CHAPTER II

LITERATURE REVIEW

In many parts of the world, especially in the tropical countries of Africa, South East Asia and South America, snake bite is a serious medical, social and economic problem. In Thailand, one of the tropical countries in South East Asia, at least 179 species and subspecies in 10 families of snakes were found (12), most of which are non-poisonous. The terrestrial venomous snakes commonly found in Thailand belong to the family Elapidae ie. cobra (Naja naja), banded krait (Bungarus fasciatus), Malayan krait (Bungarus candidus),and king cobra (Ophiophagus hannah) etc, family Viperidae, the only species found being Russell's viper (Vipera russelli), and family Crotalidae ie. green pit viper (Trimeresurus species), Malayan pit viper (Agkistrodon rhodostoma) etc. (13,14,15).

Although snake venoms are chemically heterogeneous and can therefore produce multiple simultaneous effects, the venoms of snakes in the Elapidae family are predominantly neurotoxic (2) while Viperidae and Crotalidae venoms cause mainly hemorrhages and coagulation disturbance (7).

There are at least twelve cobra species in six genera, most of which are found in Africa. Only two species, Naja naja and Ophiophagus hannah, are present in Asia , where many

subspecies of Naja naja have been recognized (2). Cobra in Thailand has been classified in the subspecies Naja naja kaouthia (13,16) (some authorities recognize it as a full species Naja kaouthia) (17,18), also known as Naja naja siamensis. It is a monocellate non-spitting cobra.

COBRA VENOM AND ITS MODE OF ACTION

Cobra venom, like the venom of most snakes, is a complex mixture. Most of the constituents (about 90%) are proteins of low molecular weight which migrate towards the cathode on electrophoresis. Several biologically active components such as neurotoxin, membrane toxins (cardiotoxin, direct lytic factor, cytotoxin etc.), phospholipase A and some other enzymatic activities have been separated from cobra venom (2).

Neurotoxin

Neurotoxin is the most toxic component of cobra venom and is the major cause of death in cobra bites. It is a postsynaptic neurotoxin , also referred to as curareform, curaremimetic or curare-like toxin since its action mimics that of curare (19,20). Neurotoxin binds to the nicotinic (cholinergic) acetylcholine receptor at the neuromuscular junction and prevents the depolarizing action of acetylcholine, producing flaccid paralysis and death due to respiratory failure in most animal species (2).

Neurotoxin is a low molecular weight basic protein with isoelectic points in the vinicity of 9.0-10.0. The toxin has been

separated into two distinct groups according to the size, usually called short and long neurotoxin. Short neurotoxins contains 60-62 amino acids and four disulfide bonds (approximately MW 7,000) whereas the long one usually has 71-74 residues and five disulfide bonds (approximately MW 8,000) (21,22). The content of neurotoxin in cobra venom varies from one species to another and there is evidence that more than one kind of neurotoxin is present even in the same venom (10).

The principal neurotoxin of Naja naja siamensis, neurotoxin 3 or T3, has been isolated and purified by Karlsson et al. in 1971 (10) by ion-exchange chromatography using Bio-Rex 70 column (or IRC-50). It is a long neurotoxin containing 71 amino acid residues in a single peptide chain cross-linked by five disulfide bridges (Figure 1) and the amount accounts for about one-fourth of the weight of lyophilized crude venom, or one-third of the total venom proteins. This is the highest content of an individual neurotoxin found in the venom of any terrestrial snake.

The neurotoxic action of cobra neurotoxin has been studied extensively. It was shown to block neuromuscular transmission by acting on acetylcholine receptor at the motor end plates of skeletal myoneural junction (3,4,5,6). However, reports on the reversibility of neuromuscular blockade by neurotoxin from different cobra species are inconsistent. The neuromuscular blockade by neurotoxin from Naja naja atra (20), Naja nigricollis (23) and Naja naja naja (24) has been reported

to be reversible, whereas neurotoxin 3 (T3) from Naja naja siamensis was a highly potent, specific and irreversible blocking agent of cholinergic receptors of neuromuscular junction (5,25). The studies also showed both pre- and post junctional activities of the toxin purified from Naja naja siamensis (5,25), Naja nigricollis (5) and Naja nivea (26).

Membrane Toxins

In addition to the curaremimetic toxins, many other basic proteins of low molecular weight have been isolated from cobra venom. Most of these compounds act on various membranes by increasing their permeability. This group of toxins are regarded as membrane toxins or isotoxins. The toxins included in this group are cardiotoxin, cytotoxin, direct lytic factor (DLF), cobramine, toxin γ, etc. (21).

Cardiotoxin is the most basic and the most abundant constituent of cobra venom, amounting to 25-55% on a dry weight basis. It is heat stable in acid pH with a molecular weight of about 6,000-7,000. It acts on various kinds of cells, causing depolarization of cell membranes and contraction of skeletal muscle (3,27,28). It blocks the axonal conduction in peripheral nerve (28), augments the systolic arrest of heart muscle (27,29) and causes local irritation (27).

Although the toxicity of cardiotoxin is not as great as crude venom, death due to cardiotoxin effects may occur within 10 minutes following an intravenous injection of heavy

dose (LD 100) of cardiotoxin. The lethality is greatly increased in the presence of phospholipase A (28) which leads to massive hemolysis and represents one of the factors contributing to lethality. Death by shock is aggravated by the potassium released from the lysed red cells which can cause cardiac standstill. Phospholipase A alone does not have the same effect.

Direct lytic factor (DLF) possess the hemolytic activity directly on washed erythrocytes of several animal species (30,31). The DLF, by itself is only weakly hemolytic, but acts synergistically with phospholipase A (30,32).

Enzymes

Many enzymes are present in cobra venom such as phospholipase A, B, C, acetylcholinesterase, phosphodiesterase, phosphomonoesterase, 5'-nucleotidase, glycerophosphatase, peptidase and hyaluronidase etc.(33,34). The toxicity of enzymes is due mainly to their actions on substrates. The destruction of the substrate itself may be responsible for the effects observed, or the pathology may be mediated through the compounds liberated during the enzymatic reaction.

Besides direct lytic factor, hemolysis by cobra venom is due to the action of phospholipase A, either "indirectly" by producing lysophosphatide from serum or added lecithin or "directly" by hydrolyzing phospholipids in the red cell membrane (33). Moreover, phospholipase A was also shown to greatly potentiate or accelerate the effects of cardiotoxin (28) and

direct lytic factor (30,32).

The role played by other enzymes in overall toxicity of cobra venom appeared to be negligible, if not entirely absent. Many of them such as 5'-nucleotidase, acetylcholinesterase have been shown to be non-toxic or nearly so (33).

Nonproteins

The nonprotein portion of the cobra venom constitutes a much smaller fraction of the whole venom and, in general, is biologically less active. Included in the nonprotein fraction are metal ions, inorganic substances and some small organic molecules including peptides, lipids, nucleotides, carbohydrates and amines. (35).

PHARMACOKINETICS OF COBRA VENOM

Absorption

The absorption of cobra venom has been studied since 1941, Barnes and Trueta (36) stated that cobra venom, with its low molecular weight toxins, is readily absorbed from the subcutaneous tissue into the blood circulation by passing through the capillary wall, whereas black tiger snake and Russell's viper venoms, whose molecular weights exceed 20,000 are absorbed by the lymph stream. Tseng et al. (8) compared the absorption of ^{131}I-labeled crude cobra venom, neurotoxin and cardiotoxin of Formosan cobra (Naja naja atra) in mice after subcutaneous injection and revealed that the absorption of cardiotoxin, as

well as crude venom was very slow; only about 30% of the injected cardiotoxin were absorbed within 4 hours, while absorption of neurotoxin was much faster, about 60% within 2 hours. Since the molecular weights of both neurotoxin and cardiotoxin of cobra venom are about the same (MW~ 7,000), the delayed absorption of cardiotoxin is likely due to the strong affinity of cardiotoxin to tissue at the site of injection.

Snake venoms introduced by the oral route are usually nontoxic but a lethal effect can be observed if a sufficiently large dose of cobra venom is administered (37). In view of the fact that both neurotoxin and cardiotoxin are basic polypeptides, absorption of cobra venom from the intact gastrointestinal tract should be extreamly slow. Moreover, the venom may be destroyed by the proteolytic enzymes of the gastrointestinal tract.

Distribution

Sumyk et al. (38) and Tseng et al. (8) utilized the autoradiographic technique to study the distribution of cobra venom in mice and rabbits. When radioiodinated cobra venom or its neurotoxin and cardiotoxin were administered, the highest concentration of radioactivity was always found in the kidney with marked localization in the cortex. Significant amounts of radioactivity, especially cardiotoxin, were also found in the liver, spleen, lung and intestine.

Cobra venom, or its neurotoxin, also shown to accumulate

on the motor end plate zone of the mouse diaphragm, provides further supports the conclusion that cobra neurotoxin blocks neuromuscular transmission by acting on the motor end plate.

The amount of radioactivity in CSF and brain, after intravenous injection of radioiodinated venom or its purified toxin, was extremly low, indicating the existence of the blood-brain barrier for these toxins. The findings were consistent with the observation that respiratory paralysis caused by cobra venom is peripheral and not central in origin (39,40,41).

Fate and Excretion

After an intravenous injection of cobra venom and its purified toxins into rabbits, Tseng et al. (8) found that the plasma level of cardiotoxin declined much more rapidly than that of neurotoxin. Cardiotoxin declined to about two-fifths of the initial level within 5 minutes and more than 90% of the injected dose disappeared from the plasma within 30 minutes, while about 20% of the injected neurotoxin disappeared from the plasma within 5 minutes. Most of the venom was found to be excreted in the urine. The excretion rate of venom is about 30% within 2 hours (8) and 70% within 5 hours (9).

The findings that cobra venom accumulates in the kidney and is excreted through the urine suggest that the kidney plays an important role in venom excretion.

TOXICITY

Toxicity of cobra venom varies among various cobra species, although there is no marked qualitative difference. It also appears that the lethal dose by different routes of administration (Sc. or IV) does not differ greatly among most warm-blooded animals except for the cat, whose skeletal muscle is highly resistant to the neuromuscular blocking action of cobra neurotoxin (42).

Among the components separated from cobra venom, neurotoxin is the most toxic, being 3-10 times more toxic than the original venom when assayed in mice (43,44).

The LD50 of the principal neurotoxin isolated from Thai cobra (Naja naja siamensis toxin 3) was found to be 170 µg/kg in mouse (45) whereas the LD50 of Naja naja siamensis crude venom was 250 µg/kg (46).

SYMPTOMS AND SIGNS PRODUCED BY COBRA VENOM

Flaccid paralysis is the outstanding feature of cobra venom poisoning. After an initial period of restlessness and excitement probably due to local irritation, the animal becomes dull and drowsy , with unsteady movement and dropping head, followed by labored breathing. Death, often heralded by asphyxial convulsion, is due to respiratory paralysis in most animals (2).

In humans, systemic signs start 15 minutes to 5 hours

after the bite. Ptosis and drowsiness are the most common and earliest neurotoxic manifestation. Dyspnea, apnea, frothy saliva, slurred speech, flaccid skeletal muscle and respiratory failure were found in decreasing order of frequency (47). In some cases, unconsciousness was accompanied by respiratory failure. Death, in severe cases, was attributed to respiratory paralysis and usually occurred in an average of 2-20 hours after the bite (47,48,49,50). The relatively rapid death in poisoning by cobra bites is probably due to the smaller molecules of the venom, allowing more speedy absorption into the blood stream (36).

In Thai cobra (Naja naja kaouthia) bites, about 54-64% of patients developed neurotoxic signs (especially ptosis) (47,51) which is much higher when compared to 13% by Malayan cobra (Naja naja leucodira) bites as reported by Reid in 1964 (48).

Besides the systemic neurotoxic effects, local swelling and necrosis are always observed. As pointed out by Reid (48), in a series of 47 identified Malayan cobra bites, 20 (43%) patients developed local necrosis while only 13% developed neurotoxic manifestation. Local necrosis was also reported in about 40% of Thai cobra bites in children (47).

THE MANAGEMENT OF COBRA BITES

First Aid and Pre-hospital Treatment

In areas where elapid snakes reside, any person suspected of being bitten by a snake should be admitted into a hospital even in the absence of a snake-bite wound. However, first aid is proven to be of value in delaying the absorption of venom and the onset of envenoming. Recently, the development of a first aid measure consisting of a firm crepe bandage application over the injected site combined with immobilization by a splint was shown to effectively delay the venom movement for a long period (52,53). Moreover, during transportation, the body in general and the bitten limb in particular should be moved as little as possible to minimize the spread of the venom (50).

Antivenine Treatment

In systemic snake-bite poisoning, specific antivenine is the most effective therapeutic agent available. When used correctly, it can reverse systemic poisoning even after hours or days after the bite. It is therefore not only safe but highly desirable as well to wait for clear clinical evidence of systemic poisoning before giving antivenine. It should not be given routinely in all cases of snake bites because it is expensive and may cause side-effects. Its misuse (eg. if given by the wrong route or in an inadequate dose) can discredit antivenine therapy. As for the route of administration, antivenine must be given intravenously since absorption from other routes is too

slow to be of value. Dosage of antivenine varies depending on the amount of venom deposited and on the potency of antivenine being used. In severe poisoning, 100-150 ml of antivenine would be a suitable initial dose. If, by then, there has been little significant improvement, additional antivenine should be considered (50).

It was suggested that when a person bitten by an identified elapid snake was admitted within 6 hours, he should be given specific antivenine even though no symptoms or signs of envenomation has been shown, since the risk of dying from the bite is greater than the hazard of anaphylaxis or serum sickness (54,55).

Nevertheless, the administration of antivenine should be taken with the greatest caution and vigilance. Reid in 1964 (48). reported that 3 of 4 patients of Malayan cobra bite receiving Bangkok antivenine (monospecific anti Naja naja horse serum from the Queen Saovabha Memorial Institute , Bangkok) which is "raw" antiserum developed both immediate (one severe, two moderate), and delayed (all moderate) serum reactions. This figure was much higher than that reported later by Mitrakul et al. in 1984 (47) who also used monospecific anti Naja naja "raw" horse serum, prepared by the Thai Red Cross. Only mild to moderate urticaria was observed in 8 of 44 patients and no other side-effects occurred. The difference may be due to the later improvement in the preparation of antiserum, the age difference of the patients, the amount of the antivenine given and the difference in the size

of the patients studied.

Monospecific antivenines are most effective and less likely to cause reactions than polyspecific antivenine (50,56). In countries where only monospecific antivenine is available, the prompt identification of unknown envenoming snakes by immunodiagnostic tests is considered to be helpful in the management of the patient. This is feasible if the time taken to carry out the assay can be shortened and the cost be reduced to an acceptable level in developing countries.

Other Modes of Treatment

Other clinical trials in the treatment of cobra bites have been used, including the treatment with neostigmine (57), corticosteroid (58) and artificial respiration (59). The effect of neostigmine is still equivocal, although it has been proven that neostigmine can reverse the paralysis produced by pure cobra neurotoxin isolated from Naja naja atra in experimental animals (60). Corticosteroid has been used in the treatment of Thai cobra bites (58) but was shown to be of no benefit for either local or systemic envenoming effects as reported in other clinical trials (48). Artificial respiration was shown to play a significant role in the management of respiratory depression caused by cobra venom (47). Some authorities in Thailand, especially in the Department of Medicine, Chulalongkorn University Hospital, Bangkok, argued that antivenine was of little value in the treatment of neurotoxic envenoming and even possibly harmful for local envenoming effect. They advocated the

management by prolonged artificial ventilation, use of corticosteroids and attempted promotion of venom excretion by forced diuresis. It was found that there was no significant difference in the mortality rate between antivenine and non-antivenine treated patients and the latter group seemed to have decreased incidence of serum reaction and lower hospital expenses (61).

IMMUNODIAGNOSIS OF SNAKE BITE

The rational management of a patient with snake bite is facilitated by identification of the offending snake. It is often impossible, however, to establish an accurate species diagnosis on the basis of the patient's history and physical signs. Many immunoassay techniques have been developed for identifying venom in tissues or body fluids of recently bitten victims which may result in improved treatment of serious snake bite poisoning. Assay systems for detecting specific venom antibody are helpful to determine the extent of the problem of the snake bite in both developing and developed countries. Such techniques are also being applied in studying the kinetics of snake bite poisoning in man and animals, in assessing the potency and effectiveness of both new and currently available commercial antivenoms, examining the possibility of protective effects induced by previous venom exposure in man, and in other important aspects of laboratory-based venom research.

Ideal requirements for assay systems detecting venom and venom antibody include high levels of sensitivity, specificity,

(the ability to differentiate accurately among venoms and venom antibodies produced by closely-related species of snakes), rapidity in obtaining a result , reproducibility, simplicity and ease of sample collection.

Development and use of assay techniques in snake venom research includes:

Immunodiffusion

This gel diffusion technique was first described by Muelling et al. (62) to successfully detect king cobra (Ophiophagus hannah) venom in the homogenate of excised tissue from the bitten site of a 17 year-old girl 8 hours after admission to the hospital. This technique has been used successfully in animals by Trethewie and Thomas (63) to detect tiger snake (Notechis scutatus) and death adder (Acanthophis antarcticus) venoms in saline extract, aspirates or tissue fluid exudates from the bite site in guinea pigs.

Greenwood et.al. (64) used this technique to identify species-specific venoms in 101 patients with snake bites using wound apirates, blister fluids, sera and concentrated urine samples collected on admission to the hospital. They were able to achieve species diagnosis in only 40% of the patients. Besides being insensitive, immunodiffusion is a slow method, requiring 48 hours' incubation period.

Immunoelectrophoresis (IEP)

Greenwood et al. (64) examined the 44 positive samples on immunodiffusion by counter-current immunoelectrophoresis (CIE) against appropriate antiserum and 40 gave positive results. The technique was more rapid; positive results were obtained within 30 minutes in patients bitten by Naja nigricollis.

Tu and Salafranca (65) used immunoelectrophoresis to examine the in vitro neutralization capacity of the sea snake antivenine (equine anti-Enhydrina schistosa) against four heterologous sea snake venoms. It appeared that the antivenine effectively neutralized these venoms; the higher the neutralizing potential of the Enhydrina schistosa antivenine, the more similar the immunoelectrophoresis pattern.

By immunoelectrophoresis, Gawade and Gaitonde (66) were able to show cross neutralization of monovalent Enhydrina schistosa antivenine against venoms of other sea snakes and to a lesser degree against venoms of Naja naja, Ophiophagus hannah and Vipera russelli. Nevertheless, it is unlikely to be of practical use in the routine assay of venoms and venom antibodies, owing to the high frequency of cross-reactions.

Immunofluorescence

The direct immunofluorescence technique was used to localize Bothrops atrox and Crotalus adamenteus venoms in tissues following intraperitoneal administration in rats (67). Both venoms appeared to be concentrated in the medulla oblongata and

cervical cord and to a lesser extent in the phrenic nerves and diaphragm. In animals injected with Crotalus adamenteus venom the kidney also fluoresced strongly, indicating possible renal excretion of the venom.

The immunofluorescence technique is reliable for detecting venoms in biopsied tissues either from the patients or from experimental animals, but is unreliable when applied to post-mortem frozen sections because of tissue autolysis. The technique is rather subjective and laborious . Large-scale screening is impractical.

Hemagglutination

Boche and Russell (68) developed a passive hemagglutination test to demonstrate both venom and venom antibody. Whole venoms were coupled on sheep erythrocytes by the bis-diazo benzidine coupling procedure. Antibody was determined directly by hemagglutination and antigen was quantitated by examining the reduction in hemagglutinin titer after absorbing with the venom of interest.

Khupulsup et al.(69) reported a passive hemagglutination test for the semiquantitation of antibody to Naja naja siamensis toxin 3 . The test was specific and the toxin-coupled erythrocytes were stable for at least 4 months at 4 c with no detectable hemolysis when tested with various elapid venoms.

The passive hemagglutination is a semiquantitative, simple, rapid and economical in vitro test. It can be used to

assay antivenom potency when extreme accuracy is not required. The problems (in some cases) are due to the instability of the coupling agent. The test is less quantitative than radioimmunoassay or enzyme immunoassay.

Radioimmunoassay (RIA)

Before the development of enzyme immunoassay, RIA was extensively used to study the venom antigens and antibodies in snake bites. Coulter et al. (70) developed a solid phase competitive RIA using ^{125}I-labelled whole venom for the detection of venoms from tiger snake (Notechis scutatus) and brown snake (Demansia texilis) . This assay was highly reproducible and was capable of detecting as little as 15 ng per ml. of circulating venom 10 minutes after subcutaneous injection of a lethal dose of Notechis scutatus venom into experimental animals. Sutherland et al. (71) successfully used this technique to identify and quantitate snake venoms in human tissues and body fluids after being bitten by an unidentified snake.

A major drawback of competitive RIA for snake venom assay is that the iodination of individual venom component can vary considerably (72) due to the heterogeneity of the snake venom. After iodination, certain components may have their antigenic sites altered, thus competing unsatisfactorily with native venom in the competitive RIA.

Coulter et al. (73) developed a sandwich RIA system, for venom antigen detection using ^{125}I-labeled rabbit IgG

antivenom. This technique increased the sensitivity of the assay ie. it could detect minute amount of whole venom and specific venom components at below nanogram level in experimental animals. It was also observed that venom reached the circulation very quickly and was excreted in the urine in large quantities. It was concluded that the urine may be the most satisfactory body fluid to examine in patients with a snake bite. This was found to be so in the majority of snake bite cases studied later (74).

Sutherland et al. (52,75) used RIA to investigate the movement of injected venom as well as to assess the efficacy of various first aid methods which retard the absorption and entry of the venom. They showed that when snake venom was injected subcutaneously into monkeys, venom could be detected in the circulation within 15 minutes and plasma venom level peaked about 60 minutes. Post mortem studies showed that very little of tiger snake venom remained at the injection site, but high concentration were found in the regional lymph nodes. Both venoms and venom components were excreted in the bile and urine. First aid using a firm crepe bandage combined with immobilization by a splint was found to delay the movement of all the major Australian snake venoms.

Although the RIA assay gives a greater sensitivity than other immunodiagnosis technique, it raises some practical and economical problems. It is a complicate and time-consuming procedure (the assay completed in 24 hours) (73). The technique is expensive and requires considerable technical expertise in the

handling of radioisotopes, the use of elaborate and expensive counting equipment and the ^{125}I-radioisotope has a relatively short shelf life. It is therefore unsatisfactory for immediate species diagnosis in snake bite.

Thus, the main role of RIA currently has in the management of snake bites is that of a retrospective means of determining the type of snake and extent of envenomation. This, in turn, leads to a better clinical understanding of envenomation by providing definite proof that a particular species of snake can cause a major pathological disturbance, for example, myoglobinuria after tiger snake bites.

Enzyme-Linked Immunosorbent Assay (ELISA)

The first description of such technique appeared in 1971 (76). The methods are analogous to those in radioimmunoassay and immunofluorescence and can be used to assay both antibodies (indirect ELISA method) and antigens (double antibody sandwich ELISA method) at a high level of sensitivity and reproducibility yet employing relatively simple and inexpensive equipments. The range of application of ELISA technique is potentially as wide as that of radioimmunoassay and may also reinforce or replace other serological tests such as immunofluorescence, hemagglutination and complement fixation. To date, the ELISA technique has been used in the area of infectious disease (77), endocrinology (78), immunopathology (78,80), hematology (81), tumor immunology (82), parasitic disease (83), drug (84) and snake venom research (85,86).

The principle of the technique depends on the fact that antibodies or antigen can be linked to an enzyme, the complex of which retaining both immunological and enzymatic activity. In these assays, the antigen or antibody is usually attached to a solid-phase support to allow for easy separation of bound and free reagents. It has been found that both antigens and antibodies can be covalently attached to particulate materials such as cellulose and polyacrylamide, and that satisfactory passive adsorption can also be obtained in tubes, beads, disks or wells of microtiter plate made of nylon, polystyrene, polyvinyl, or polypropylene. The enzyme used in ELISA is neccessary to have high activity and stability, be cheap, available in a pure form and have a substrate reaction that can be easily measured. The most satisfactory enzyme found to date are horseradish peroxidase, glucose oxidase, alkaline phosphatase and β-galactosidase. These are usually linked to antibodies or antigens by means of glutaraldehyde or periodate (87).

In the snake venom research, ELISA has been successfully applied to the detection of several snake venoms and venom antibodies by Theakston et al. in 1977 (85). The assay was very sensitive; it can detect venom level down to 1-5 ng/ml in rat and mice injected intravenously or subcutaneously with 1 LD50 of venom. Moreover, it gave good specificity, without cross-reaction among 14 different types of venom from clinically important snakes found in Africa, South East Asia, Europe, North America and Australia. Specific antibody could be detected in human serum more than 2 years after an accidental bite by Echis

carinatus (85).

Although the time taken to carry out the venom assay was reduced to 3 hours by using microtiter plates precoated with the IgG fraction of specific antiserum (85), it was generally considered to be too slow to be useful for clinicians in treating severely poisoned patients with specific antivenom.

For rapid diagnosis, Coulter et al. (88) developed a rapid and sensitive ELISA for the detection of snake venom. By using Protein A (Protein A sepharose CL-4B) purified rabbit IgG antivenom and the enzyme horseradish peroxidase (HRPO), the positive results could be obtained within 30 minutes at a sensitivity of 2 ng/well of venom or within 90 minutes at a sensitivity of 0.5 ng/well of venom.

Sutherland et al. in 1979 (89) carried out a trial of rapid ELISA kits for the detection of Australian snake venoms. Generally the kit had performed well, especially with swabs taken from the bite site where venom levels were obviously high.

More recently, Chandler and Hurrell (90) also developed a new modified ELISA kit suitable for field use. By using precoated tubes and the urease enzyme conjugated antibody, results were observed visually for color change on the tubes within a test time of 30-40 minutes. The sensitivity of the test was 5-10 ng/ml. This technique demands a high cost of £ 11 per kit and makes it unlikely to be used in poor or developing countries where snake bite is a serious medical problem.

In a study carried out in Malaysia, Dhaliwal et al. in 1983 (91) reported a study of a rapid micro-ELISA test which could be completed and read visually within 35-45 minutes to distinguish between the common cobra (Naja naja) and viper bite (Agkistrodon rhodostoma or Malayan pit viper). This is beneficial since on many occasions the clinical signs and symptoms are not specific to the cobra or viper.

The prompt identification of the snake involved in snake bites by this technique allows selection of the correct monospecific antivenine and may decrease the need to administer a large volume of polyvalent antivenine in patients. This might, in turn, result in a reduction of the incidence of both immediated and delayed reactions caused by injection of a large volume of polyspecific antivenoms.

In Thailand, where only monospecific antivenines are available, the species diagnosis is important. Viravan et al.(51) confirmed the value of ELISA technique in the diagnosis of acute and past envenoming by the Thai cobra (Naja kaouthia). The venom was found in 22 of 58 suspected cobra bites, 8 of 33 patients with only local envenoming and 14 of 20 with local plus systemic (neurotoxic) envenoming, the mean venom concentration being 33 times greater in the later group. Serum antibody was present in patients bitten previously from 1 month to 7 years. The study confirms the impression that cobra bites are the principal cause of neurotoxic envenoming in the Samut Prakan province of Thailand. However, the attribution of one fatal case of Bungarus

fasciatus venom in this study and a previous report of Bungarus candidus venom in the serum and tissue fluid aspirate from the bite site of two severe neurotoxic patients in Thailand (92), suggests that Bungarus bite should be suspected if the patients fail to respond to cobra antivenine.

The use of ELISA in detecting specific venom antibody in previously snake-bite victims is helpful in studying sero-epidemiology of snake bite in many parts of the world where the frequency of poisoning by co-existing medically important species is unknown and where biting species are generally unidentified on clinical grounds. In snake bite victims, natural venom antibody could develop within 1-2 months after the bite despite therapeutic antivenine being given. Venom antibody may wane with time. It is more likely to be detected in the first 10 years after snake bite poisoning than the later period (93). However, in some cases, antibodies were still present up to 40 years after the original bite (93,94). The more often an individual was bitten, the higher the antibody was observed (95) and the titer of venom antibody declined with an approximate half life of 2-3 years (51).

ELISA technique can also be applied to assess antivenine potency. Theakston et al. (96) reported the usefulness of an ELISA test as an in vitro ED50 test in screening the potency of antivenines from both commercial and research sources. The ELISA test was comparable to the conventional in vivo mouse ED50 assay. The method is rapid, cheap, simple, economic in amounts

of venom used, and most importantly, greatly reduced the need for live animals.

Other applications of ELISA technique include assessing the extent of active immunization in animals and man, examining the specificity of antibody to isolated venom fractions (97) and monitoring monoclonal antibody production.