DEVELOPMENT OF NANOPARTICLE BASED IMMUNODIAGNOSTIC KIT FOR DETECTION OF ENVENOMATION FROM MEDICALLY IMPORTANT SNAKES

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Submitted By

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Under the Guidance of

Dr. Mugdha Potnis- Lele

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CERTIFICATE

This is to certify that the thesis entitled **"Development of nanoparticle based immunodiagnostic kit for detection of envenomation from medically important snakes"** which is being submitted herewith for the award of the Degree of Vidyavachaspati (Ph.D.) in Biotechnology of Tilak Maharashtra Vidyapeeth, Pune is the result of original research work completed by **Shri. Balasaheb Suryakantrao Pawade** under my supervision and guidance. To the best of my knowledge and belief, the work incorporated in this thesis has not formed the basis for the award of any Degree or similar title of this or any other University or examining body upon him.

Dr. Mugdha Potnis- Lele
Research Guide

Place: Pune
Date: 08/11/2016

DECLARATION

I hereby declare that the thesis entitled "**Development of nanoparticle based immunodiagnostic kit for detection of envenomation from medically important snakes**" completed and written by me has not previously formed the basis for the award of any Degree or other similar title upon me of this or any other Vidyapeeth or examining body.

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ABBREVIATIONS AND DEFINITIONS

Ab

Antibody

ACV-Abs

Purified Anti-CV Antibodies

ARV-Abs

Purified Anti-RV Antibodies

Antidote

A medicine or remedy, which prevents or counteract with the effects of toxic material.

Ag

Antigen

Adjuvant

A substance, which enhances body's immune response to an antigen.

Antivenom

A purified fraction of immunoglobulins or immunoglobulin fragments from the hyperimmune plasma of animals that have been immunized against snake venom or a snake venom mixture along with the adjuvant.

ASVA

Anti Snake Venom Antibodies

BSA

Bovine serum albumin

CFA

Complete Freund's adjuvant

Cross- reactivity

The ability of an antivenom raised against a venom, or a number of venoms, to react and neutralize the lethal effects of the venom of a closely related species though it is not included in the immunizing mixture.

Conspecific

Belongs to same species or group.

CV

Cobra venom

Da

Dalton

ED_{50}

The effective dose 50 is the quantity of antivenom that protects 50% of test animals injected with a specified number of LD_{50} venom.

EDTA

Ethylene diamine tetra acetic acid.

ELISA

Enzyme-linked immunosorbent assay

Envenomation

The introduction of venom into a body of victim by means of the bite or sting of a venomous animal.

EV

Echis venom

F $(ab)_2$

Bivalent fragments obtained by pepsin digestion of immunoglobulin of hyperimmune plasma.

Fc fragment

The crystallizable fragment of an immunoglobulin molecule composed of the constant region of the heavy chains.

g

Gram

GMP

Good manufacturing practices

h

Hour

HBPCL

Haffkine Biopharmaceutical Corporation Limited

Heterospecific

Belongs to a different species or group.

HRP

Horseradish peroxides

Hyperimmune plasma

Plasma containing antibody at desired level

Hyperimmunization

Repeated immunization of animal with single or mixture of venoms along with a suitable adjuvant to produce long lasting antibodies or higher neutralizing antibody titer against the lethal and other deleterious components in the immunogenic toxins.

IFA

Incomplete Freund's adjuvant

IgG

Immunoglobulin G

IgE

Immunoglobulin E

IU

International unit

i.v.

Intravenous (ly)

Immunization process

A process of producing long-lasting and high-titer neutralizing antibody responses against the lethal and other deleterious components of the immunogen.

Immunoglobulin

Antibody molecule generated by immunizing an animal (most often a horse or sheep) using the antigen with or without adjuvant.

kDa

Kilodalton

KV

Krait venom

l

litre

LD_{50} (Lethal $dose_{50}$)

The quantity of snake venom injected intravenously/subcutaneously that causes death of 50% of animal population in a group after a specified period of time (usually 24-48 h)

LFA

Lateral flow assay

LPS

Lipopolysaccharides

Lyophilization

A process by which material is rapidly frozen and dehydrated under high vacuum.

M

Molar

MAb

Monoclonal antibody

ml

Milliliter

mm

Millimeter

mg

Milligram

MW

Molecular weight

Mol

Mole

Monospecific antivenom or antivenin

Antivenom that obtained by fractionating the plasma from animals immunized with a single species of venom.

NA

Not applicable

NC

Nitrocellulose

ND

Not determined

nm

Nanometer

NS

Normal Saline

OD

Optical density

PBST

Phosphate- buffered saline containing Tween 20

Plasma

The liquid portion remaining after the separation of the cellular elements from blood collected in a receptacle containing an anticoagulant, or separated by continuous filtration or centrifugation of anti-coagulated blood in an apheresis procedure.

Plasmapheresis

Procedure in which whole blood is removed from the donor, the plasma is separated from the cellular elements by gravity separation or centrifugation, and at least the red blood cells are returned back to the donor.

Polyspecific antivenom

Antivenom that obtained by fractionating the plasma from animals immunized with the mixture of venoms.

Primary immunization

The immunization of unexposed fresh animals to the immunogen to attain the desired immune response.

rpm

Revolutions per minute

RV

Russell's viper venom

s.c.

Subcutaneous

SD

Standard deviation

SDS–PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

SE

Standard error

SEDIA

Snake envenomation detection immunoassay

SSAb

Species-specific antibody

TMB

Tetra methyl benzidine

TLC

Thin layer chromatography

Tris

Tris (hydroxylmethyl) aminomethane

Quarantine

A period of enforced isolation and observation of new animals before induction on immunization program mainly to avoid the possibility of an infectious disease among animals.

µg

Microgram

Venom

The toxic secretion of a specialized venom gland of snakes is delivered through the fangs and provokes deleterious effects. Venoms usually comprise many different proteins and enzymes.

WBCT

Whole blood clotting test

WHO

World health organization

ABSTRACT

Snakebites are the professional hazard primarily disturbing the countryside residents of a tropical and subtropical area, which comprises mainly emerging and underdeveloped nations. As stated by the World Health Organization (WHO), about 2,00,000 snakebite cases and about 35,000 to 50,000 deaths occur per year in India due to snakebites. In the world, more than 3,596 snake species observed out of which only 768 found venomous. In India, about 305 snake species observed out of which only 52 considered as venomous. Moreover, four species considered medically significant, as they are responsible for the majority of deaths in India includes Russell's viper, Saw-scaled viper, Indian cobra, and Common krait.

Fatalities and permanent impairments from venomous snakebites can overwhelmed with the early and reasonable administration of the effective remedy, snake antivenom to envenomed victims, with all required supportive medical treatments, considering the possible side effects of the antivenom. Generally, snake antivenom contains specific immunoglobulins obtained by hyper-immunization of equines with venoms. The adverse reactions of snake antivenom are due to the heterogeneity and product safety profile of the product. Many cases of snakebite does not result in systemic envenomation also the severity is very variable, as average venom injected depends on the species, size, mechanical efficiency, control of venom discharged by the snake. Considering above facts, it becomes crucial to determine the severity of snake envenomation and identification species for the appropriate medical management of the snakebite victims.

The present available physical and biochemical tests do not clearly diagnose the snake envenomation and species of snake. Since long time, investigators have been involved in developing a simple, rapid, sensitive, and reliable test for identification of offending snake species and its envenomation. Over the time, ELISA turns into the choice of technique for detection of the snakebites, but it has some inherent limitations such as it requires time, laboratory, and technical staff support. Snake venom is a complex mixture of enzymatic, non-enzymatic proteins, polypeptide toxins, and non-toxic proteins, having a differing mode of action. The snake venoms contain several similar proteins, between the species and it increases in closely associated species.

Anti snake venom antibodies (ASVA) manufactured by hyper-immunization of animals using native venoms. Antibodies raised in animals have the ability react with most of the venom antigens injected. The generated antibodies demonstrate cross reactivity with the heterospecific venoms, if snakebite detection assay developed using these antibodies, may create uncertainty in the analysis of results due to cross reactivity.

To remove such cross-reactive antibodies, the preparation, and application of species-specific antibodies (SSAb's) is essential in snake species detection using venoms. The SSAb's are generated by passing the affinity purified ASVA through the conspecific venom columns to remove all non-specific antibodies and the eluted antibodies reacted with heterospecific venom columns to remove all cross reactive antibodies to generate SSAb's. The ELISA test performed using heterospecific venom confirms the specificity of the SSAb's. The SSAb's are utilized today for snake venom detection in many formats and have potential in the manufacture of lateral flow assay (LFA) for snake venom detection. LFA is the easiest test and normally for testing, it does not require any chemicals and pretreatment of sample. The application of test samples like urine, saliva, blood, or any liquid to the sample pad provides results within 5 to 10 minutes (min) and the result interpretation is by naked eyes in many formats. Currently, there is no reliable, fast, and field detection test available in India, where snakebites are rampant. Accordingly, development of LFA for detection of snake venoms has tremendous potential to save lives of the snakebite victims by early detection of snake envenomation.

To encounter the detection problem in snake envenomation, the study was undertaken to develop gold nanoparticle (GNP) based LFA for Russell's viper venom (RV) and Indian cobra venom (CV) detection on a strip. For the development of LFA, purified polyvalent ASVA (equine) conjugated with 25 nm GNPs and applied to the conjugate pad. The RV and CV SSAb's are impregnated on nitrocellulose (NC) membrane at relevant test zones. All the LFA components were assembled to develop, snake envenomation detection immunoassay (SEDIA), which observed selective, rapid, and sensitive to detect snake venom up to 0.1 ng/ml and demonstrated results in 10 min. The SEDIA device observed specific to the conspecific venom and has immense potential to become a field test for detection of snake envenomation.

CHAPTER 1

INTRODUCTION

1.1 Epidemiology of snakebite

Snakebite is a neglected; yet fascinating community health issue leads to life-threatening medical emergencies (Kanchan et al., 2012; WHO, 2010; Kasturiratne et al., 2008). The majority of the snakebite occurs in the subtropical and tropical regions, which chiefly includes emerging and undeveloped countries. Snakebites are the professional hazard mostly affecting the rural population working as agricultural workers, farm workers, anglers, snake handlers, and especially those involved in agricultural and plantation work (Alirol et al. 2010). Snakebites result in the fatalities or chronic disabilities to many active younger persons, therefore has socio–economic implications where it occurs commonly (Theakston et al. 2003; WHO, 2010; Warrell, 2005).

The figures of snakebite injuries and deaths are not trustworthy because of improper reporting systems in the most part of the world, especially in emerging and undeveloped countries. Chippaux J. P. (1988) reported that about 80-percent snakebite victims in Africa first seek advice from the traditional practitioners prior to taking treatment from a medical center. In the Asian sub-continents, the occurrences of snakebites documented inadequately and generally, the data based on government hospital admitted victims. The obtained data is unreliable and misleading as all the snakebite victims may not seek treatment in hospitals but by traditional, herbal or Ayurvedic practitioners (Chippaux 2008; Gupta Y.K. and Shah Peshin S., 2014; Warrell D.A., 1989).

The WHO stated that, there are about 2,00,000 cases of snakebites per year in India and about 35,000 to 50,000 deaths occur due to snakebites. Chippaux (1998), in his study, using references of 114 publications and hospital records or health authority reports hypothesized that the overall snakebites per year may exceeds five million with a snakebite death of 1,25,345 per year in the world. This comprises about forty lakh cases of snakebites, twenty lakh envenomings, and one-lakh deaths per year in Asia region. Fig. 1.1 indicates that, Southeast Asia, South Asia, and sub-Saharan Africa region have highest snakebite deaths (Kasturiratne A. et al., 2008).

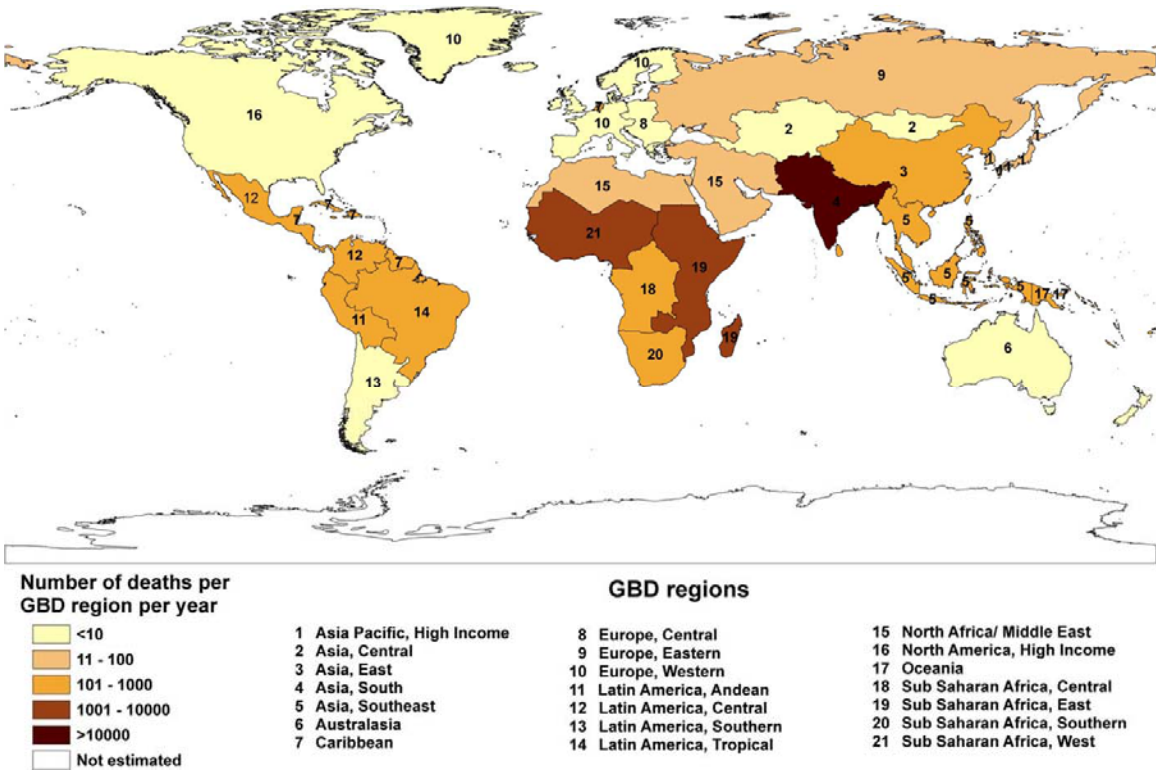

Fig 1.1: The global burden of diseases (GBD) – deaths due to the snakebite (Anuradhani Kasturiratne, 2008)

1.2 Worldwide presence of snakes

Snakes are distributed worldwidely except the Arctic regions. Snakes are classified in phylum *Chordata*, order *Squamata*, and suborder *Serpentes.* In the world, more than 3,596 snake species observed, out of these only 768 found venomous. India contains about 305 snake species among them only 52 are venomous. The Common krait (*B. caeruleus*), Indian cobra (*N. naja*), Saw-scaled viper (*E. carinatus*), and Russell's viper (*D. russelii)* are accountable for most of the snakebites and fatalities in India and therefore considered as medically important (Brunda et al., 2006; R. Whitekar, 2006; Lock & Wellehan, 2015; Uetz 1995; WHO, 2010,2005; Simpson & Norris, 2009).

1.2.1 Indian cobra

Fig.1.2: Indian cobra

Cobra is the important venomous species as it is distributed all over India. From the ancient time cobras are worshiped in India and people have a lot of myths about this snake. The Indian cobra belongs to the Elapidae family, and its venom affects the nervous system. The cobra has an impressive and large hood containing a specific mark. Cobra frequently observed in close proximity of the population in piles of rubble, termite mound, heaps of straw, building foundations, firewood, and farm lands targeting the farmers and farm workers mainly. Frogs, toads, and rodents are the choice of prey of a cobra. The fully developed adult cobra is about 2.2 m in length. The dry weight of average venom injected per strike by Indian cobra is around 60 mg. The quantity of venom generally depends on the mechanical efficacy of the bite, control of venom ejection by the snake (Meenatchisundaran & Michael, 2009; WHO, 2010, 2005).

1.2.2 Common krait

Fig1.3: Common krait

Common krait is an important venomous snake species found all over India. The snake belongs to family Elapidae, mainly affecting the nervous system and very toxic in nature amongst snakes found in India. The krait venom neurotoxins prevent the release of acetylcholine to the neuro-muscular junction. The snake is nocturnal, shy, hides himself during the day, generally observed in a burrow of a rat or mouse, in a termite heap, or underneath of rubbish. Blind worms, small mouse, small snakes, and lizards are the main prey of this snake. The snake contains bluish black or black color body with white bands and the fully developed snake is up to 1.750 m in length. Krait bites generally occur at night mainly to the persons sleeping on the ground or floor. The fang marks by the krait are inconspicuous and almost painless, and there were no local signs of envenoming. Due to above reasons, victims often misdiagnosed, which delays the envenomation treatment and may result in more fatalities due to this snake. The average dry venom injected per strike by the kraits is around 20 mg and the human fatal dose of this venom is around 6 mg (Warrell, 2010; Meenatchisundaran & Michael, 2009).

1.2.3 Russell's viper

Fig1.4: Russell's viper

The Russell's viper commonly observed in all parts of India except the desert areas of Rajasthan and in Assam and other far northeastern states. In India Russell's viper is one of the most significant snake, as it is responsible for the maximum number of snakebites and deaths. The snake belongs to family Viperidae and primarily hemotoxic in nature. Russell's viper venom has many constituents cumulatively liable for local tissue damage and hemotoxic symptoms like hemorrhages, renal failure, hematuria, anemia hyper, and edema. Russell's viper generally found in the cover of thorny and thick plants, leaf garbage, and thick tall grass associated with agri-fields and the rodents are the main prey of this snake. The snake is about 1.8 m in length and having heavyset body with a big head, elongated fangs, and very deleterious venom. The quantity of dry venom sufficient for the death of human is around 15 mg and the snake injects around 63 mg of venom per strike (Theakson R.D.G. & Reid H.A., 1983).

1.2.4. Saw-scaled viper

Fig. 1.5: Saw-scaled viper

The Saw-scaled viper widely distributed in India except wet and heavily forested parts, northeast, Andaman, and the Nicobar Islands. The snake belongs to family Viperidae and like Russell's viper the venom of this snake is also hemotoxic in nature. The snake generally feeds on arthropods such as spiders, crickets, grasshoppers, and scorpions as well as small frogs and mice. Saw-scaled vipers normally reside beneath the rocks. Its average length is about 0.30 m. On an average, this snake can inject around 40 mg of venom per strike and the fatal dose for humans is around 6.65 mg (Warrell, 2010; Meenatchisundaran & Michael, 2009; Theakston, R.D.G. and Reid, H.A., 1983).

1.3 Families of venomous snakes

1.3.1 Elapidae

This family includes snakes like Cobras, King cobra, Kraits, Sea snakes, and Coral snakes. The venoms of these snakes mainly acts on the nervous system of the victim.

Snakes belonging to this family are generally thin, long, with scales on the top (dorsum) of the head. Some cobras form a hood, comprises a mark and has comparatively short fangs. The snake belongs to family are responsible for numerous snakebites in India and world widely. In India, many of the bites are from four snakes also called as Big 4's, two of them Indian cobra and Common krait belongs to this family.

1.3.2 Viperidae

The snakes belonging to this family are thick, comparatively short, and contain a characteristic patterns on dorsal surface of the body and head. The snakes have folding and fairly long fangs, which become straight at the time of the bite. The venom of these snakes is mainly hemotoxic in nature. Worldwide, most of the incidences of snakebites are due Viperidae family snakes. There are two main subfamilies of these snakes, typical vipers (Viperinae) and pit vipers (Crotalinae). Viperinae subfamily includes snakes like such as Saw-scaled viper, European adders, Russell's viper, and Gaboon viper. The Saw-scaled viper and Russell's viper snakes are the cause for most of the snakebites and are included in the Big 4's of India.

1.3.3 Colubridae

Snakes belongs to family Colubridae are more diverse and have global presence except the Antarctics, they have back-fangs and only a some of them are life - threatening.

1.3.4 Atractaspididae

Snakes belong to family Atractaspididae observed in the Middle East and Africa. These snakes are mostly inoffensive and very few cases of snakebites reported due to these snakes.

1.4 Venom composition

Snake venom is a complex mixture of enzymatic, non-enzymatic proteins, nerve growth factors, polypeptide toxins, and non-toxic proteins, having a differing mode of action. The actual mode of action of venom not fully understood. The snakes belonging family

Elapidae, the main toxic factor is a neurotoxin while amongst the snakes belonging family Viperidae; the main toxic factor is haemorrhagins. Considering dry weight the snake venom contains about 90% protein. The venom contains a large number of different proteins and enzymes. Which includes phospholipase A2, digestive hydrolases, L-amino acid oxidase, hyaluronidase, DNAase, phosphomono-and diesterases, 5'-nucleotidase, NAD-nucleosidase, antifibrin, peptidases, thrombase, snake venom metalloproteases (SVMP), and activators or inactivators of physiological processes (Biardi et al., 2012; Fox, 2013; Kanchan et al., 2012; Markland & Swenson, 2013; Warrell, 2010). These proteins classified according to their mode of action on various organs.

1.4.1 Neurotoxins

Neurotoxins are one of the major lethal components of snake venom, present mainly in hydrophid and elapid snake venoms. The cobratoxin, bungarotoxin, and crotoxin are some examples of neurotoxins. These toxins bind to acetylcholine receptors present at the postsynaptic sites preventing binding of acetylcholine, resulting in paralysis of a variety of organs and the condition becomes life threatening.

1.4.2 Cytotoxin

The most important cytotoxins are haemolysin, which disrupts the red blood cells; haemorrhagins mainly affects blood vascular systems, cytolysins affecting the liver, kidney cells, and cells from the bite site. Leucolysin mainly affects the endothelial cells.

1.4.3 Cardiotoxins

Cardiotoxins usually observed in cobra venom causing cardiac asystole and bradycardia. It affects the contraction of skeletal muscle cells, epithelial cells, and prevents the aggregation of platelets. Unusual cardiotoxicity observed in krait venom.

1.4.4 Proteases

Proteases damage the vascular endothelium also responsible for anticoagulant, local

inflammation, fibrinolytic, and necrosis activity.

1.4.5 Hyaluronidase

Hyaluronic acid is an intercellular substance present in epithelial, connective, and nervous tissues of humans. Hyaluronidase allows faster diffusion other fractions of venoms throughout the tissues by depolymerization of hyaluronic acid.

1.4.6 Phospholipase A2 (lecithinase)

Phospholipase A2 stimulates the release of lysolecithin, acts as a powerful hemolytic factor, which hydrolyzes the Phospholipids. It damages the red blood cells, platelets, skeletal muscle, peripheral nerve endings, leucocytes, vascular endothelium, mitochondria, and other membranes. Phospholipase A2 damages the membranes of mast cell and releases active material called as autocoids like 5-hydroxytryptamine, kinins, and histamine, which contribute to local side effects such as bruising and inflammation.

1.4.7 Cholinesterase

Cholinesterase generally observed in elapid (Krait and Cobra) snake venom. Cholinesterases are responsible for hydrolysis of acetylcholine present in neuro-muscular junctions to acetic acid and choline, which impairs transmission neuro-muscular signals.

1.4.8 Phosphodiesterase

Phosphodiesterase is the main cause decrease in blood pressure during envenomation.

1.4.9 L-amino acid oxidase

This enzyme catalyzes the deamination of L-amino acid. The biological activity includes induction of edema, apoptosis, and platelet aggregation (Kanchan et al., 2012).

1.5 Snakebite envenomation

1.5.1 Snake venom apparatus

The venom apparatus of the snake contains two salivary glands, situated underneath and at the back of the eye; ducts connect these glands to grooved or hollow fangs. In Saw-scaled viper and Russell's viper, the fangs are elongated and movable, which consists of a duct from bottom to the tip of the fangs. The fangs pull back against the crown of the mouth at resting condition. In cobra and krait, the size of the fangs is smaller as compared to Viperids and they are fixed in position (WHO, 2010, 2005).

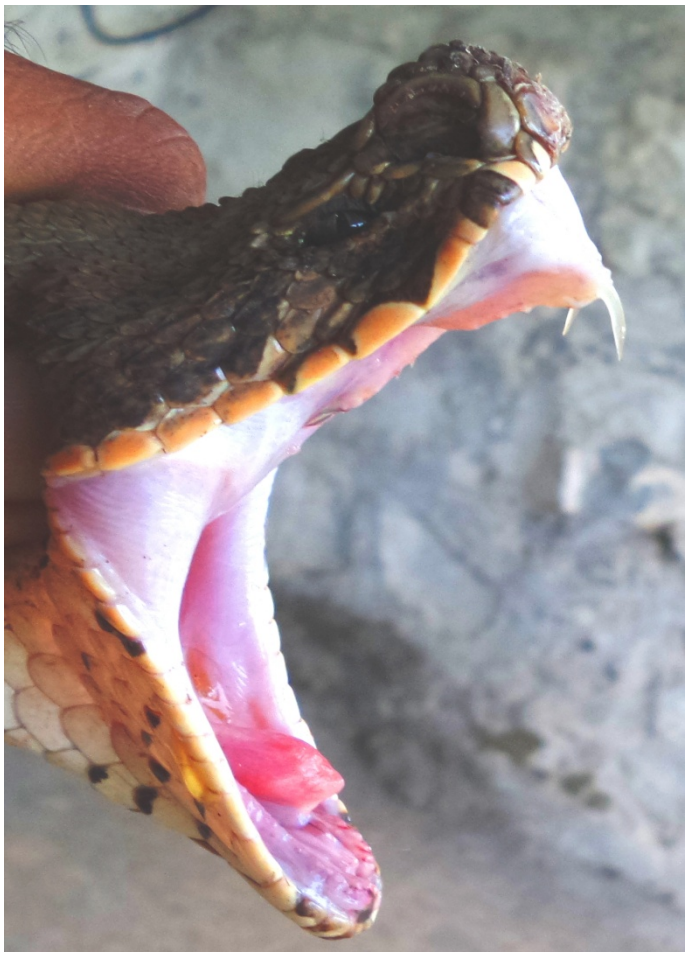

Fig. 1.6: Snake venom apparatus

1.5.2 Snake envenomation

The average venom injected by the snake into the victim is very variable, which depends on the size and species of the offending snake, the mechanical efficiency for a bite, control of venom ejection, severity of penetration of fangs in the skin and signs of any repetitive strikes by the snake. Snakes inject more venom to the second victim than first during rapid succession. Nevertheless, Russell's viper tries to inject nearly all venom during the first strike. Snakes do not inject venom most of the time during the bites, such bites without injection of venom are called as dry bites. However, some bites of venomous snakes may result in the injection of sufficient venom to cause clinical effects. The bites of Malayan pit vipers (50%), Russell's vipers (50%), Indian cobra (30%), and Saw-scaled vipers (5%-10%) do not result in envenomation. (Theakston et al., 2003; Harris et al., 2010; Tun-Pe et al., 1991; Kanchan et al., 2012).

1.6 Snakebite treatment

The rural population of developing countries like India is unaware of treatments of snakebite, most of the snakebite patient go towards the traditional healers. The traditional healers treat a patient with herbal medicines, whose efficacy was not scientifically proven and also the great confidentiality maintained by them about medicine and treatments. Some of the patients cured increasing the popularity of the healer, but the reason behind it may be the bite was a dry bite. It should be taken into consideration that, if the patient is envenomed, the traditional treatment may become life threatening due to incorrect or delay in treatment. Snake antivenom is the only remedy for snake poisoning. Deaths and permanent injuries due to envenomation conquered with quick and reasonable utilization of the snake antivenom to the envenomed victims only, with all required supportive treatments; considering the potential side effects associated with snake antivenom and its shortage world widely (Simpson et al., 2009; Kularatne et al., 2011). Specific treatment with antivenom inclusive of supportive measures depends on the composition of venom, whether venom is hemorrhagic, or neurotoxic in nature, and the related clinical manifestations.

1.7 Snake antivenom

Emil Adolf von Behring revealed the antitoxin a century back and his colleagues demonstrated the capability of serum separated from the blood of the animals hyperimmunized to the Tetanus and Diphtheria toxin to treat the Tetanus and Diphtheria infection respectively. The era of passive immunization began with this break through development of the 19th century. In 1895, Dr. Albert Calmette succeeded in developing antivenom against the monocle cobra (*N. kaouthia*). During further studies, it was understood that venom changes from species to species and require specific antivenom.

In 1932, Haffkine Institute, India developed polyvalent antivenom against four medically important snakes of India viz. Russell's viper, Indian cobra, Saw-scaled viper, and Common krait. Most of the antivenom manufactured commercially today is by traditional technology using equines. The equines become the choice of animal for the production of various antivenoms as they are easy for handling, survive in all climates, yields large quantities of serum and also the technique of purification of horse antibodies are well-standardized (Chatterjee et. al, 1968; Meenatchisundaran & Michael, 2009). The equines can withstand high doses of toxins/venoms and ensure fast seroconversion to produce more antigen specific antibodies. The antibodies produced by equines are more glycosylated and cause strong immunogenic reactions in humans and animals (Malasit et. al, 1986; Theakston et. al, 2003; Bawaskar, 2004; Punde, 2005).

Several attempts have been made to manufacture antiserum in other animals, but with limited success, as no standardized antivenom manufacturing process developed yet. Concern given towards that some diseases that may transfer from the source animal; prions may limit the use of sheep as an animal for the production of therapeutic sera (Paul et al., 2007; Peres et al., 2006).

1.7.1 Fractionation of Antibodies

To minimize possible side effects and antigenicity of the antibody, normally antibodies cleaved to yield $F(ab)_2$ fragments. The $F(ab)_2$ fragment of antibody does not contain the Fc fragment, one of the major antigenic part of an antibody. The cleavage of the Fc fragment decreases the molecular weight of the antibody, the $F(ab)_2$ can migrate faster in the various tissues than the IgG proving rapid and effective neutralization of venom toxins *in vivo*. WHO guidelines, to manufacture snake antivenom mentioned two protocols for manufacturing of ASVA, both includes cleavage of antibody to yield $F(ab)_2$ using pepsin at low pH (3.0-3.3), followed by either precipitation of unwanted protein using Ammonium sulphate or Caprylic acid (Nudel et al., 2012; Wolf D.K., 2008).

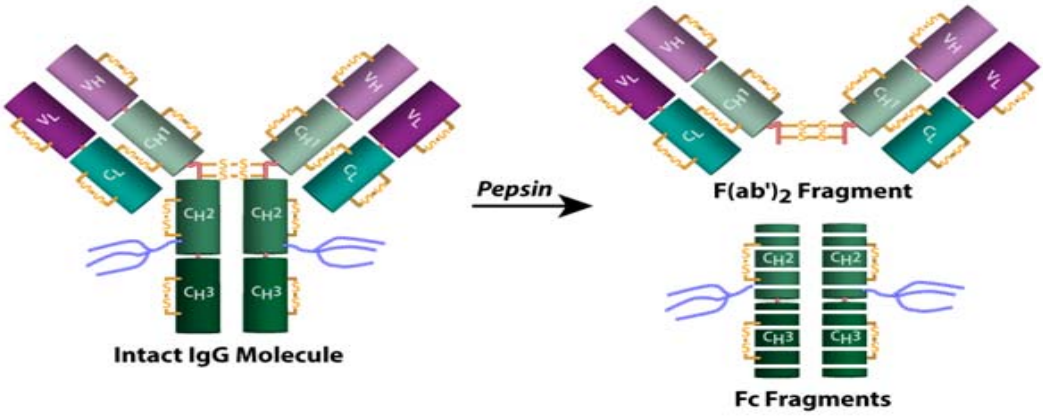

Fig 1.7: Enzymatic cleavage of antibody (Encapsula Nanoscience, 2013)

1.7.2 Fractionation protocol for $F(ab)_2$

The plasma obtained from the immunization of equines following immunization protocol were subjected to fractionation procedures which comprise enzymatic digestion by Pepsin at pH 3.2 for 3 hours, and ammonium salt graded precipitation to remove unwanted proteins includes following main steps. There is a continuous enhancement in manufacturing procedure, in order to prepare effective and safe antivenom. The best antivenom is that which demonstrates the maximum potency, purity with the lowest protein content.

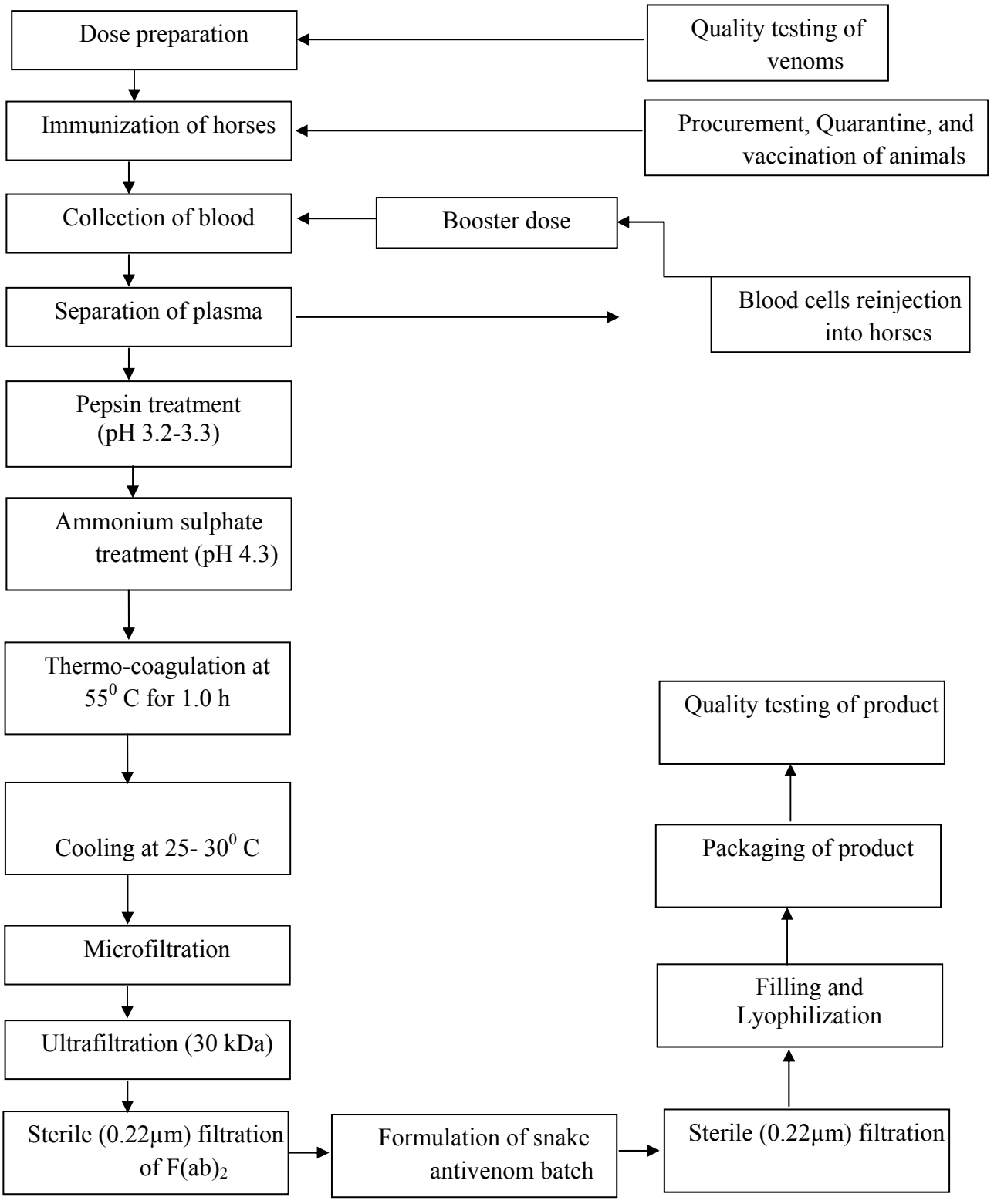

Fig. 1.8: Process flow diagram of manufacturing snake antivenom

1.7.3 Side effects of antivenom

More than 10% of the victims receiving antivenom suffer from antivenom related reactions. The high incidence reactions are due to heterogeneity and the product safety profile of antivenom. The antivenom reaction was principally classified by the WHO into three types of reaction, based on the time required to express the clinical symptom, I) Early anaphylactic reactions, ii) Pyrogenic reactions, and iii) Late serum sickness reactions (Warrell, 2010).

1.7.3.1 Early anaphylactic reactions

The early anaphylactic reactions usually occur within 10-180 min after the administration antivenom. The clinically non severe reaction includes itching, urticaria, abdominal colic, dry cough, nausea, fever, vomiting, tachycardia, and diarrhea. Some patients may develop angio-oedema, hypotension, and bronchospasm, considered as severe anaphylactic reactions. These reactions are based on two different mechanisms, IgE-mediated and non-IgE mediated.

The IgE-mediated reactions avoided or controlled by the administration of Adrenaline intramuscularly. These reactions generally occur in patients previously exposed to the antivenom, leads to the formation of IgE antibodies against antivenom proteins. At the second time of exposure to the antivenom during envenomation, IgE binds with high-affinity receptors, i.e. FcεRI located on mast cells and basophils to induce the cell degranulation that releases mediators of anaphylaxis e.g. leukotrienes, histamine, platelet activating factors, causing increasing vascular permeability and vasodilation leads to anaphylaxis. One more source of IgE stimulation is the traces of antibiotics in the antivenoms, used during treatment of equine infections.

The non-IgE mediated reactions occur without any previous history of antivenom administration by the patients. To decrease these reactions, improvement in antivenom manufacturing is necessary, The extra load proteins can be avoided by specific purification antibodies and cleavage of a Fc fragment of IgG which is a the main reason for the

complement activation which leads to the cascade of anaphylactic reactions (Warrell, 2010; Selvanayagam & Gopalakrishnakone, 1999; Simpson & Norris, 2009).

1.7.3.2 Pyrogenic (endotoxic) reactions

The reactions include chills, headache, fever, myalgia, nausea, and increase in heart rate, vasodilatation, and a fall in blood pressure. These reactions start within 1-2 hours after the administration of antivenom. These reactions arise by pyrogenic contaminants added during the manufacturing antivenom. The pyrogenic reactions initiated by the gram-negative bacterial cell membrane components called as lipopolysaccharides (LPS). The standard procedure of management includes administration of adrenaline, IV fluids, antipyretics, and physical cooling of the patient. To minimize the possibility of pyrogenic reactions, it becomes essential to avoid microbial contamination throughout the manufacturing process, strict adherence to the GMP, and guidelines given by WHO for manufacturing of snake antivenom.

1.7.3.3 Late (serum sickness type) reactions

These reactions usually appear within 7-10 days after administration of antivenom. Clinical symptom includes nausea, fever, vomiting, itching, diarrhea, recurrent urticaria, arthralgia, lymphadenopathy, myalgia, mononeuritis multiplex, periarticular swellings, proteinuria, nephritis, and not often in encephalopathy. To treat the late serum sickness reaction usually corticosteroids and antihistamines are preferred (Alirol et al., 2010; Kanchan et al., 2012; Warrell, 2012).

1.8 Diagnosis of snakebites envenoming

The identification of offending snake species is essential for management of the snakebite as symptoms shown by the patient varies from species to species (neurotoxic or hemorrhagic), and the amount of venom injected.

1.8.1 General examination

During envenomation common symptoms observed is nausea, abdominal pain, vomiting, drowsiness, weakness, bleeding, prostration hemorrhages, hypotension, shock, heaviness of the eyelids, systemic bleeding, blood clotting defects, cardiovascular abnormality, and urinary irregularity like passage of dark brown/black urine (Hung et al., 2003; Warrell, 2005). The symptoms varies in wide range, are not enough to identify the snake species properly and the severity of envenomation.

1.8.2 Examination of bitten part

Local signs at bitten part are fang marks, pain, bleeding, inflammation, necrosis, and infection at the bite site. Kraits are more active during the night, a majority of bites occur in the night. Bites by krait are almost painless, negligible swelling at the bite area, and no prominent bite marks increase difficulties in identification snakebite.

1.8.3 Biochemical tests

1.8.3.1 20-min whole blood clotting test (20WBCT)

This is one of the widely used informative test for detection of envenomation as it is very easy to perform and inexpensive than other methods. In this test, patient's blood collected in clean, dry vessel and kept for 20 min at the ambient temperature without disturbing the vessel, if the blood not clotted after 20 min and runs out; the patient has incoagulable blood, may be due to venom (Sano-Martins et al., 1994).

1.8.3.2 Urine examination

The color of the urine of the envenomed patient may change to red, pink, brown, or black; red cell casts indicate glomerular bleeding. Microscopic examination of urine can confirm the presence of erythrocytes in the urine. Considerable proteinuria is an early indication of the increase in capillary permeability may be due to Russell's viper envenoming (Kanchan et al., 2012; Dong et al., 2003, 1991)

These tests do not clearly indicate the snake envenomation and species. Since a long time, investigators are involved in developing a rapid, simple, sensitive, and reliable test for identification of offending snake species and its envenomation. Moreover, it is very difficult for the medical practitioner to distinguish between snakes due to similar coloration and appearance. Considering above facts, the identification of envenoming and species of snake has become important in the snakebite management.

1.8.4 Immunological techniques

Many serological methods like radioimmunoassay, immunoelectrophoresis, immunodiffusion, and enzyme-linked immunosorbent assay (ELISA) are available for identification the species of snakes. Over the time, ELISA turned into the choice of technique for detection of the snakebites, as first described by Theakston *et al*. (1977) and subsequently reviewed and used for species-specific identification of snake envenomation without reflecting any cross-reactivity within species (Theakston, 1983; Dong et al., 2004; Selvanayagam and Gopalakrishnakone, 1999; Hung et al., 2003; O'Leary et al., 2006).

1.9 ELISA test

ELISA is a diagnostic test used for detection of the antibody or antigen from the test sample. In the ELISA, an enzyme-labeled antibody used to detect the antigen, antibody, and antigen-antibody complexes. Today ELISA extensively used in the field of diagnostics, to detect the absence or presence of antigens or antibody. There are lot of progress in ELISA technique to increase the rapidity, sensitivity, specificity, and simplicity. In hematological studies, ELISA becomes a valuable technique. ELISA extensively reviewed for sensitivity, field application, and identification snake species using venom without ambiguity in results (Brunda and Sashidhar, 2007). ELISA principally categorized into three formats viz. indirect ELISA, direct ELISA, and sandwich ELISA.

1.9.1 Direct ELISA

The direct ELISA term indicates that detection of antigen using the primary antibody bound to the enzyme. In direct ELISA, micro-titre wells coated with the antigen and allowed to react with enzyme labeled antigen specific antibody. The presence of enzyme is detected by the addition of substrate in the wells. The direct ELISA test is very fast compared to other formats also has very fewer background signals, but this test is relatively less sensitive than other formats.

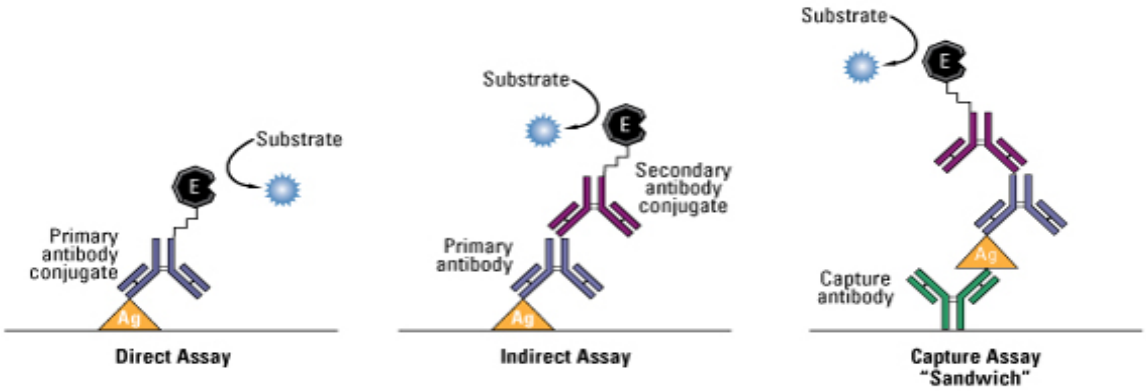

Fig.1.9: Different formats of ELISA (Thermo scientific, 2015)

1.9.2 Indirect ELISA

The indirect ELISA term indicates that an ELISA in which the pre-coated antigen reacts with the added primary antibody and binds specifically and the presence of primary antibodies detected by primary antibody specific secondary antibodies labeled with the enzyme. The Secondary antibodies generated against the primary antibodies in other species of animal by the process of immunization. The indirect ELISA has following advantages.

1) The Indirect ELISA comprises of two antibody–antigen reactions; the first reaction occurs amongst the antigen coated wells and the primary antibody. The second reaction occurs amongst the primary antibody and the enzyme labeled secondary antibody. In this test, the secondary antibodies instead of primary antibodies are enzyme conjugated which

amplifies the signal of enzyme-substrate reaction results in increasing sensitivity of the assay.

2) As the secondary antibodies are species specific, many researchers can use it working on that species although the antigens of interest are different.

1.9.3 Sandwich ELISA

This ELISA called as "sandwich" since the antigen of interest is sandwiched between the primary and capture antibody. The sandwich ELISA can detect both antibody and antigen. The sandwich ELISA is nearly similar to that of the Indirect ELISA, the only difference is the antigen captured actively by the capture antibodies coated in the wells.

The ELISA has advantage over other tests, as it is specific and sensitive to detect a protein even in nanogram & picogram quantities. ELISA has a choice of different formats, easy to standardize, and validate.

1.10 Species-specific antibodies for venom detection

The snake venom is a mixture of several proteins, inorganic, and organic material. Studies confirmed that closely associated species contains several common proteins, which varies according to the season, individuals, and environment (Dong et al., 2003; Sharma et al., 2015). Selective detection of snake venom becomes more critical due to its complex nature. During immunization when native venom used as an immunogen it leads to the generation of polyclonal ASVA against the majority of the proteins injected, which demonstrates cross-reactivity with other snake venoms (Isbister et al., 2010; Selvanayagam et al., 1999; J. F. Gao et al., 2013; Casewell et al., 2010). Application of these antibodies for the detection of species leads to uncertainty in the results, and it becomes essential to remove the cross-reacting antibodies. The SSAb's are generated by allowing to pass the affinity purified ASVA through the conspecific venom columns to remove all non-specific antibodies and the eluted antibodies reacted with heterospecific venom columns to remove all cross reactive antibodies to generate SSAb's. (J. F. Gao et al., 2013; Ibrahim et al., 2013; Selvanayagam and Gopalakrishnakone, 1999). However, today SSAb's are in use for several ELISA based tests also; it has a great prospective for the development of the LFA for snake venom detection.

1.11 Lateral Flow Assay

LFA is the easiest test and normally for testing, it requires no chemicals. The application of test samples like urine, blood, saliva, or any liquid to the sample pad provide results within five to ten min and the result interpretation is by naked eyes in several formats (Wong and Tse, 2008). The LFA results are qualitative mainly representing presence or absence of the analyte of interest, impart an edge over ELISA. LFA is emerging as a field application test, initially applied for early pregnancy detection using the urine. Now the use of LFA extended to diverse fields like agriculture, veterinary, industrial testing, molecular diagnostics, environmental safety and health, food industry, forensic science, and many more (Ju et al., 2010; Bogdanovic et al., 2006; Rong-Hwa et al., 2010; Ching et al., 2012; Sajid et al., 2014; Shyu et al., 2002; Fang et al., 2011).

Advantages of LFA

- LFA is specific, sensitive, rapid, and mainly easy for use.
- It requires the sample in small quantities and in many formats sample pre-treatment not required.
- It can be stored at room temperature for 12-24 months.
- The device is cost-effective as requires reagents in small quantities.
- The manufacturing of LFA needs relatively small investment and area.
- The results of LFA are easy to understand and interpret.

1.11.1 Components of LFA

I) The membrane

The membrane permits binding and retention of proteins over the shelf life at control and test lines. Once the sample is added to the sample pad of the device, the sample driven by capillary flow to the conjugate membrane and further to the membrane, allow it to flow constantly, providing sufficient time to react with test and control lines, and finally flow the excess label, reactants, and fluids to the wicking pad without any background noise. The

choice of material for LFA is a Nitrocellulose (NC) membrane. It has certain characteristics that include, high protein-binding capacity, low cost, correct capillary flow, easy to handle, and the available range of products with different pore sizes, and wicking rates. NC membranes used for LFA have nominal pore sizes from 8 to 15 microns. The polyvinylidene fluoride (PVDF) membranes and nylon membranes are some of the alternatives for NC membranes but have limited success.

II) The conjugate pad

The conjugate pad provides space for conjugate for its whole shelf life, and when the sample exposed to the conjugate pad, it allows the analyte to react with conjugate and releases the complex efficiently and consistently to the NC membrane. The materials generally used as conjugate pads are polyesters, glass fibers, or rayons. The conjugate pads need pretreatment as most of the materials are hydrophobic and it becomes the necessary to treat them to make them hydrophilic. The addition of conjugated antibodies to the treated conjugate pad is a crucial step for the performance of the LFA. Two methods are mainly used; the I^{st} method involves preparation of the conjugate pad by immersing it into the solution and the II^{nd} method involves dispensing solution on the conjugate pad using dispensers. GNPs mainly used as labels for detection.

III) The sample pad

It is one of the most important part of the LFA system, which accepts, treats the sample to make it compatible for assay and release it to the conjugate pad. Sample treatment includes a suitable change in pH, removal of particulates, binding to the assay interfering components, and release of treated analyte to the conjugate pad. The material of sample pad depends on the sample processing requirements. Generally, glass fiber, cellulose, and rayon used as materials for the sample pad.

IV) The wick

It is the most crucial part of the LFA system. Generally, the material of wick is high-density cellulose as cellulose has high absorptive capacities. The wick functions to pull the fluid by capillary action hold it and does not allow fluid to release in a back direction into the assay strip.

V) Labels for detection

There are several existing labels for LFA systems; the selection of labels essentially depends on the LFA application and available instrumentation. Today for LFA colloidal Carbon, liposomes, bioluminescent markers, upconverting Phosphors, fluorescent probes, etc. used as labels for detection, but due to some inherent advantages, the Colloidal GNPs are most widely used in LFA's.

A) **Colloidal gold**

Thomas Graham coined the term colloidal in 1861, which is a Greek word for 'glue'. Today most of the LFA available commercially are using GNPs as detection systems as it is inexpensive and very easy to prepare. The visualization of GNPs is by naked eyes and does not require any sophisticated instrumentation. In 1857 Faraday was the first to do studies for preparation of GNPs and study its properties. The GNPs composed of an elemental gold core surrounding with a negative, ionic double layer of charges. The colloidal GNP firstly used for immunoelectron microscopy in 1971 by Faulk and Taylor and was produced by the reduction of ionic gold with white phosphorus. Later Frens described a simple Trisodium citrate reduction method for producing a GNPs of uniform and controllable size that sets the stage for the extensive use of GNPs as a marker in the biological fields. In 1981, Leuvering *et al*. described the use of GNPs to conjugate with antibodies for the preparation of diagnostic immunoassay.

B) Preparation of GNPs

The basic principle behind the preparation of GNP solution is the conversion of ionic gold to metallic gold in a controlled manner, for these reducing agents like white phosphorus, Sodium borohydride, Ethyl alcohol, Ascorbic acid, and Trisodium citrate are used. The Sodium citrate reduction procedure is simple and most widely used, developed by Frens. In

this method, the ionic gold solution heated to boil and the rapid addition of the sodium citrate solution, immediately, ionic gold starts being converted to metallic gold atoms until the supersaturation. Simultaneously metallic gold atoms start aggregating called as nucleation, which contains central icosahedral structure of gold cores containing 11 atoms, which forms nucleation sites, and this process is extremely quick. The remaining suspended gold atoms start binding to nucleation sites until removal of all atoms from the solution. The produced nucleation sites determine the number of particle. The higher no of nucleation sites in the solution forms smaller GNPs.

VI) Antibodies

Since decades, antibodies are in use for clinical diagnosis of samples in many immunological techniques and the majority of the LFAs use antibodies for recognition of analyte, the antibodies used for application at test and control lines on NC membranes. Many LFAs utilizes antibodies for conjugation with labels. Antibodies have an ability to specifically recognize and bind to the target analyte. Antibody, which binds specifically target analyte, is called as the primary antibody but the one, which is used to bind a target containing an antibody or another antibody, is known as a secondary antibody. For the development of robust LFA, high affinity and specific antibodies for the target antigen is the necessity. The polyclonal and monoclonal antibodies (MAbs) having some inherent advantages and disadvantages, the selection of antibodies has the highest importance.

In several LFA systems, MAbs used to recognize the target analyte have some benefit than the polyclonal antibodies, but for some LFAs, use of polyclonal antibodies is imperative. The polyclonal antibodies being used for LFA must be affinity purified for consistent and reliable results.

VII) LFA architecture

LFA is an Immunochromatographic method; it is a combination of paper chromatography and antigen–antibody reaction. Typically, LFA consists of many components each one serves a different function but components are interdependent.

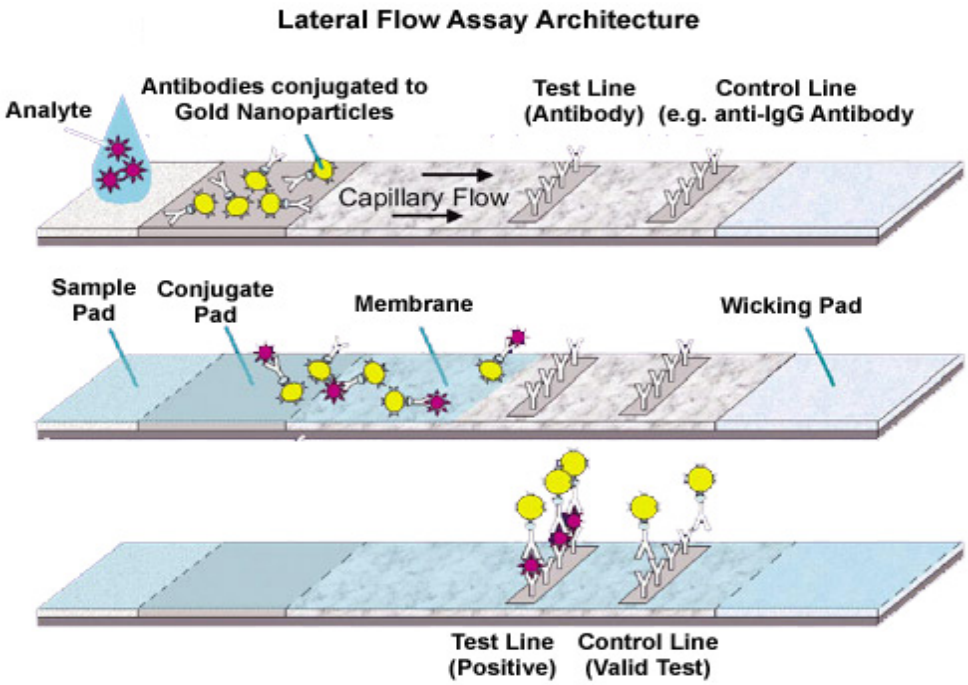

Fig1.10: LFA architecture (Cytodiagnostics.com, 2015)

The first component of the LFA is sample pad. Sample pad receives the sample and makes it compatible for further reactions. The next part of the device is a conjugate pad, typically contains analyte specific antibodies or antigen conjugated with labels. When the sample arrives at the conjugate pad, it initiates the antigen-antibody reaction between the sample and the conjugate. The specific antigen-antibody attaches to each other and nonspecific remains free. The complex migrates to the NC membrane, generally consist two types of lines i.e. test line and control line. At the test line, analyte specific antibodies are present and they capture the antigen-antibody (GNP labeled) complex and indicate detection the analyte. The control line contains antibodies against primary antibodies (GNP labeled) and confers the validity of the test. The remaining analyte /conjugate leaves the NC membrane due to capillary action and gets trapped in the wicking pad; it captures all liquid and does not allow the liquid to go in a backward direction (Wong & Tse, 2008).

1.12 Scope of the study

1.12.1 Problem associated with snakebite envenomation detection

As discussed earlier Snakebite is a neglected, yet the fascinating community health issue leads to life threatening medical emergency. Most of the snakebite occurs mainly in emerging and underdeveloped nations. The WHO anticipated that, about 2,00,000 snakebite cases and approximately 35,000 to 50,000 citizens die due to snakebite per year in India.

Fatalities and permanent impairments from venomous snakebite overwhelmed with an early and reasonable administration of ASVA to the envenomed victims, with all required medical treatments, considering the possible side effects of the antivenom. Generally, snake antivenom contains specific immunoglobulin's obtained by hyper-immunization of equines with venoms. The adverse reactions by snake antivenom are due to heterogeneity and the product safety profile of the product. In many cases, snakebite does not result in systemic envenomation also the severity is very variable, as average venom injected depends on the size, species, control of venom discharged by the snake, mechanical efficiency, and many snakebites are dry bites. Considering above facts, it becomes crucial to determine the severity of snake envenomation and identification species for the appropriate management of the snakebite victims.

The present available physical and biochemical tests do not diagnose the species and snake envenomation clearly. Since a long time, investigators are occupied in developing a simple, rapid, reliable, and sensitive test for identification of offending snake species and its envenomation. Over the time, ELISA turns into the choice of technique for detection of the snakebites, but it has some inherent limitations such that it requires the laboratory, technical staff support and requires more time and hence not suitable for evaluation of envenomation before of ASVA administration.

In this scenario, development of reliable, rapid, and field detection method for snake envenomation has an enormous prospective from the public health point of view for reasonable utilization of snake antivenom and avoiding its possible side effects.

1.12.2 Scope of the present work

To overcome the snake envenomation detection problem, this study focused on the development of rapid, reliable and field diagnostic testing method for snake envenomation detection.

The LFA is a simple, rapid test and generally does not require chemicals for testing and emerged as a test of field application, first used for detection of pregnancy. Today the use of LFA has expanded to many areas including agriculture, diagnostic, veterinary, industrial testing, environmental health and safety, molecular diagnostics, forensic science, food industry, and many more (Bogdanovic et al., 2006; Ching et al., 2012; Rong-Hwa et al., 2010; Sajid et al., 2014; Fang et al., 2011; Ju et al., 2010; Shyu et al., 2002).

In our study, the attempt made to develop GNP based LFA for the RV and CV detection on a device using SSAb's of venoms. The objectives of this study are as follows

I. To generate antibodies against detoxified venom and detection of its neutralizing ability.

II. To purify antibodies generated by affinity chromatography.

III. To standardize ELISA test for antibodies and venom detection.

IV. To detect cross reactivity of antibodies against other venom.

V. To generate GNPs and its conjugation with antibodies.

VI. To develop and validate LFA for detection of venom.

VII. To detect snake envenomation in experimental animals.

CHAPTER 2

LITERATURE REVIEW

2.1 Snakebite epidemiology

Alirol E. *et al.*(2010) stated that, the actual burden of snakebites remains controversial, although there are many attempts in various parts of the world to find out the actual cases and deaths due to snakebite. According to WHO in India more than 2,00,000 incidences of snakebites occur per year and moreover 35,000 to 50,000 deaths due to snakebites Gaitonde B.B. *et al*. (1980) noted that in Maharashtra has the maximum numbers of snakebites, upto 70 bites per one lakh people and deaths are about 2.4 per lakh people /year). National Health profile 2015, published by Central bureau of health intelligence, Ministry of health and family welfare, India estimates as low as about 1,34,980 snakebite cases and 1,180 deaths in the year 2013. The Maharashtra state has about 14,723 cases of snakebites and about 20 deaths due to snakebites. Andhra Pradesh has the highest no. of snakebites about 26680 and about 107 deaths due to snakebites.

It was stated by WHO and many other researchers that the deaths due to snakebites remained unnoticed as in many developing and underdeveloped countries, as the snakebite victims are treated by the traditional healers with the use of herbal medicines and death occurs in their dispensaries. Many die before reaching the hospitals and their deaths remained unnoticed to the authorities remain unnoticed due to improper reporting systems in these countries. In many countries, the data available about the snakebite mortality based on patients admitted to the government hospitals.

According to Warrell D.A., (2012), snake venom is a complex mixture of proteins containing hundreds of proteins, which varies from species to species. The variability in proteins depends on the heterogeneity of the species and has diverse biological action on the body during envenomation.

Snake antivenom is the only remedy for snake poisoning. The antivenom is mainly manufactured by hyperimmunization of the equines. The treatment with antivenom requires supportive treatment depends on the venom, whether venom is hemorrhagic, or neurotoxic in nature, and their clinical manifestations. Meenatchisundaran, S. and Michael, A., (2009) reported that snake antivenom is a precious life saving drug having scaricity across the world. As the antivenom contains equine immunoglobulins against the venom, it acts as a foreign protein and have some adverse effects therefore, given to the envenomed patient only. Generally snakebites are

identified by bite marks, general examination of the patient, biochemical tests, 20 min WBCT and immunological methods like radioimmunoassay, immunoelectrophoresis, immunodiffusion, and ELISA. The ELISA is a method of choice for detection of snake envenomationas it has some advantages over the other methods. (J. F. Gao et al., 2013; Selvanayagam and Gopalakrishnakone, 1999; Ibrahim et al., 2013; Casewell et al., 2010; Theakston, 1983; Dong et al., 2004)

2.2 ELISA for snakebite detection

Brunda *et al*. (2006) used venom specific egg yolk antibodies for detection of Indian cobra venom in biological samples according to him there are many methods for detection of snake venom amongst all these methods ELISA is the ideal method of choice due to its sensitivity and specificity. The use of chick IgY reduces interferences and provides an added advantage in immunoassays. In addition, the use of IgY provided an advantage in reducing the non-specific binding. The time required for the test was about 4 hours.

Dong *et al*. (2003) developed an ELISA kit for specific detection of venom from four common snakes found in South Vietnam. Lyophilized whole venoms of *C. rhodostoma, N. naja,* T. *popeorum, and O. hannah* was used for immunization. The IgG antibodies were purified by the protein A affinity chromatography and checked for cross reactivity with heterospecific venoms by ELISA and it was observed by him that antibodies have the cross reactivity with the heterospecific venoms. To remove the cross-reactive antibodies, immunoaffinity chromatography was performed using different venom bound affinity columns. The obtained SSAb's shown no cross reactivity with heterospecific venom. Further, these antibodies used for preparation of venom detection kits, observed specific and sensitive to detect venoms. The time required for testing was approximately 30 min.

As seen from the above ELISA test is a method of choice due to sensitivity and specificity, but it has some inherent limitation such as, time required is 30 min to four hrs and technical staff required to perform the test. Since snakebites mainly occur in rural areas, facilities to perform the test are scares, also the high cost of the test. Snakebite is an emergency where no doctor can wait until the completion of the test.

2.3 Lateral flow assay

Sajid M. *et al.* (2014) pointed out in review that recently, more investigations focused on the development of LFA for various types of samples. LFA has lots of advantages over other serological methods like easy test procedure, less sample quantity, rapid results, less expensive, field test, and for analysis of result no expertise required.

Ying Ju *et al.* (2010) used colloidal gold based LFA for early detection of *Streptococcus suis* serotype 2 (SS2), a gram-positive bacterium responsible for severe infections such as pneumonia, septicemia, meningitis, and sudden death mostly in pigs and humans. For a generation of polyclonal antibodies, rabbits immunized with inactivated, SS2 bacteria. The antibodies were isolated by affinity chromatography and conjugated with 25 nm GNPs. The conjugate was added to the pre-treated conjugate pad and dried at 37^{o} C for 2 h. The test line coated on NC membrane with 2.0 mg/ml rabbit antibody against SS2 and the control line coated with 0.5 mg/ml goat anti-rabbit antibodies. All the components assembled and then cut into 4-mm-wide strips. The results obtained within 5–10 min sample application and the developed LFA specifically detected the analyte of interest.

Literature review demonstrates that venom specific antibodies are utilized in development various ELISA based assays for snake venom detection. LFA has many advantages over other serological methods and used for detection of many bio-molecules using specific antibodies. Considering above points, venom specific antibodies may be used to develop LFA for snake envenomation detection and LFA has potential to become a method of choice, as it is a rapid sensitive and field test.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Venoms

The venoms (lyophilized) of snakes made available by Haffkine Biopharmaceutical Corp. Ltd. (HBPCL), Maharashtra, India. Venoms provided by HBPCL were a pool of venoms collected from various regions of Maharashtra. These venoms used for commercial manufacturing of antivenom. The venoms provided by HBPCL were reconstituted and lyophilized in aliquots, and maintained at -20^{o} C.

3.1.2 Animals

Swiss albino mice (male) weighing 18 - 20 g and rabbits (New Zealand white) weighing 2.0 - 3.0 kg maintained at HBPCL. The animals domiciled at room temperature, provision of food and water with dark/light cycle maintained during the experimentation.

3.1.3 Materials

I. CFA and IFA - DIFCO, USA.
II. BSA and Bentonite powder - HiMedia, Maharashtra, India.
III. Anti-horse (rabbit) IgG - horseradish peroxidase (HRP) and Anti-rabbit (goat) IgG - HRP - Merck, India.
IV. Tetramethyl benzidine/Hydrogen peroxide (TMB/H_2O_2) substrate- Merck, India.
V. Amicon centrifugal filters - Merck, India.
VI. Mabselect and CNBr-activated Sepharose 4B - GE Healthcare Bio-Sciences Corp. USA.
VII. MaxiSorp Nunc flat bottom 96 well plates - Nunc, Denmark.
VIII. Gold Chloride ($AuCl_4$) 49% - Sigma, Bangalore, India.
IX. NC membrane, conjugate pad, sample pad - mdi membranes, India, provided by Oscar Medicare Pvt. Ltd. Delhi, India.
X. All other reagents and chemicals used were of analytical grade.

3.2 Estimation of protein content

For determination of the sample protein, Bradford's (1976) method was used. For the determination of antibody protein, immunoglobulin (equine) used as a standard. Venom protein content was determined using BSA as a standard. For preparation of standard graph 10 to 100 µg protein diluted in 0.1 ml with 0.15 M NaCl buffer as mentioned in (Table 3.1). The sample dilution adjusted according to standard graph range. Bradford's reagent 5 ml, added to each tube including blank, mixed and incubated for five min. The absorbance taken at 595 nm wavelength. The sample protein content was evaluated from the standard graph.

3.2.1 Reagent preparation

I) Stock solution preparation

10 mg of standard protein dissolved in 10 ml of 0.15 M NaCl buffer.

Sr.	Stock solution (µl)	Buffer (µl)	Volume (µl)	Final concentration (µg)	Bradford's reagent (ml)
1	-	100	100	Blank	5.0
2	10	90	100	10	5.0
3	20	80	100	20	5.0
4	30	70	100	30	5.0
5	40	60	100	40	5.0
6	50	50	100	50	5.0
7	60	40	100	60	5.0
8	70	30	100	70	5.0
9	80	20	100	80	5.0
10	90	10	100	90	5.0
11	100	-	100	100	5.0

Table 3.1 Preparation of dilutions for standard calibration curve to estimate protein

3.3 Determination LD_{50} of snake venom

The LD_{50} of snake venoms determined by the method suggested by WHO (2010). For new venoms to avoid more usage of experimental animals as recommended by the guidelines LD_{50} range finding study was performed using single mouse (18-20 g) per dilution, 0.5 ml injected intravenously. The deaths of mice examined to find out the range of lethality from 0% to 100% and the same range used for determination of venom LD_{50}. To find out the LD_{50} of venom for statistically significant results requires minimum five mice per dilution, in our study; we used six mice group weighing in the range of 18-20 g per dilution. Each mouse was injected with 0.5 ml of diluted venom intravenously (i.v.), prepared in NS solution. The deaths of mice recorded upto 24 hrs and the minimum amount of venom, which kills 50% of animals in the group (LD_{50}), was determined. (Reed, L. and Muench, H., 1938; Sells, 2003).

3.4 SDS –PAGE profiles of venoms and hyperimmune plasma

SDS–PAGE of venoms, hyperimmune plasma, and affinity purified IgG's were performed as procedure mentioned by the Laemmli (1970).

3.4.1 Preparation of reagents

I) 30% Acrylamide solution

Bis - acrylamide 0.8 g and Acrylamide 29.2 g dissolved in ultra pure water and diluted to 100 ml.

II) 1.5 M Tris HCl

Tris base (18.15 g) added in ultra pure water and pH adjusted to 8.8 with concentrated HCl and diluted with ultra pure water up to 100 ml.

III) 0.5 M Tris HCl

Tris base (6.05 g) added in ultra pure water and pH adjusted to 6.8 with concentrated HCl and diluted with ultrapure water up to 100 ml.

IV) 2X Laemmli sample buffer

The Laemmli sample buffer was prepared by taking 4.75 ml ultra pure water and adding 1.25 ml Tris HCl (0.5 M) , 0.5 ml β-mercaptoethanol, 1.0 ml Glycerol, 2 ml SDS (10% w/v), and 0.5 ml Bromophenol blue (0.5%) produce 10 ml.

V) Running Buffer

Glycine 15.014 g, SDS 1.0 g, and Tris Base 3.02 g added to the ultra pure water dissolved completely, pH adjusted to 8.3 and diluted to produce 1000 ml.

VI) SDS- PAGE gel preparation

Reagent Name	Resolving gel (12%)	Resolving gel (10%)	Stacking gel (4%)
1.5 M Tris Buffer pH 8.8	2.5 ml	2.5 ml	-
1.5 M Tris Buffer pH 6.8	-	-	2.5 ml
30% acrylamide	4.0 ml	3.35 ml	1.34
10% APS	0.05 ml	0.05 ml	0.05 ml
20% SDS	0.05 ml	0.05 ml	0.05 ml
Ultra pure water	3.4 ml	4.05 ml	6.05 ml
TEMED	0.007 ml	0.007 ml	0.01 ml

Table 3.2 SDS- PAGE gel preparation

VII) Fixing reagent:

10.0 ml Acetic acid (glacial) and 40 ml Ethanol dissolved in ultra pure water to produce 100 ml.

3.4.2 Reagents for silver staining

I) Sensitizing reagent

Sodium thiosulphate 0.2 g and Sodium acetate 6.8 g dissolved in ultra pure water. In the solution 30 ml absolute ethanol and added 0.5 ml Glutaraldehyde (25%) solution, the final volume produced to 100 ml with ultra pure water.

II) Silver nitrate reagent

0.25 g of Silver nitrate dissolved completely in ultra pure water and 40.0 µl Formaldehyde (37%) solution added, to make a final volume to 100 ml with ultra pure water.

III) Developing reagent

2.5 g Sodium carbonate dissolved in ultra pure water and 50.0-µl Formaldehyde (37%) solution added , to make a final volume to 100 ml with ultra pure water.

IV) Stopping reagent

1.5 g EDTA dissolved and the final volume made to 100 ml with ultra pure water.

3.4.3 Procedure

I. The gels casting unit was cleaned, 12 % resolving gel was prepared as mentioned in (Table 3.2) and added to casting gel unit with care to avoid air bubbles.
II. After 30 min, the stacking gel was prepared, poured carefully on resolving gel. Immediately comb was inserted avoiding air bubbles and allowed to solidify.
III. The comb removed carefully; the gel assembled in electrophoresis unit and added running buffer to equilibrate the gel.
IV. Samples treated with reducing Laemmli sample buffer at 95 °C for 2 min.
V. Five µg of protein sample and five-µl standard protein marker (broad range) was loaded carefully in the wells of the gel.

VI. Electrophoresis performed using an electrophoresis system (WEALTEC Corp., USA.) at ambient temperature. The current flow maintained at 20 mA till the dye front migrates to bottom.

VII. Washing given to the gel with ultra pure water, placed in fixing reagent on a shaker for 30 min, and again washed the gel with ultra pure water.

VIII. The gel was transferred to a sensitizing reagent on a shaker for 30 min and washed the gel 2 to 3 times with ultra pure water.

IX. Transferred the gel to a silver reagent for 20 min on the shaker and washed again 2 to 3 times.

X. Bands developed by adding developing reagent and the reaction terminated by adding stopping reagent.

XI. The profile of proteins captured on the Gel doc system (GeNei, India).

XII. Rabbit plasma (Monovalent) and antibodies purified by affinity chromatography treated with sample buffer (native) and proteins separated on 10% gel, for native sample buffer β mercaptoethanol not added, all the composition was same as mentioned in reducing sample buffer and the same procedure followed as mentioned above.

3.5 Detoxified venom preparation

All appropriate precaution taken at the time of venom handling, since lyophilized venom powder is highly antigenic in nature and may lead to cause severe side-effects including allergy and respiratory distress. The venom preparation area having separate air-handling unit and all the venom activities strictly performed in the biosafety cabinet. During handling of venoms, the appropriate gowning procedure followed with all necessary safety equipment. The detoxification method mentioned by Brunda *et al.* (2006), followed for detoxification of RV and CV. In brief, for the preparation of venom stock solution 1 mg/ml lyophilized venoms reconstituted with sterile normal saline. Venom stock solution heated at 60^{o} C for 60 minutes and immediately cooled for 10 min, and the process repeated two times. Detoxified venoms prepared freshly for immunization.

3.6 Immunization

Immunization of rabbits carried out as mentioned by Brunda *et al.* (2006) with some modifications. New Zealand white (male) rabbits weighing about 2.0 to 3.0 kg were chosen for the generation of antibodies against RV and CV, respectively. The dose was prepared by considering LD_{50} values of the venoms. To avoid contamination of microorganisms and reducing the microbiological load, dosages were prepared under strict aseptic environment.

During preparation and administering of dosages any direct contact to venoms and adjuvants was avoided, as some adjuvants contains inactivated *M. tuberculosis* and mineral oil, which can produce severe manifestations. The first immunization dose of rabbits was prepared using detoxified venom 100 µg per Kg body weight and CFA in equal amounts by following instructions. To obtain an emulsion of CFA repeated plunging was performed using sterile syringe ($26^{1/2}$ gauge) untill a whitish emulsion produced and its consistency was checked by drop test. The immunization of rabbits was carried out by shaving area on each side of the abdomen. The area was thoroughly disinfected with 70% ethanol and injected 0.5 ml sub-cutaneously (s.c.) on each sides of abdomen. Subsequent two dosages of venom using IFA repeated with 3 weeks interval. The IFA emulsion was prepared similarly as described for CFA emulsion. Further, two subsequent venom dosages were prepared and injected by using native venoms as mentioned in Table 3.3 using 2% w/v solution of Bentonite in equal amounts. It was reported that absorption of antigen to Bentonite would enhances the immunogenicity of the antigen. The use of different adjuvant changes the presentation of the antigen resulting in better seroconversion. Additional, two booster venom doses of native venoms containing 100µg per Kg body weight were prepared in NS and administered. Blood samples from the ear vein of immunized rabbits were collected after 10 to12 days of immunization. The tubes for blood collection was prepared by adding 5% w/v Sodium citrate. The blood collected and centrifuged for 5 minutes at 5000 g to get clear plasma. The clear supernatant collected and filtered using 0.45 µ syringe filters and stored at 2-8 ^{o} C. Indirect ELISA was performed for monitoring of antibody levels in the plasma (Brunda et al., 2006; Pawade et al., 2016).

Sr. No	Time (Weeks)	Venom(µg)	Adjuvant	Dose volume
1	1st	400 (Detoxified)	CFA	2 ml
2	3rd	400 (Detoxified)	IFA	2 ml
3	6th	400 (Detoxified)	IFA	2 ml
4	9th	200	Bentonite	2 ml
5	12th	400	Bentonite	2 ml
6	15th	400	Nil	2 ml
7	18th	400	Nil	2 ml

Table 3.3 Plan for immunization of rabbits

3.7 Plasma purification

The Mabselect is an affinity medium contains protein A (recombinant) produced in *E. coli.* The protein A particularly binds to the IgG at Fc region. The antibodies from the rabbit hyper-immunized plasma were separated using Mabselect™, as per the manufacturer instructions. To avoid cross-contamination of antibodies, the purification performed on separate columns manually for both monospecific plasma samples (Paul et al. 2007; Pla et al. 2012).

3.7.1 Reagent Preparation

I) Buffer A

0.283 g Sodium phosphate, 0.877 NaCl dissolved in 75 ml ultra pure water; the pH of the solution was adjusted to 7.2 and diluted to 100 ml.

II) Buffer B

2.941 g sodium citrate dissolved to produce 100 ml with ultra pure water and pH was monitored which should be between 3.0 - 3.6.

III) Tris HCl 1M

Tris base (12.1 g) added to dissolve in 70 ml ultra pure water, the pH of the solution was adjusted to 9.0 with concentrated Hydrochloric acid and diluted to 100 ml with ultra pure water.

3.7.2 Protocol for affinity purification

I. The buffer B was titrated with 1 M Tris-HCl to obtain neutral pH. Collection tubes prepared by taking 200 µl of 1 M Tris-HCl.
II. Rabbit plasma was diluted 1:1with buffer A.
III. Column washed with eight column volumes with the buffer A to remove storage buffer and equilibrated with ten volumes of buffer A.
IV. The diluted plasma sample was applied to the column and kept for 10 min.
V. Washing given to the column until no traces of protein observed in the washing.
VI. Elution buffer added to the column, allowed to react and elution collected in collection tubes.
VII. The column regenerated with elution buffer and stored in buffer A.
VIII. The collected elution desalted and concentrated using Amicon® Ultra - 4 10K centrifugal filter devices.

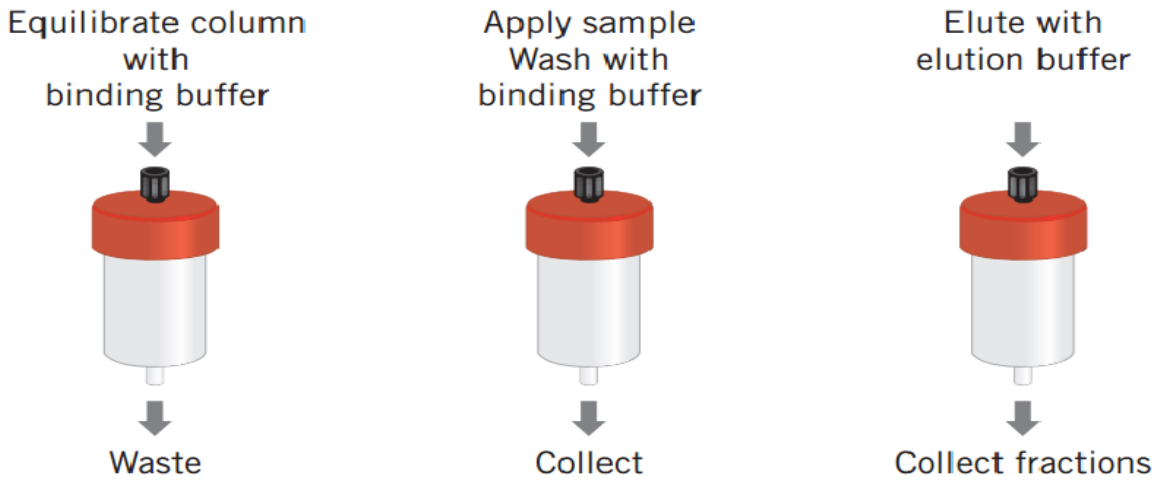

Fig. 3.1 Schematic diagram for affinity purification of monospecific plasma (GE Healthcare Bio-Sciences Corp. USA**.,** 2015)

3.8 Determination of ASVA by ELISA

The antibody titer of plasma was determined using the indirect ELISA method. Checkerboard analysis applied to optimize of Indirect ELISA (Pawade et al., 2016; Rial et al., 2006; Salvi et al., 2010, 2013; Wild, 2001).

3.8.1 Preparation of reagents

I) Carbonate buffer

2.930 g of $NaHCO_3$, 1.590 g of Na_2CO_3 allowed to dissolve in 750 ml of ultra pure water, the pH adjusted to 9.6 and diluted to 1000 ml.

II) Phosphate buffer saline, and Tween 20 (PBST)

1.500 g Na_2HPO_4, 8.0 g of NaCl, 0.200 g KCl, and 0.200 g of KH_2PO_4, dissolved completely in 750 ml of ultra pure water, the pH of the solution adjusted to 7.4, added slowly 0.5 ml of Tween 20, and diluted to 1000 ml with ultra pure water.

III) Blocking solution

1 g BSA dissolved in 100 ml PBST

IV) 1N H_2SO_4

0.54 ml of concentrated H_2SO_4 carefully added to ultra pure water to produce 20 ml.

3.8.2 Procedure

I. Venom diluted to two µg/ml using a carbonate buffer. The ELISA plate coated with venoms by adding 100 µl above diluted venom to the wells and incubated for 18 hrs at 2-8^{o}C.

II. To remove traces of unbound venom, three washings given to the plate using PBST, on ELISA plate washer (BioTek, USA) .

III. Two hundred µl of blocking buffer added to the wells and kept at RT for 60 min. The blocking buffer traces removed by washing (three times).

IV. The wells were applied with 100 µl of diluted (1:10000) equine ASVA samples and incubated for 30 mins at RT followed by three washing cycles using PBST.

V. The wells were added with a 100µl solution of anti-horse IgG-HRP conjugate (1:20000), incubated at RT for 30 minutes, and to remove traces of all unbound secondary antibodies the wells were washed five times with PBST.

VI. Afterwards, the wells were applied with 100 µl of TMB/H_2O_2 and incubated for 15 min in the dark.

VII. Finally, to terminate the enzymatic activity, the wells added with 100 µl of 1N H_2SO_4. The developed color in the wells was measured using an ELISA reader (BioTek, USA) at 450 nm. To determine rabbit ASVA, same protocol was followed using anti-rabbit IgG-HRP secondary antibodies. All the analysis performed in triplicate.

3.8.3 Standardization of ELISA for ASVA determination

To Standardize ELISA for determination ASVA potency linearity, specificity, and the range of the method was assessed. The standard ASVA provided by HBPCL, Pune. The plasma diluted in such a manner that the potency ranges from 0.025 to 0.2 mg/ml. Generally, the same range of potency considered for immunization and manufacturing. For the assessment, each dilution was prepared in triplicate and the same dilutions of normal plasma used as blank at each point. The optimized Indirect ELISA protocol followed during entire experimentation. The ELISA results analyzed by Graphpad Prism-6 software.

3.9 Detection of RV and CV by sandwich ELISA

The indirect sandwich ELISA carried out as stated by Selvanayagam *et al.*,(1999) with minor changes and the Checkerboard method was used for optimization of ELISA (Gilliam et al., 2013; Pawade et al., 2016; Salvi et al., 2010, 2013).

I. Equine ASVA was diluted five µg per ml with coating buffer. The wells of ELISA plate was coated by adding 100 µl above diluted sample and incubated for 18 hrs at 2-8^{o}C. Consecutively three washings were given using ELISA washer.

II. Two hundred µl of blocking solution was added to the wells and kept for 60 mins. The traces of blocking buffer removed by washing three times using PBST.

III. The wells applied with 100µl of diluted venom samples and incubation given for 30 min at RT, followed by three washing cycles using PBST.

IV. The wells allowed to react with 100 µl (1:1000) diluted purified ASVA (rabbit) for 30-min at RT, followed three washing cycles.

V. In the wells added with the 100 µl solution of anti-rabbit IgG-HRP conjugate (1:20000), incubated at RT for 30 minutes, and to remove traces of all unbound secondary antibodies the well were washed five times with PBST.

VIII. Afterwards, the wells were applied with 100 µl of TMB/H_2O_2 and incubated for 15 min in the dark.

IX. Finally, to terminate the enzymatic activity, the wells added with 100 µl of 1N H_2SO_4. The developed color in the wells was measured using an ELISA reader. All the analysis performed in triplicate and the values showed are blank corrected.

3.9.1 Standardization of sandwich ELISA for detection of RV and CV

To standardize indirect sandwich ELISA, for detection of RV and CV linearity, specificity, and the range of the test was evaluated. The plates coated with ASVA allowed to react with RV and CV in triplicate. The logarithmic dilutions (0.01 to 1000 ng/ml) of venoms applied separately using BSA as a blank at each point. All the procedure followed as mentioned in the indirect sandwich ELISA procedure for both venoms and checked for parameters like standard deviation, linearity, range, and R^2 values analyzed by Graphpad Prism-6 software (Pawade et al., 2016).

3.9.2 Assessment of cross-reactivity

For assessment of efficiency and cross-reaction between ARV-Abs and ACV-Abs, sandwich ELISA was performed. Conspecific and heterospecific venoms applied to pre-coated plates in triplicate with graded dilutions from 0.01 ng/ml to 1000 ng/ml. The ARV-Abs and ACV-Abs (1:1000) added in respective cross-reactivity experiments. The optimized sandwich ELISA protocol followed for cross-reactivity of affinity purified antibodies and SSAb's of both venoms (Pawade et al. 2016; O'Leary et al. 2007).

3.10 SSAb's preparation by immuno-affinity chromatography

3.10.1 Preparation of column

The method described by Dong L.V. (2003) and J. F. Gao *et al*., (2013) used to isolate the RV and CV SSAb's (RV-SSAb's and CV-SSAb's). Briefly, the resin was swollen in 1.0 mM HCl (pH 3.0) medium for about 1 hr at RT with occasional gentle side to side stirring. The separated slurry was then equilibrated with coupling buffer (Sodium bicarbonate 0.1 M, with NaCl 0.5 M, pH adjusted 8.3). 20 mg of snake venom diluted in coupling buffer, applied to the slurry and incubated overnight at 2-8 ^{o}C on a horizontal rotator. The slurry extensively washed with coupling buffer. The unoccupied sites were blocked with the help of 0.1 M Tris HCl-pH 8.0 at RT for two hrs, followed by three alternate washings with

acetate buffer (0.1 M), NaCl (0.5 M)-pH 4.0 and Tris HCl (0.1 M), NaCl (0.5 M)-pH 8.0. The final wash given using Tris HCl (0.1 M) buffer and slurry filled into the column. The column equilibration performed using 20 mM PBS, pH 7.0 and stored at 2-8 ^{o}C until use.

3.10.2 Immuno-affinity purification of antibodies

ARV-Abs and ACV-Abs applied to the conspecific venom columns and permitted to react with the venom. The columns washed with PBS buffer pH 7.0 until no traces of protein observed in the washings. The venom-specific antibodies eluted by adding 0.1 M Glycine (pH 2.8), allowed to react, elution was collected in collection tubes and containing the previously determined amount of 1 M Tris HCl as described in protein-A affinity protocol.

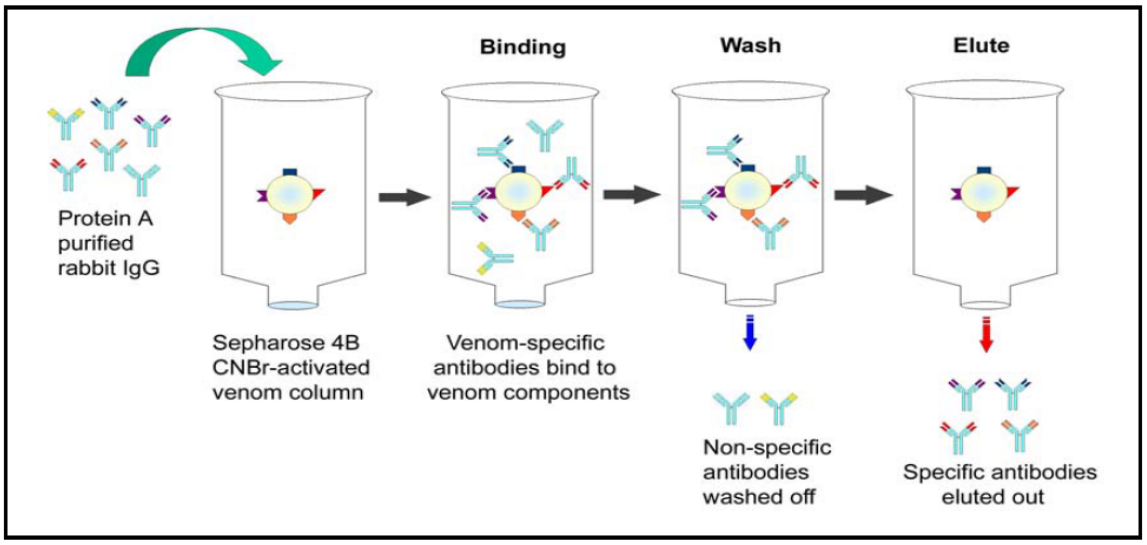

Fig 3.2 Schematic representation of immunoaffinity purification, Protein A purified rabbit IgG was applied on Sepharose 4B CNBr –activated conspecific venom-A column. Venom specific IgG was captured and unbound components are washed, venom specific IgG eluted by the use of low pH buffer (Dong L.V., 2003).

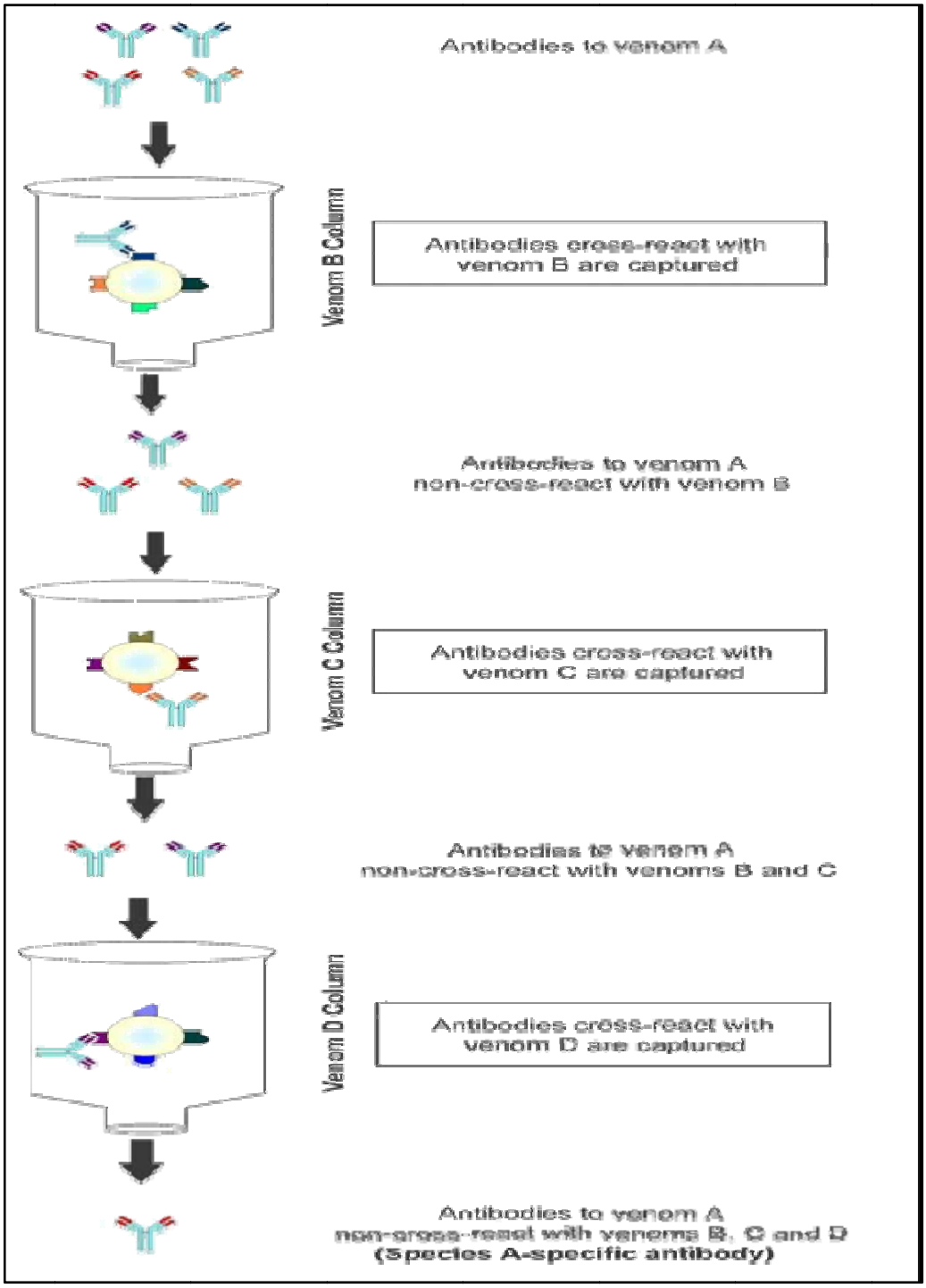

Fig. 3.3 Preparation of SSAb's. For purification of SSAb's, the conspecific venom immunoaffinity purified antibodies passed through heterospecific venom columns to remove cross-reacting antibodies by binding. The unbound part contains SSAb's to the conspecific venom (Dong L.V., 2003).

3.10.3 Preparation species-specific antibodies (SSAb's)

For the preparation of SSAb's, the immunoaffinity-purified antibodies were loaded on heterospecific venom column to adsorb cross-reacting antibodies and collected unbound fraction. The collected fraction passed through next heterospecific venom columns as shown in the schematic diagram of SSAb's preparation (Fig. 3.3) to yield SSAb's. The antibodies subjected to desalting, concentration using Amicon® Ultra - 4 10K centrifugal filter devices as per manufacturer's instruction and stored at 2-8^{o}C.

3.11 Synthesis of GNPs

The 25 nm GNPs prepared by the method illustrated by Grabar *et al*. 1995. For the preparation of GNPs, care was taken to avoid contamination, all the glasswares used for synthesis were washed with 0.22 µm filtered ultra pure water. For preparation of $HAuCl_4$ solution and Sodium citrate solution, used 0.22 µm filtered ultra pure water. To prepare 0.01 % $HAuCl_4$ solution, 20 mg of $HAuCl_4$ dissolved in 200 ml ultra pure water. The solution heated and allowed to boil, rapidly added 2 ml Tri-sodium citrate (1%), the solution stirred continuously which initially formed blue color and then changed to a cherry red. The solution boiled additional for 10 min, and care taken during boiling that vapors to condensate and maintains the volume of the solution. Followed by stirring of the solution for 15 min; cooled to RT and stored at 2-8 ^{o}C in an amber glass bottle. The gold solution analyzed on an UV spectrophotometer (UV1800, Shimadzu Corporation, Kyoto, Japan) to find out the maxima (λ max) and the OD of the solution (Grabar et al., 1995; Pawade et al., 2016)

Fig. 3.4 Preparation of the 25 nm GNP solution

3.11.1 Determination of the amount of antibodies requires to stabilize GNPs

The minimum quantity of antibodies require for stabilizing GNPs evaluated as stated by Slot and Geuze, (1984) with minor modifications. Briefly, GNP and antibody solution taken and pH maintained to 7.0 using carbonate buffer. In each tube, two ml of GNP solution was taken and 0.2 ml of antibody solution containing protein concentrations from 100 to 700µg/ml added, mixed well and after 1 min, 2 ml of 10% w/v NaCl added to each tube. The stability of GNP solution was observed by taking the absorbance using

spectrophotometer at 580 nm. The minimum quantity of protein required to stabilize the GNPs was determined by the point from which there is no decrease in the OD of the solution. After addition of the NaCl, GNPs start aggregating if not stabilized by proteins. For the final conjugation experiment, added 10 % excess antibodies to the stabilization point (Slot and Geuze, 1984).

3.11.2 Preparation of GNP probes

Antibodies conjugated with GNPs by the procedure illustrated by (Hung et al., 2014) with some changes.

I. The purified ASVA (equine) diluted to 1 mg/ml.
II. The pH of GNPs was adjusted to 7.0 using carbonate buffer.
III. The determined quantity of antibodies added to the gold solution and stirred constantly for 1 hr.
IV. The available unbound sites on GNPs blocked by the addition of BSA to make a final concentration in the solution to 1% and the solution centrifuged on the fixed rotor centrifuge at 30000 g for 30 min (Remi C-24BL, India).
V. To pellet dissolved in PBS, containing 1% BSA, 2% sucrose (pH 7.4) and kept at 2-8 ^{o}C.

3.12 LFA preparation

An LFA device contains of four key components; conjugate pad, NC membrane, sample pad, and absorbent pad. An NC membrane provide sites for capture antibodies. The LFA device prepared and optimized according to (Posthuma -Trumpie et al., 2008) with some changes.

I. The SSAb's and anti-equine antibodies diluted to one mg per ml with carbonate buffer.
II. NC membrane having pore size 10 μm applied with different concentrations of antibodies at control and test lines, for optimum concentration determination, which subsequently finalized to one μl per cm.

III. TLC spotter (Linomat IV, Camag, Berlin, Germany) used to plot the control and test lines with anti-horse antibodies and SSAb's of CV and RV with their respective positions as shown in the schematic diagram (Fig. 3.5). Care was taken to avoid any contact with NC membrane, as these membranes are delicate and may be damaged or contaminated, giving background noise, which may create ambiguity in the results.

IV. These membranes desiccated in an oven at 37 $\pm$ 1^{o} C for one hr separately, to immobilize antibodies on the surface of the NC membrane.

V. The unoccupied sites on the membrane was blocked by applying solution 1% BSA in PBS for 30 min with gentle rotation. To remove the unbound BSA, the membranes washed two times with PBS. The membranes dried in the oven completely at 37 ^{o}C for two hrs.

VI. For the preparation of conjugate pad, the pads (PT-R7) immersed in the solution of GNPs conjugated with antibody, followed by complete drying in the oven at 37 ^{o} C for two hrs.

VII. The sample pad (GFB-R7) was pretreated and used without any further treatment.

VIII. The LFA device assembled by placing other components on plastic backed NC membranes, the adhesive protecting paper was removed and absorbent pad was placed in the direction of flow as shown in (Fig. 3.5) by overlapping five mm to the NC membrane.

IX. The treated conjugate pads kept towards the test line ending, by overlaping five mm to the NC membrane.

X. The sample pad kept at one end of the plastic backing in such a way that it overlaps five mm to the conjugate pad.

XI. On assembling all the components on plastic backing of the NC membrane, the strips prepared by cutting the membrane in three mm width vertically.

XII. These strips accommodated in a plastic cassette and the device kept at RT, protected from humidity and sunlight until use.

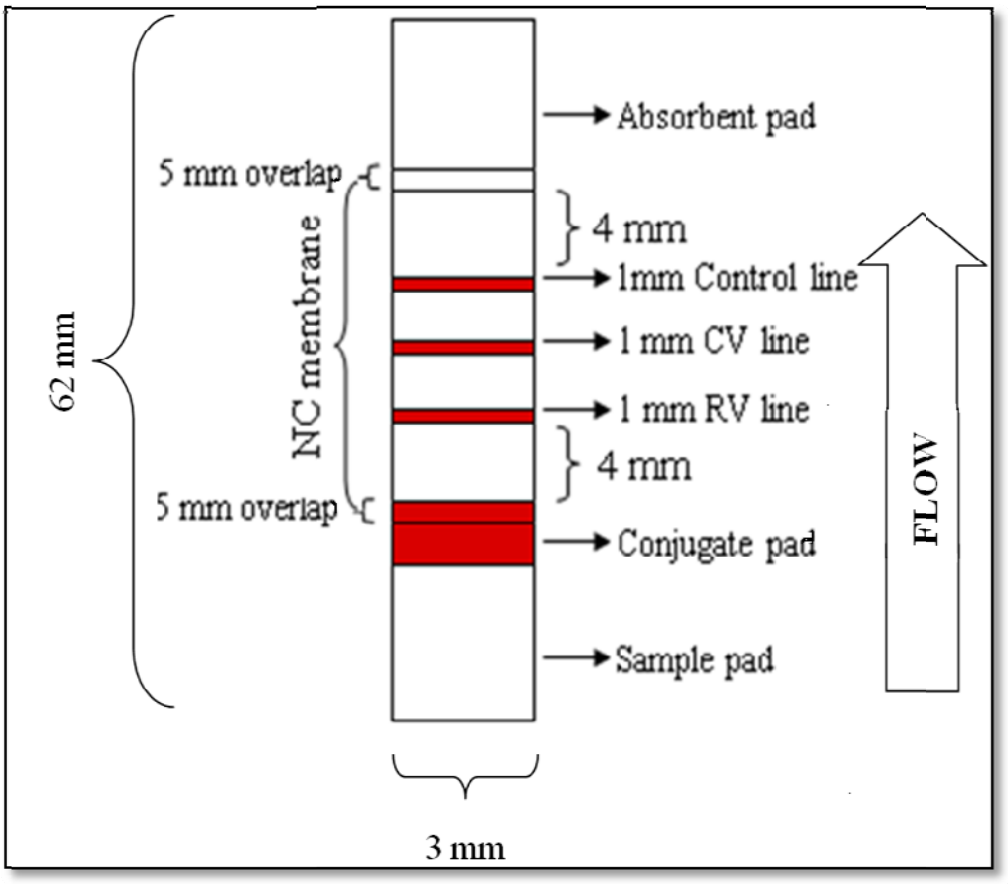

Fig. 3.5 Schematic diagram of the SEDIA strip.

3.13 Assay performance of SEDIA device

3.13.1 Specificity

To determine the specificity of SEDIA device, all venoms (CV, RV, EV, and KV) were spiked individually using PBS buffer such that the final concentration of venom in the solution was 1000 ng/ml. To check specificity and cross-reactivity of the SEDIA device, applied diluted samples (about 100 µl). The results evaluated by the visualization of red lines at the respective test and control regions.

3.13.2 Sensitivity

To find out the sensitivity of the SEDIA device, a logarithmic dilution (1000 ng/ml to 0.01 ng/ml) of CV and RV venoms prepared in PBS. All the simulated samples applied to SEDIA device (about 100 µl) to demonstrate the sensitivity of SEDIA. Strip 1 was applied with PBS and device 2 to7 with a serial dilution of venom respectively.

3.14 Experimental envenomation

Swiss albino mice used to simulate the experimental envenomation. In brief, concentration venom adjusted in NS such a way that it should contain two LD_{50} of venom per dose. The diluted RV and CV injected 250 µl subcutaneously to the group of Swiss albino mice weighing 20-22 g and concern given towards minimum pain to the animal during the entire experiment. The sample was collected from the heart of the mice by cardiac puncture method after 30 mins of envenomation (Group1,n=3), and after 60 mins (Group II,n=3). Anticoagulant 5% w/v solution sodium citrate added to the tubes to make a final volume to 5% of the total volume. These tubes centrifuged to separate the plasma on the fixed rotor centrifuge at 5000 g for 5 minutes. The plasma is stored at 2-8 ^{o}C until use. The samples were diluted 1:1 with PBS and 2-3 drops (100µl approx) applied to the SEDIA device with the help of dropper to assess its functionality. The sandwich ELISA of the samples was performed using one hundred µl of the sample to estimate the amount of venoms from the samples using the standard curve and OD obtained plotted on a graph to estimate the amount of venom (Brunda et al., 2006).

CHAPTER 4

RESULTS

4.1 Characterization of venoms

The four native venoms were assessed in reducing condition using SDS–PAGE (12%). The bands were visualized by silver staining of the gels (Fig.4.1). It was observed that RV has more number of prominent bands distributed below 97.4 kDa. The KV contains lower molecular weight proteins and a band near 66 kDa, probably of Bungarotoxin. During immunization, it was observed that RV was more immune responsive than the CV. The reason behind this may be high molecular weight proteins are more antigenic compared to the lower ones. The figure 4.1 demonstrates that each venom has the distinct banding pattern, nevertheless, some bands overlapped between venoms representing equivalent molecular weight proteins and may be the basis for the cross-reaction among venoms as established during further studies.

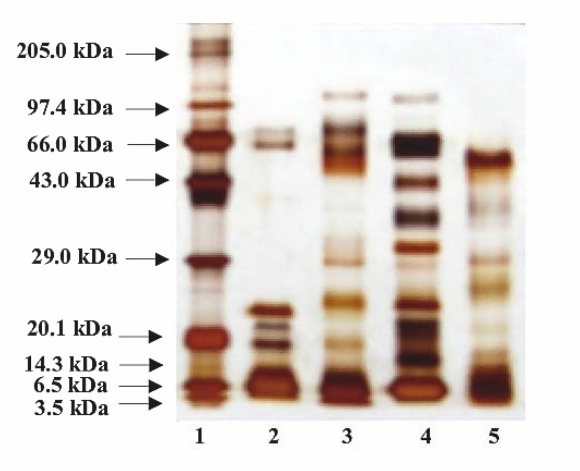

Fig. 4.1: SDS–PAGE (12%) of venoms. Lane 1 contains the standard molecular weight protein marker, whereas Lane 2: KV, Lane 3: CV, Lane 4: RV, and Lane 5: EV; the arrows indicate the standard molecular weight bands in kDa.

4.2 Protein content and LD_{50} of the venom

The amount of protein was estimated by Bradford's method. The protein content of the venom varies from species to species. It observed that RV and CV has more protein content as compared to KV and EV (Table 4.1). Although the KV have less protein content, it was more toxic compared to other venoms. During LD_{50} determination, all deaths occurred

within 12 hrs of injection. The deaths due CV and KV are mainly due to neurological effects such as flaccid paralysis of hind limbs, disorientation, and respiratory failure. The deaths of mice inoculated with EV and RV are mainly due to hemorrhagic effects, bleeding gums, and blood in the urine.

Sr. No	Venom	venom protein (g/g)	LD_{50} / mouse (SD)
1	CV	0.91	7.52 (0.18)
2	RV	0.86	8.33 (0.39)
3	KV	0.72	2.90 (0.09)
4	EV	0.79	10.59 (0.25)

Table 4.1: Determination of LD_{50} and protein content of the venoms. LD_{50} values are average values (n=3) and SD values indicated in the bracket.

4.3 Immunization monitoring

For the beginning of immunization, the LD_{50} of venoms taken into consideration and only sub-lethal doses of venoms injected subcutaneously. Immunization by means of detoxified venom permitted larger amount of antigen with no any harmful effects on physical conditions of the animal. During immunization monitoring, we observed that although the immunization carried out using detoxified venoms, generated antibodies have activity against the native venoms. The rate of seroconversion increased after 6 weeks of immunization, persisted till 18^{th} week and maintained thereafter, which proved that the immunization with a combination of adjuvant gave better seroconversion. The change in antigen presentation led to yield more neutralizing antibodies. CV observed less immune responsive than RV, as it contains more number of lower MW proteins and this is the reason generally CV antibodies are used as a marker for immunization in manufacturing of polyvalent ASVA. Indirect ELISA used for monitoring of the immuno-conversion of the RV and CV, the red line represents CV and blue lines represent the RV and SD values shown by error bars. (Fig.4.2)

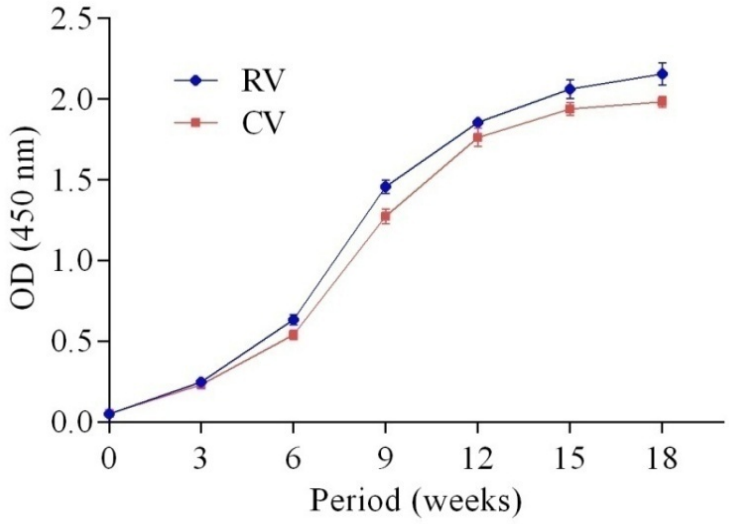

Fig. 4.2: Monitoring of immunization: anti-RV and anti-CV antibodies. The indicated values of OD are mean values (n=3), error bars indicates the SD.

4.4 Purity of antibodies

Fig. 4.3 demonstrates the protein profiles of monovalent plasma (rabbit), ARV- Abs and ACV-Abs resolved using SDS–PAGE (10%) in native conditions. The silver staining procedure carried out for visualization of the bands. The rabbit monovalent plasma shows multiple bands, mainly two distinct bands around 205 kDa and 66 kDa, which are suggestive of IgG and BSA respectively. In affinity purified samples, only a single band around 205 kDa, indicative of IgG. This confirms that, in affinity purification only antibodies separated from other proteins. The unbound section ascertained to have no activity against the venoms. The eluted IgG's were further tested for their functionality against the venom. These antibodies remained functional in the process of affinity chromatography and showed activity against the venom.

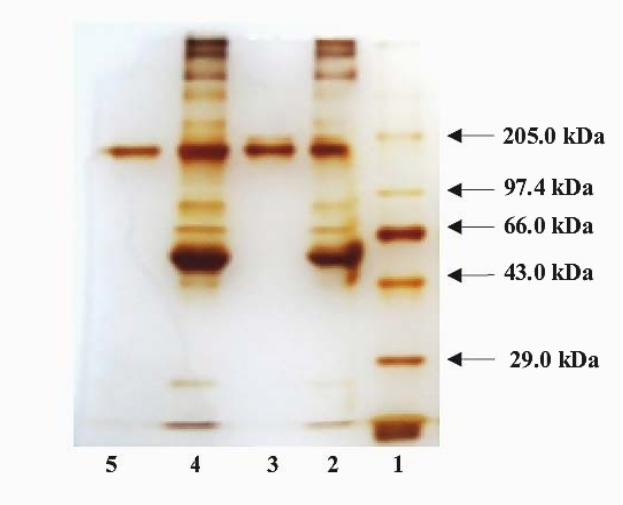

Fig. 4.3: SDS–PAGE (10%) of purified antibodies and hyper-immunized plasma samples. Lane 1 contains standard molecular weight protein marker, whereas Lane 2: anti RV monovalent rabbit plasma, Lane 3: ARV-Abs, Lane 4: anti CV monovalent rabbit plasma, and Lane 5: ACV-Abs; the arrows specify the standard proteins. Monovalent plasma shows multiple bands whereas, purified antibody samples contains a band of IgG and confirms the purity of samples.

4.5 Indirect ELISA optimization for determination of ASVA

For the optimization of ELISA Standard ASVA and normal plasma were tested at different dilutions ranging from 1:1000 to 1:15000 were allowed to react with different concentration of coating venoms from one to five µg per ml. For secondary antibody dilutions viz. 1:5000, 1:10000, and 1:20000 were considered. All these dilutions were optimized using Checkerboard analysis. The criteria for selection of dilutions were that blank OD should be below 0.1 and positive samples having potency 0.1 mg/ml should have OD around 1.0. It was observed that two µg per ml of venom used for coating, primary antibody diluted to 1:10000 and secondary antibodies at dilution 1:20000 gave better results. The optimized Indirect ELISA protocol and followed during entire experimentation.

4.6 Standardization of ELISA for ASVA potency determination

To Standardize ELISA for ASVA potency determination specificity, linearity, and the range of the method was evaluated. The standard plasma diluted in such a manner that the potency was in the range from 0.025 to 0.2 mg/ml. The result interpretation carried out using Graphpad Prism 6 software by linear regression parameter. The specificity, accuracy, and linearity ascertained during standardization. The obtained R^2 values for CV and RV were 0.985 and 0.982 respectively. The SD values for CV and RV are 0.029 to 0.044 and 0.012 to 0.022, respectively (Fig. 4.4)

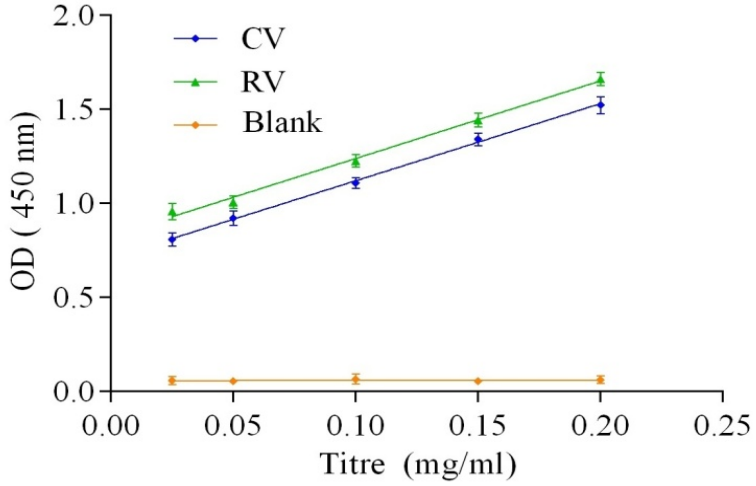

Fig. 4.4: The estimation of ASVA using ELISA. Two µg per ml of venom used to coat the Micro ELISA plates using a carbonate buffer and added with dilutions of ASVA ranging 0.025 - 0.2 mg/ml. Normal equine plasma used as a blank. The OD values are the average values (n=3), error bars indicates the SD.

4.7 Sandwich ELISA optimization for the venom estimation

Optimization of indirect sandwich ELISA was performed by the Checkerboard analysis. It was observed that the ELISA selectively detects RV and CV venoms. The BSA used as blank, gives OD equivalent to blank at all stages of dilution. The sandwich ELISA was sensitive to detect the venom about 0.01 ng/ml and the graph observed linear from 0.1 to 1000 ng/ml for CV and RV venoms indicating the suitability of the method for

envenomation detection of snakebite patients. The SD values for anti-CV and anti-RV antibodies were inside 0.020 to 0.049 and 0.038 to 0.058, respectively. The R^2 values for anti-CV and anti-RV antibodies were 0.997 and 0.996, respectively (Fig. 4.5). The result interpretation was carried out using Graphpad Prism 6 software.

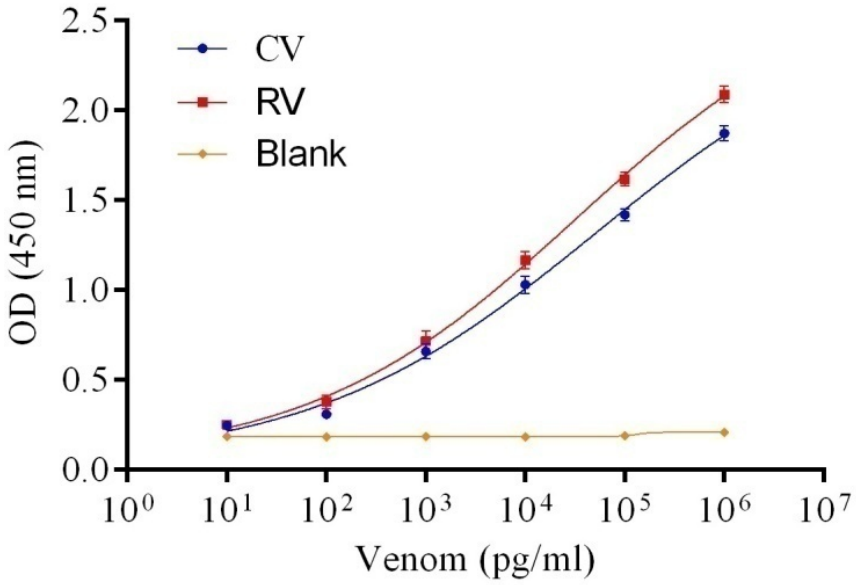

Fig. 4.5: Calibration plot of sandwich ELISA for estimation of the RV and CV. ELISA wells coated with horse ASVA using a carbonate buffer. The wells incubated with venom ranging from 0.01 ng /ml to 1000 ng/ml and BSA used as a blank. The OD indicates average values (n=3) error bar indicates the SD.

4.8 Assessment of cross-reactivity of affinity purified anti-RV and anti-CV antibodies.

Indirect sandwich ELISA, used to assess the cross reactivity monovalent affinity purified antibodies with heterospecific venom. To estimate the cross reactivity standard ASVA (equine) used as a capture antibody. The dilutions of conspecific and heterospecific venom ranging from 0.01 to 1000 ng/ml were analyzed during the study to evaluate the cross-reaction between ARV–Abs and ACV–Abs. The result showed that antibodies strongly responded to conspecific venom. Also, it was observed that snakes belonging to the same family have more cross reactivity, ACV-Abs shown maximum cross-reactivity to KV and ARV – Abs to EV, The cross reactivity with heterospecific venom is significant till 1 ng/ml dilution. The observed cross-reactivity of antibodies may give ambiguous results in

selective detection of snake venom without removal of cross-reacting antibodies by immuno-affinity chromatography. Therefore, the protein A purified antibodies were passed through conspecific and heterospecific venom bound columns to get the RV and CV SSAb's.

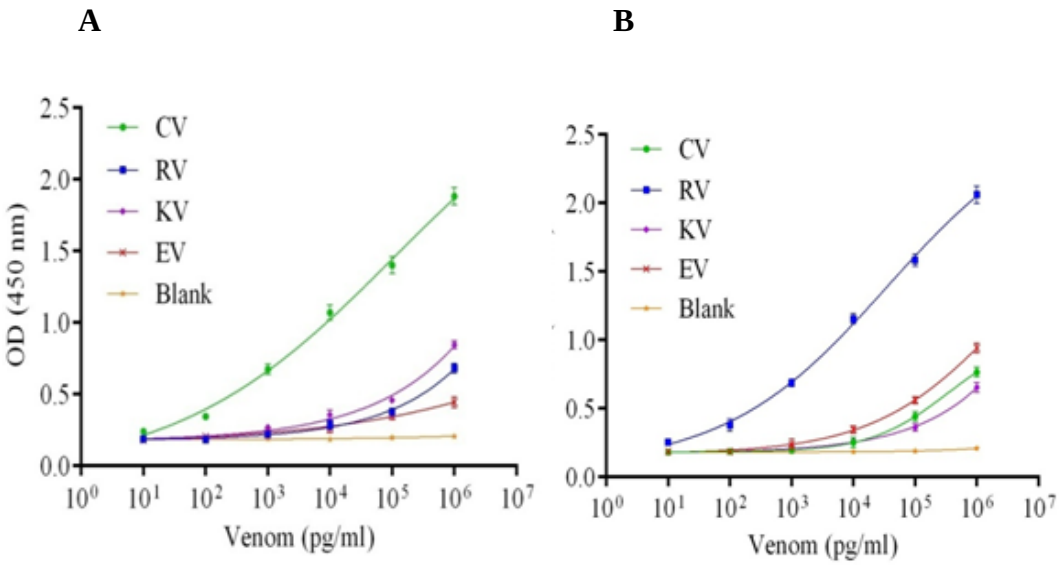

Fig. 4.6: Cross-reactivity evaluation using sandwich ELISA. To evaluate the cross reactivity ASVA (equine) used as a capture. The wells incubated with venoms (CV, KV, RV, and EV), the quantity of venoms ranges from 0.01 to 1000 ng/ml. The figure (A) indicates cross-reactivity of ACV-Abs (rabbit) with conspecific and heterospecific venoms and fig (B) represent the cross reactivity of ARV-Abs (rabbit). The OD values are mean values (n=3) error bar indicates the SD.

4.9 Specificity determination of CV and RV SSAb's

Indirect sandwich ELISA used to assess the specificity of SSAb's. To estimate the specificity standard ASVA (equine) used as a capturing antibody. The dilution of conspecific and heterospecific venom ranging from 0.01 to 1000 ng/ml analyzed during the study to evaluate the specificity of RV and CV SSAb's. The result showed that antibodies strongly responded to conspecific venom and SSAb's shown no cross-reactivity with heterospecific venom for all the dilutions selected during the study indicates that SSAb's

are specific for conspecific venom (Fig. 4.7). This result confirms the use of SSAb's in snake envenomation detection tests.

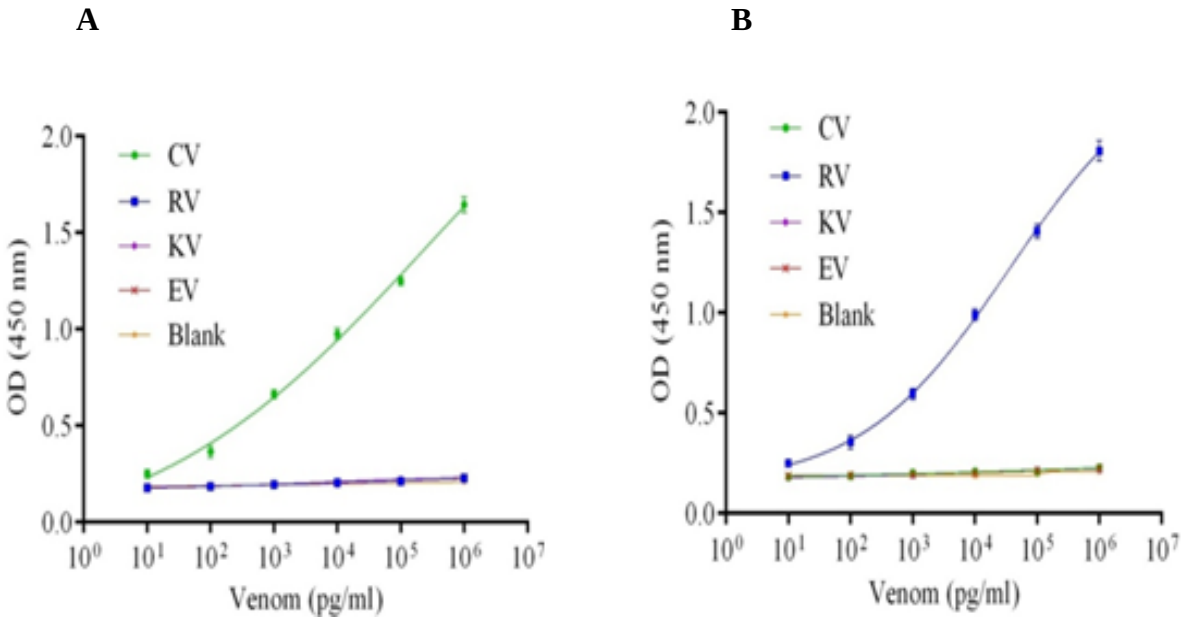

Fig. 4.7: Specificity evaluation of SSAb's using indirect sandwich ELISA. To evaluate the specificity of SSAb's the equine ASVA used as a capture. The wells incubated with venoms (CV, KV, RV, and EV), the quantity of venoms ranges from 0.01 to 1000 ng/ml. Figure (A) indicates specificity of ACV-Abs and fig (B) represent the specificity of ARV-Abs. The OD indicates the average values (n=3) error bar indicates the SD.

4.10 Determination of binding capacity of GNP solution.

The minimum quantity of antibodies to stabilize the GNPs determined according to protocol mentioned by Slot and Geuze, (1984). The pH of GNPs and antibodies adjusted to 7.0. In each test tube added 2 ml of gold solution and 0.2 ml of antibody solution containing protein concentrations from 100 to 700 µg/ml and mixed well. After 1 min, 2 ml of 10% w/v NaCl added to each tube (Fig. 4.8). The stability of gold solution was observed by taking the absorbance at 580 nm wavelength. It was observed that 20 µg/ml proteins required to stabilize the GNPs as OD values stop decreasing from test tube, no. 4 and for conjugation experiment 10% excess antibodies taken.

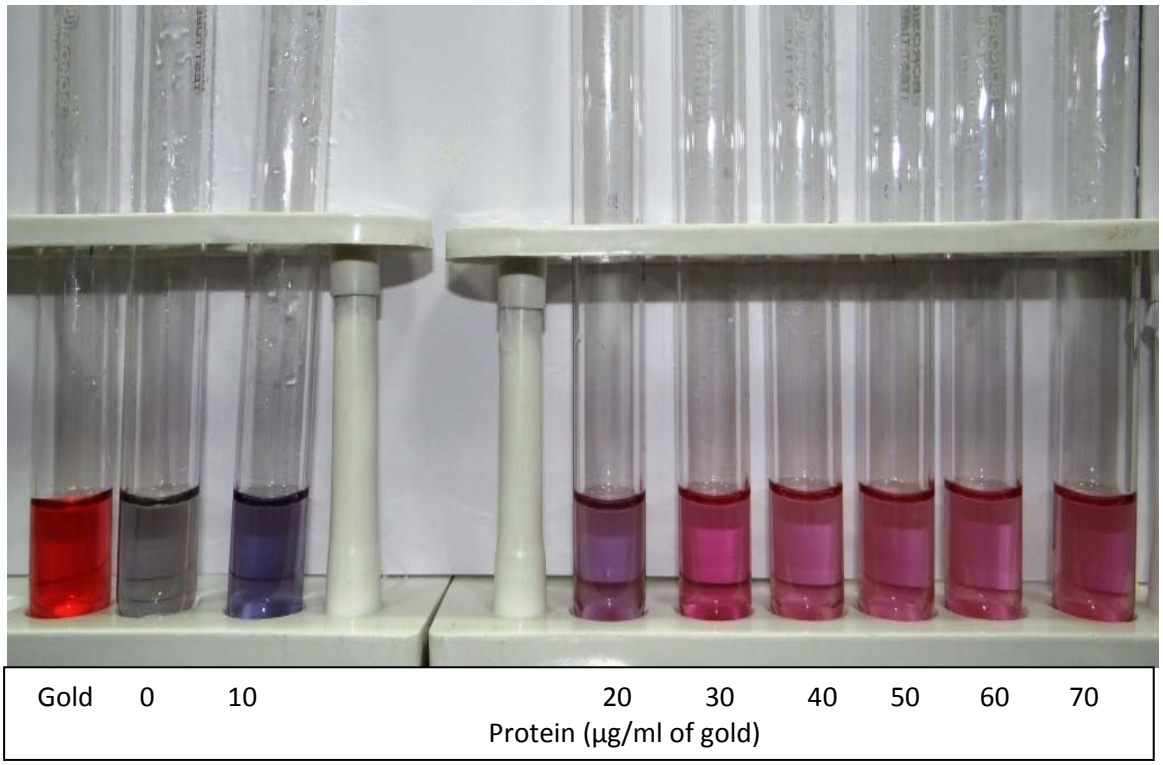

Fig. 4.8: The estimation of the quantity of protein required to stabilize the dispersed GNPs. The first test tube contains the control GNP solution. The test tube 2 to 9 contains 2 ml GNP solution incubated with increasing concentration of proteins and consequently, 2 ml NaCl (10% w/v) added to observe the color change.

4.11 Performance of the SEDIA device

For the preparation of SEDIA strips, the NC membrane used about 10-µm size and gave excellent results. The pre-treated sample pad and the conjugate pads used; the concentration of GNPs optimized on the conjugate pad. For impregnation of antibodies on NC membrane concentration analyzed from 0.5 to 2 mg/ml by manual application and it was observed that 1 mg/ml was the optimum concentration. After optimization, one µl/cm solution was applied at the test and control lines using TLC spotter (Linomat IV, Camag, Berlin, Germany). The strips manufactured and assembled in cassettes. The devices packed, stored at ambient temperature, and protected from humidity and light.

4.11.1 SEDIA results interpretation

The result interpretation of SEDIA shown in (Fig. 4.9). The test region immobilized with SSAb's of CV and RV. A positive result indicated by the observation of the red lines at corresponding test regions on the membrane, the lines produced due to the capture of gold conjugated antibody and venom complex. In the absence of venom, gold-conjugated antibodies cannot capture at the test regions. In the control region, immobilized anti-horse antibodies, which captures the gold conjugated antibodies and gave a red line with or without in the presence of venom indicating the validity of the device. If the control region does not produce red line, the test considered as invalid.

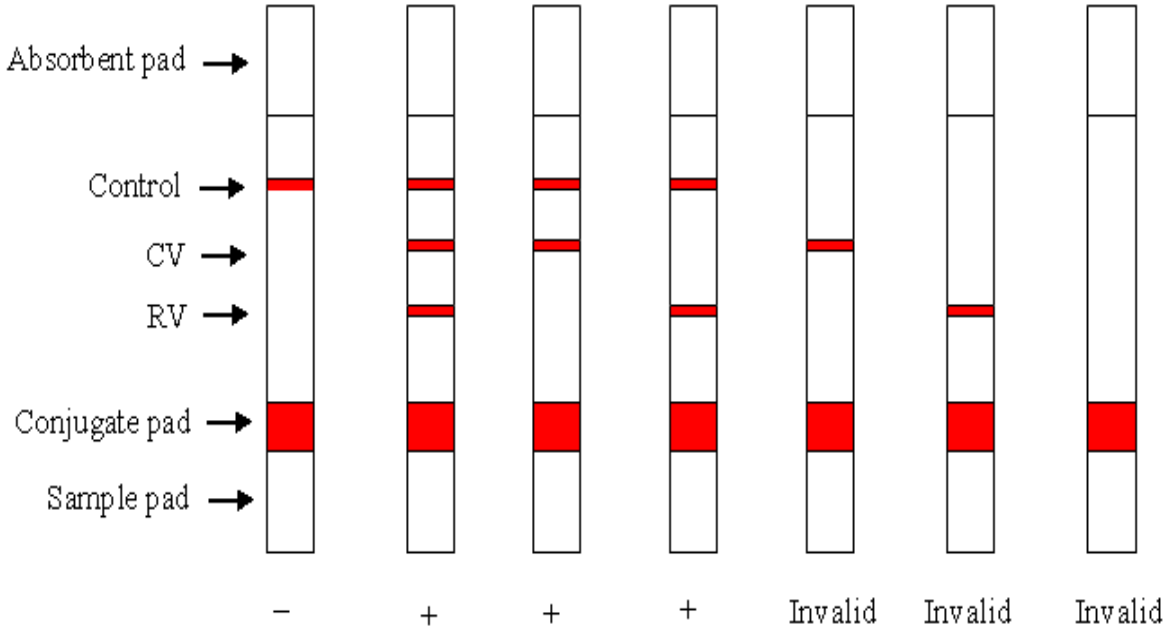

Fig. 4.9: Schematic representation for interpretation of SEDIA results

4.11.2. Specificity

To determine the specificity of SEDIA device, all venoms (CV, RV, EV, and KV) spiked individually using PBS buffer such that the final concentration of venom in the solution was 1000 ng/ml. To ensure specificity and cross-reactivity of SEDIA strips, applied diluted

sample (100 µl). The results obtained by the visualization of red lines at control and the respective test region. The results of test obtained within five to ten min. All SEDIA devices demonstrated its validity by producing red lines at all control regions (Fig. 4.10). In the strip 1, where only PBS was added showing red line at control region only, representing the negative control. The device 2 was added with a mixture of RV and CV in PBS, a red line at control as well as both test regions was obtained indicating the device can identify a mixture of venoms, also displaying the positions of lines on a device. The device 3 and 4 inoculated with PBS containing CV and RV, respectively, and the red lines observed at their respective test zones. The SEDIA device did not show any cross-reactivity between CV and RV. In device 5 and 6 the red lines observed at the control region, representing the definite specificity of SEDIA device for the RV and CV.

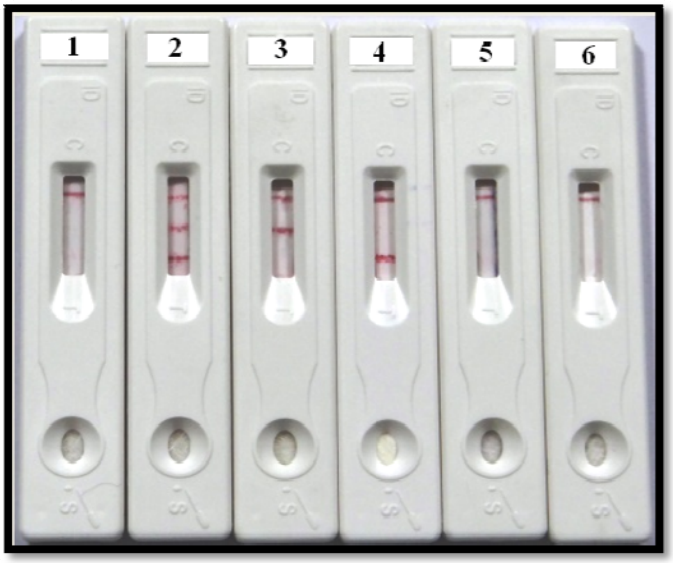

Fig. 4.10: Specificity evaluation of the SEDIA device using different venom spiked (1000 ng /ml) samples. The control region developed red lines in all tests indicating the validity of the device. The device 2 contains a mixture of the RV and CV venom hence developed color in all regions. The device 3 and 4 applied with the CV and RV venom spiked sample and exclusively showing red lines at respective test zones. The device 5 and 6 applied with heterospecific venoms spiked samples of KV and EV and shown only control line indicating specificity of SEDIA device.

4.11.3. Sensitivity

Assessing sensitivity of SEDIA is the most crucial part in detection snake envenomation as the levels of venom in serum are very low. According to Hung et al. (2003), during snake envenomation detection of cobra bites in Taiwan, victims with severe systemic envenomation had a concentration of venom in the serum between 228–1270 ng/ml and for a mild systemic envenomation, concentration of venom in the serum between 0–24 ng/ml. Therefore, it becomes mandatory for snake envenomation assay, to detect venoms in nanograms. To assess the sensitivity of the SEDIA device, a logarithmic dilution (1000 to 0.01 ng/ml) of CV and RV venoms prepared in PBS. All the simulated samples were applied to the SEDIA strips (about 100 µl) to demonstrate the sensitivity of SEDIA. Device 1 was applied with PBS and device 2 to7 with a dilution of venom respectively. As device, 1 applied with PBS, hence no capture of the conjugate at test region resulted in development of the red lines only at control region. The intensity of the red line is dependent on the concentration of antigen in the sample and results are reproducible. Device 7 shown only the control line therefore sensitivity of SEDIA device is about 0.1 ng/ml (Fig. 4.11).

Fig A

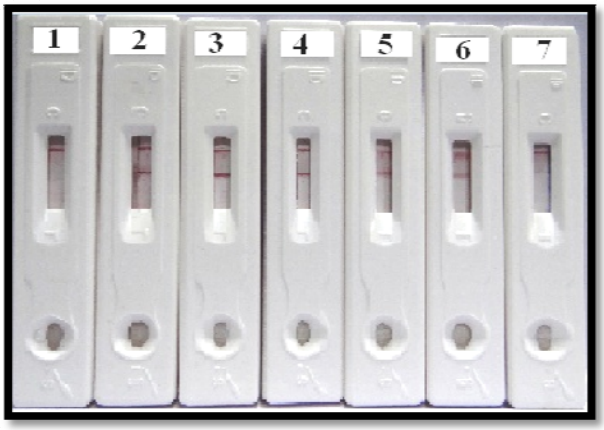

Fig B

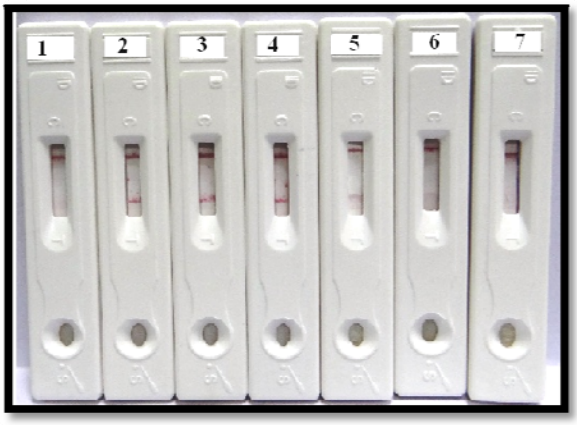

Fig. 4.11: Sensitivity determination of SEDIA device. A logarithmic dilution (1000 ng /ml to 0.01 ng/ ml) of CV and RV venoms prepared in PBS and applied on SEDIA device. Fig. (A) and (B) representing the sensitivity CV and RV respectively. Device 1: negative control, the device 2 to 7 applied from left to right with decreasing logarithmic dilutions of spiked venom samples. In both figures device 7 shown only the control line, therefore sensitivity of SEDIA device is 0.1 ng/ml.

4.12. Evaluation of venoms from experimental envenomation

The RV and CV venoms injected s.c. in Swiss albino mice for systemic envenomation. The blood samples were collected after 30 and 60 min of duration (Fig 4.12). Plasma separated and diluted equally with PBS. The samples tested using SEDIA and sandwich ELISA for comparison of the results (Table 4.2). Device 2 and 3 applied with the CV samples and device 4 and 5 with the RV samples produced a prominent line at the respective test region. The results of SEDIA compared with sandwich ELISA using the same samples and there was 100% agreement between both results.

Fig. 4.12: Experimental envenomation determination by the SEDIA. The device 2 and 3 representing results of CV and the device 4 and 5 for the RV envenomation produced the red lines at test as well as control zones, while device 1 produced a red line at control zone only; confirms the efficiency of the SEDIA device to selectively detect experimental envenomation.

Time period	CV		RV	
	ELISA (µg/ml)	SEDIA	ELISA (µg/ml)	SEDIA
30 mins	0.022	+	0.021	+
60 mins	0.020	+	0.019	+

Table 4.2: Comparison of experimental envenomation detection. The values indicated in the table are mean ELISA values, the + symbol represents the red line at the test zones on the SEDIA device

CHAPTER 5

DISCUSSION

AND CONCLUSION

Snakebite is a neglected community health issue of India. The deaths due to snakebites remained controversial and the true scale monitoring of death and injuries of snakebites are not available. The deaths noticed by the authorities are underestimated, as WHO estimated that there are around 35,000-50,000 deaths due to snakebite in India. The variation in estimates is due to inappropriate reporting systems; conventional healers give treatment to many of the snakebite victims, and many of them lose their life before being admitted to the hospital and remain unnoticed. The records of snakebite deaths primarily based on victims admitted to the government hospitals. In emerging countries like India, the data reporting systems are improper, due to that the deaths outside hospitals remain unnoticed; this may be the main cause for the underestimation of the deaths due to snakebite in India. Snakes are scattered in the world except Arctic regions. In India, about 305 snake species observed among them 52 are venomous and the majority of the snakebites are due to four medically significant snake species called as India's Big 4.

Deaths and serious injuries due to snakebites can conquered with early identification and treatment of the victims. The antivenom is the only effective remedy against snakebite, but equal consideration given to the possible adverse drug reactions due to the antivenom. In India to cure snakebite victims, generally, polyvalent ASVA is used, which has the venom-neutralizing activity for four medically significant snakes of India. Although the ASVA is polyvalent, the supportive treatment varies and plays an important role during snake envenomation treatment resulting in the saving of lives and serious injuries to the victims (Warrell D.A., 2012). Many authors reported that use of specific monovalent ASVA is ideal, as it has antibodies against particular venom, which efficiently neutralizes particular venom. The monovalent antiserum avoids the unnecessary burden of non-specific antibodies present in polyvalent antiserum to the victim resulting in the use of less antiserum. Monovalent ASVA treatment is cost saving than polyvalent antiserum as it avoids unnecessary administration of non-specific antibodies resulting in a saving of valuable ASVA, which has shortage world widely. In many regions of the world, polyvalent ASVA is preferred for treatment of snakebite victims as no standard methods is available to identify the snakebite envenomation and species prior to the administration of ASVA. For practical application of monovalent ASVA, there is a need to develop a trustworthy method

to detect snake envenomation at the early stage. Although, after extensive research for development of a suitable method to identify the offending snake species, we are not able to develop a reliable, specific, rapid, and cost effective method for field application, which will provide a key tool for serving effective utilization of antivenom and snakebite management (Selvanayagam Z. E. et al., 2002).

Snakebite envenomation identification is a crucial part of snakebite treatment, as several bites do not leads to systemic envenomation mentioned by Sharma *et al*. (2004). In many snakebite cases, signs, and the symptom expressed by the victims are due to fear of snakes, several non-poisonous snakes lookalike to poisonous snakes to guard themselves against the predator. In India, snakebites identified mainly based on symptomatic approach, bitten part examination, signs, and the symptoms shown by victims, also using biochemical tests like urine examination and 20-min WBCT. For medical practitioners, it becomes difficult to identify the actual envenomed victims using available tests (Warrell, D.A., 2010, 2012; Alirol et al., 2010). The above-mentioned test does not give clear indications of the severity of systemic envenomation and offending snake species. Various serological tests to are available for identification of the systemic envenomation, but ELISA turns into a method of choice for the identification snakebite envenomation. The use of ELISA for venom detection was first reported by Theakston *et al*. (1977), and afterwards widely applied for snake species identification without conferring any cross-reaction (Steuten et al., 2007; Selvanayagam Z.E. and Gopalakrishnakone, 1999).

To understand better about the snakebites, it is important to know about the composition of snake venom. Many authors confirmed that the venom contains numerous proteins and it varies greatly from one species to other. The variation among venoms may be due to the change in season, habitat, and available prey. In this study, the SDS-PAGE of CV, KV, RV, and EV venoms (Fig.4.1) confirmed that venoms have specific protein patterns, and contains some proteins, which resembles each other. Similar molecular weight proteins or same antigenic parts of dissimilar proteins may add the degree of cross-reaction between the venoms. Many research articles illustrated that there is a significant cross-reaction between monovalent antiserum of two different species; these cross-reacting

antibodies can hinder the specificity of the test. (Selvanayagam Z.E. et al., 1999; Dong L.V. et al., 2003; Gao J. F. et al., 2013).

In this study to raise antibodies against snake venom, immunization of rabbits carried out by using the detoxified venoms. The detoxified venoms in the immunization process minimized harmful side effects of the venom and allow to use a more amount of antigen per dose, which resulted in a rapid enhancement of ASVA. Although, the detoxified venom was utilized for the immunization process, generated antibodies have strong neutralizing activity towards the native venom samples (Fig.4.2). During immunization, relatively large molecular weight proteins are more immunogenic compared to lower ones. The CV observed less immunogenic than RV, SDS-PAGE profiles depict that CV contains lower weight proteins than RV (Fig.4.1).

In the results, ARV-Abs and ACV-Abs confirmed cross reactivity towards the heterospecific venoms (Fig. 4.6). The figure (Fig. 4.6) showed that ARV-Abs have strong cross reactivity against EV and ACV-Abs against KV; the reason behind this cross reactivity may be the snakes related to the same family have more similar proteins or common epitopes on different proteins resulting in a high degree of cross-reaction. In view of this, it becomes a necessity to obtain SSAb's against particular venom. Antibodies purified from hyper-immune plasma by the process of protein-A affinity chromatographic purification. Protein-A selectively separates IgG's from whole plasma, during purification all IgG's were separated which contains the specific, nonspecific and cross reacting IgG's may contributes the cross-reactivity among the venoms. In advance, to eliminate the non-specific and cross-reacting IgG's, immuno-affinity chromatography was carried out using CNBr activated Sepharose 4B beads bounded to conspecific and heterospecific snake venoms separately. The specificity and efficiency of resulting antibodies (RV & CV SSAb's) to conspecific snake venoms depicted in (Fig.4.7). In the present study, it was observed that obtained SSAb's are sensitive and specifically detected the conspecific venom.

Today, there are many ELISA based assays are available to detect snake venom from snakebite victims, but these assays require more time, therefore not useful for snake venom detection before administration of ASVA. In view of this, In Australia CSL developed SVDK kit to identify snake venoms from victims; it requires 30-40 min to demonstrate the results. The time required for SVDK is less but there is a scope to reduce time by use of advanced immunological methods. The methods like LFA are sensitive also specific and suitable for field application. The results of LFA obtained within least time, at present, LFA plays a vital role in the field diagnostics like identification of pregnancy, HIV, Malaria, Dengue, and more (Wong and Tse, 2008; Wild, 2001). Due to above facts, we here tried to apply LFA platform for selective and rapid detection of snake envenomation.

Generally, Most of the commercially available LFA platforms utilize two different MAbs for impregnation and conjugation for detection of single antigen. The application of MAbs for envenomation detection has restrictions as snake venom composed of many proteins, only some of them are characterized, and there is huge seasonal and geographical variation amongst the venoms of snakes belongs to same species. Application of MAbs may hamper the sensitivity as the circulating venom in the blood of victims is very low, in addition to the venom composed of numerous proteins, and their amount in the venom can vary. So it becomes incredibly difficult to develop sensitive and reliable LFA for detection of snakebite using MAbs. To encounter this problem, LFA was developed by using purified specific polyclonal antibodies. Use of SSAb's amplified the sensitivity of the assay as it includes only venom specific antibodies. Beside this, the use of two different species antibodies for impregnation and conjugation enhanced the sensitivity, the reason may be different species reacted differently with the same venom and antibodies generated recognize antigen in a different way leads to increase in sensitivity of SEDIA.

The developed SEDIA to detect the RV & CV on a single device observed selective to detect RV and CV at respective test zones and sensitive to detect venom as low as 0.1 ng/ml (Fig 4.11). The device able give results between five to ten min. The conjugate cannot get capture in the test zones in the absence of venom. A control zone was impregnated with anti-horse antibodies and the conjugate in presence or absence venom giving red line at

control zone, representing the validity of the assay. The test becomes invalid in the absence of a red line at control zone (Fig. 4.9). The experimental envenomation performed in Swiss albino mice by introducing a specified quantity of venoms and blood was collected after particular duration to obtain plasma samples. The diluted plasma applied to SEDIA device to verify the functionality. The SEDIA distinctively detected RV, CV, and showed 100 % conformity between SEDIA and ELISA results (Fig.4.12 and Table 4.2).

In conclusion, for the management and detection snakebite, the SEDIA has an immense potential to become a technique for field application, which will help in avoiding unnecessary utilization of valuable antivenom by the identification of only envenomed victims. Early detection and specific supportive treatment can imparted due to SEDIA, may result in the saving of lives of victims. This work illustrates a process prototype; in the Indian scenario, the application of SEDIA achieved by the inclusion of EV and KV venom detection on a single device with further advancements and case studies.

CHAPTER 6

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PUBLICATION

Research Article

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Abstract

Abstract published in **The 5th solanaceae genome workshop**, 12-16 October 2008 Cologne Germany. Neeru Jain, Rashmi Gehi, **Balasaheb Pawade**, Rupali More, Sameer Agrawal. Fingerprinting of parents and evaluation of genetic purity of commercial hybrid seeds in tomato and chilly using microsatellite markers (SSRs)

Conference Attended

1. National virology conference attended on **"Viruses: Surveillance, Identification, and Management"**, organized by Vidya Pratishtan's School of Biotechnology, Baramati, Pune, on 23rd to 25th January, 2012.
2. Conference attended as a observer on **"Good Manufacturing Practices(GMP) Regulatory Inspection using Quality Risk Management Approach (Basic)**, organized by Central Drug Standard Control Organization (CDSCO) ,Ministry of health and Family welfare, India in collaboration with World Health Organization (WHO), Pune, on 18th to 22nd November 2013.