-Umbarila et al., 2019; Bahraoui et al., 1988).Moreover, snake-bite deaths are more common than due to scorpions. Although to eliminate the less efficacy of such small molecule antivenoms it is better to find out potential toxic components from the venom. Another unusual class of antibodies lacking the light chains and the first constant portion of the heavy chain was obtained from camelid serum. This type of antibody often referred to as nanobodies or heavy-chain antibodies comprises only the variable domain of a classical antibody molecule (VHH) but is still able to neutralize antigens (Kunz et al., 2017). Nanobodies can be easily produced on large scale and also hold promising advancements over conventional antivenom such as high specificity to venom toxins, highly thermostable, and fewer chances of immunogenic reactions in humans because of high sequence identity (Fernandes et al., 2021). The very small size of such molecules enables their better distribution throughout the body which is crucial for removing toxicological effects at the cellular level. But the usage of nanobodies is still in the naïve stage and to bring it to the clinical level, extensive studies should be performed.

The high mono-specificity of the above-mentioned mAbs is not favorable in the case of highly complex snake venoms containing multiple antigens. It is, therefore, required to use broadly neutralizing mAbs that are able to act against numerous similar or dissimilar antigens (Ahmadi et al., 2020).This kind of mAbs has been generated against *Najanigricolis, N. mossambica*, and *N. melanoleuca*, and subsequently assayed with cell line viability tests only. To make its neutralizing capacity effective to be used in humans, it has to be used in several other combinations of venoms depending upon the tropical countries. Apart from a cell line, mice models should be employed with LD_{50} of venom combinations. It would be beneficial over the conventional antivenom due to its less cross-reactive properties and thus will reduce the chances of serum sickness.Although there are successes in using mAbs as neutralizing agents against animal-derived toxins, the difficulties in identifying specific isoforms of toxins and the complexities of snake venoms are discouraging the extensive usage of mAbs against snake venom toxins. These situations still possess resistances in generating toxin-specific mAbs to treat the systemic pathophysiology in snakebite envenomation (Harrison et al., 2011).

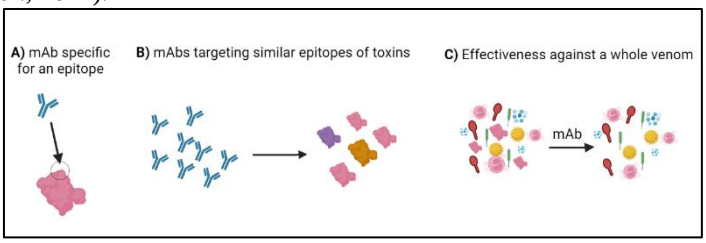

Fig.4. Efficacy of mAb-based antivenom

VII. Recombinant Antivenom

The rapidly emerging fields of recombinant technology allow the insertion of a gene of interest into a heterologous host genome and investigate the consequent expression of that gene product. Different protein expression systems have been employed for the purification of specific gene products and used in numerous biotechnological interests. The protein products can be further injected into larger animals either for the production of vaccines or for the generation of specific antibodies in the body that can be collected and used for therapeutic purposes.

With the advent of more efficient gene delivery methods, they are being used together with gene optimization techniques resulting in an enhancement of the levels of protein expressions. These strategies along with the beginning of using DNA vaccines have been proving to elicit more effective cellular immune responses (Yan et al., 2007) and also induce the production of a higher number of antibodies (Yadava and Ockenhouse, 2003; Smith et al., 2004).

The very promising protocols of genetic immunization may have a better solution to the above-mentioned limitations in developing the most effective snake antivenom for the medically important snakes of India. This would be an easier approach to present correctly folded toxins from snake venom to a host with the concurrent production of most probable neutralizing antibodies against the toxin.Structural conformations and immunogenic properties of toxins within whole venom are very easy to determine. Inducing antibody production against particular toxin by combining it with other carrier molecules may enhance the immunogenic property. Development of suitable expression systems for such recombinant toxins and re-introducing them into large animals would increase the availability of antivenom compared to the present scenario.

The *Escherichia coli* system has been widely used to study the expression of toxic components including α-neurotoxic protein of the Mexican coral snake, *Micrurus laticollaris* (Carbajal-Saucedo et al., 2013), SVMPs from the broad-banded copperhead, *Agkistrodon contortrix laticinctus (Selistre-de-Araujo et al., 2000)*, post-synaptically acting neurotoxins from *Pseudonaja textilis* (Gong et al., 1999). The snake venom toxins are very complex polypeptides having correctly folded conformation established by disulfide bonds, that are again necessary for eliciting their toxic effects. Therefore, the use of a prokaryotic host imposes several limitations in the production of such polypeptide toxins. The toxins cannot be produced in higher quantities, there is a high chance that the molecules would not be correctly folded and hence, become less active (Selistre-de-Araujo et al., 2000; Moura-da-Silva et al., 1999) To

overcome the issues, eukaryotic host systems, such as *Pichia pastoris*, has been shown to be more effective and may be an alternative approach for the expression of recombinant toxins of snake venom (Pinyachat, 2011).

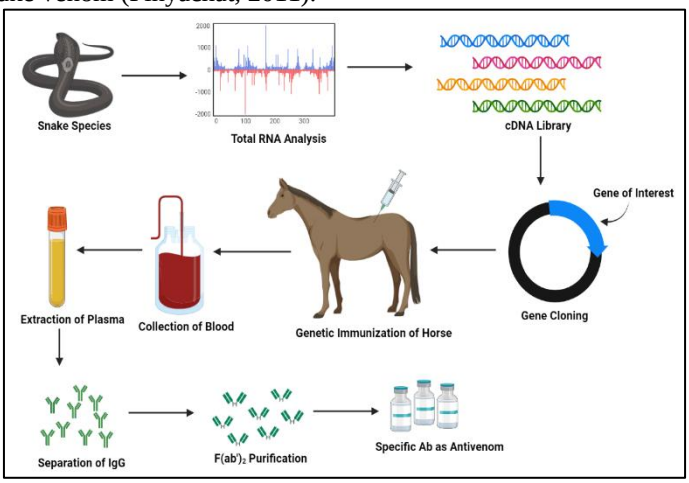

Fig.5. Schematic representation for the production of recombinant antivenom

The first DNA immunization strategy as an attempt to develop antivenom capable of neutralizing the toxicological effects elicited by the snake venom was done using the Jararhagin of *Bothrops jararaca*. The specific carboxyl-disintegrin and cysteine-rich domains of this metalloprotease were targeted in this approach and a significant reduction of around 77% in the hemorrhagic lesion was found. It was later found that the immunoglobulins have high reactivity to the components of snake venoms of a particular species which promotes the potential use of this strategy to produce toxin-specific antibodies with polyspecific cover (Harrison et al., 2003). This type of early work demonstrated the probability of using DNA immunization as an alternative approach for the next generation of antivenom therapies.

Although it is not effective to target a single toxin within the snake venom, it was found that targeting a specific toxin is able to reduce the toxic effects caused by the whole venom. The work done with *Echis ocellatus* that display a large molecular diversification of SVMPs far exceeding that observed in the case of *B. jararaca* implicated that an adequate neutralization of the toxicological activities caused by a certain venom would be accomplished by the production of antibodies targeting the antigenic epitopes present in most of the diverse SVMP isoforms (Francischetti et al., 2004; Junqueira-de-Azevedo and Ho, 2002; Kashima et al., 2004; Wagstaff et al., 2006).There has been works with cloning and cDNA sequence analysis from the total RNA extracts of a venom. In such cases, immunopotentiators are also used to increase the antibody titer. Co-administration of such venom toxins and potentiators have shown increased production of antibodies in mice models decreasing the venom-induced tissue damage (Ramos and Ho, 2015).

This clearly indicates that using the bioinformatics analysis of the venom components and even the venom gland transcriptome can be a great way to enhance the neutralization efficacy of the snake antivenom.

The initial works paved the way for new antivenom therapeutics, but still, there is no record of using recombinant toxins to synthesize the antivenoms. The snake venoms are very complex mixtures of diverse toxic components, therefore, targeting only one toxin and making antibodies against it may not be always beneficial for the neutralization of pathogenesis. To produce an adequate amount of recombinant toxins, the host must be feasible enough, but this is again very costly. By developing an appropriate database regarding the most abundant epitopes in the complex mixture of venom and using bioinformatic tools the sequence tags may be identified, that can direct the future progress in this domain of antivenom production.

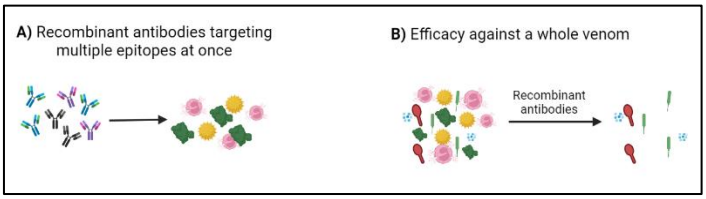

Fig.6. Efficacy of recombinant antivenom

CONCLUSION

Innovation of new antivenom for the future is not so simple. Purification of the toxins from crude venom is very complex and it does not allow the collection of enough toxins for immunization studies. Most of the elapids deliver a very small quantity of venoms per bite, yet they are medically important as they are responsible for significant mortalities in tropical and sub-tropical countries including India. Heterologous expression systems may be a better alternative for the production of important toxin molecules. Future research should be done on developing a much compatible and cost-effective antivenom having no harmful side effects or least number of side effects. Selection of most abundant epitopes from specific snake venom and developing cDNA libraries for the medically important snake species are yet to be focused. More types of recombinant toxins may be generated to neutralize the whole venoms of elapids, vipers, and colubrids. The antivenom strategy involving DNA immunization can be a great tool for antivenom development in the near future. Several aspects involving this strategy are yet to be dealt with and further studies should be done to make it available clinically.

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