THE DEVELOPMENT AND LIMITATIONS OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE CLINICAL EVALUATION OF COPPERHEAD (*AGKISTRODON CONTORTRIX*) SNAKEBITE

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Bachelor of Science

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Pullman, Washington

1985

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 1987

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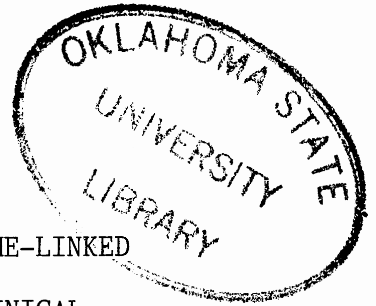
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ACKNOWLEDGEMENTS

I would like to express my sincere thanks to Dr. Charlotte L. Ownby for her guidance and for allowing me the opportunity to expand my knowledge of animal toxins. I would also like to thank Dr. George Odell and Dr. Deborah Claypool for acting on my committee and Michael Smith, Terry Colberg, and Mary Bober for their friendship. I would like to especially thank Dr. Russell Allen for supplying clinical samples and for financial support that allowed me to complete this work. Finally, I would like to thank my parents and family for all the encouragement and support they have given me throughout my life.

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NOMENCLATURE

BAEE	N-benzoyl-L-arginine ethyl ester
cm	centimeter
ELISA	enzyme-linked immunosorbent assay
I-131	Iodine-131
IgG	Immunoglobulin G
LD/50	lethal dose 50%
mg	milligram
ml	milliliter
NaCl	sodium chloride
NaN_3	sodium azide
NaOH	sodium hydroxide
ng	nanogram
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
SDS	sodium dodecylsulfate
Se-75	Selenium-75
TAME	p-toluene-sulfonyl-L-arginine methyl ester
Tween 20	polyoxyethylene-sorbitan monolaurate

CHAPTER I

INTRODUCTION

In the United States, venomous snakes are responsible for 6,000-8,000 human bites each year (Russell, 1969). Because of the availability of medical treatment and the use of antivenom, the mortality from snakebite in the U.S. is low with fewer than twelve deaths per year (Minton, 1971). However, the local damage caused by the venoms, including hemorrhage, edema, and myonecrosis continues to be an important clinical concern and can result in the loss of function of the envenomated region.

The treatment for snakebite is varied, but depends on factors such as the diagnosis of the bite due to a venomous snake, the amount of venom injected, and the length of time elapsed before reaching medical assistance (Klauber, 1956). The diagnosis of venomous snakebite is often difficult and depends on both the presence of clinical signs of envenomation and the positive identification of the snake. However, in many cases the snake responsible for the bite is not seen or is misidentified by the patient. In these cases the accurate identification of the snake responsible can be extremely difficult especially in areas where multiple species of venomous snakes inhabit the same region.

In the state of Oklahoma, U.S.A., there are seven species of venomous snakes, all of which belong to the family Viperidae, subfamily

Crotalinae. Copperheads (Agkistrodon contortrix) are common throughout the eastern two thirds of Oklahoma and bites by these snakes represent a high percentage of the total number of snakebites in the state (Parrish, 1980). Although rarely fatal, copperhead bites can produce significant local damage which includes primarily edema, erythema, and subcutaneous hemorrhage. The treatment for these cases usually consists of fluid therapy and corticosteroids; the use of antivenom is generally not required. On the other hand, bites by other crotaline snakes found in Oklahoma, such as rattlesnakes, can produce both local and systemic effects resulting in death, and the use of a polyvalent antivenom is often recommended for serious cases. The administration of the antivenom, however, increases the risk of hypersensitivity to the horse serum which is especially important if the victim should receive a future bite and require serotherapy. Therefore, although the treatment for snakebite is based on clinical signs, a more conservative treatment may be employed, especially with regard to the administration of antivenom, if the snake responsible is identified and known to produce less serious bites in humans. Since copperhead bites are usually not life-threatening and do not require the use of antivenom, it would be beneficial in treating snakebite in the U.S. to identify the snake responsible as either a copperhead or other crotaline snake. This is especially important in areas of the country with large populations of copperheads and the snakes represent a high percentage of snakebite cases.

Clinical assays have been developed which can detect and identify specific venoms in snakebite victims where the identity of the snake was not known. The most commom method is to use an immunoassay to identify

the venom in body fluids such as serum, urine, and bite site washings. Immunodiffusion, radioimmunoassays, and enyme-linked immunoassays have all been used with varied success in detecting the presence of venoms (Muelling et al., 1957; Coulter et al., 1978; Theakston et al., 1977). However, each of these assays has limitations that must be considered in a clinical application. Immunodiffusion is relatively easy and does not require sophisticated laboratory equipment but is less sensitive than other immunoassays and can only detect immune complexes in microgram concentrations. Radioimmunoassays have a high sensitivity, detecting venom in nanogram concentrations, but require the use of reagents that are labile and cannot be stored for long periods of time. Enzyme-linked immunoassays also have high a sensitivity, however, non-specific binding and cross-reactions can be a problem (Ho et al., 1986a).

The detection of specific venoms in serum and urine from snakebite victims using an enzyme-linked immunosorbent assay (ELISA) has been reported (Coulter et al., 1980; Ho et al., 1986b). The limitations of the assay based on possible interactions between snake venoms and human serum components and variations in serum and urine from individuals, however, have not been thoroughly investigated. The purpose of the following investigation, therefore, was to develop an ELISA to detect the presence of copperhead venom in serum and urine from snakebite victims and investigate possible limitations of the assay in clinical applications. Cross-reactions between antisera to copperhead venoms and venoms from taxonomically related snakes was also investigated to determine how this may influence the identification of snakes involved in snakebite in the United States.

CHAPTER II

LITERATURE REVIEW

Venomous snakes in the United States belong to either the subfamily Crotalinae or the family Elapidae. The Crotalinae or pit vipers are represented by the true rattlesnakes, genus *Crotalus*, the ground rattlesnakes, genus *Sistrurus*, and the copperheads and water moccasins, genus *Agkistrodon*. The Elapidae are represented by two genera of coral snake, *Micruoides* and *Micrurus*. In the U.S., the majority of serious human bites are caused by crotaline snakes; the coral snakes, although possessing a potent neurotoxic venom, are responsible for only about 1% of the total number of snakebites (Parrish, 1980).

Snake venoms are complex mixtures composed primarily of proteins that possess a wide range of biological activities (Tu, 1977). Snake venoms have historically been classified as either hemotoxic or neurotoxic to describe their primary mode of action on the cardiovascular system or nervous system, respectively (Minton, 1974). Because of their complexity, however, specific venoms have been found that produce deleterious effects on numerous organ systems of the body. For example, the venom of the African spitting cobra (*Naja nigricollis*), a predominantly neurotoxic venom, has been shown to also produce local damage such as myonecrosis and hemorrhage in humans (Reid, 1964). Conversely, the venoms from rattlesnakes are considered to be primarily

hemotoxic although the Mojave rattlesnake (Crotalus scutulatus scutulatus) produces a venom that causes little hemorrhage and contains high concentrations of a presynaptic neurotoxin (Castilonia et al., 1980).

Copperheads and Their Venoms

Copperheads (Agkistrodon contortrix) are small, crotaline snakes that inhabit the central and southeastern regions of the United States. In the springtime, copperheads can be found basking during the daytime on rocky outcroppings in grassy fields and along small streams. As the summer progresses, however, they become more active in the evening hours and eventually will become nocturnal. At this time they can be found crossing roads and in open meadows in search of food which includes primarily rodents, birds, lizards, and amphibians. It is also at this time that many people are bitten by copperheads. However, since other snakes, including both venomous and non-venomous species, are also active at night during summer, it is often difficult to identify the snake as a copperhead if a bite should occur.

The biological activities of copperhead venoms have not been as thoroughly investigated as those of venoms from other snakes. Copperhead venoms have been shown to produce considerable local tissue damage such as myonecrosis, edema, and hemorrhage (Mebs et al., 1983) although they are generally not as toxic as other crotaline venoms. Minton (1974) reported an intravenous LD/50 of 10.0 mg/ml for southern copperhead (A. c. contortrix) venom which is approximately 10 times less toxic than the venom of the cottonmouth (A. piscivorus), a closely related snake. Moran

et al. (1979) fractionated northern copperhead (A. c. mokeson) venom into 9 components using carboxymethyl cellulose chromatography. One of the fractions was responsible for 65-70% of the lethality of the whole venom and had a subcutaneous LD/50 of 1-2 mg/kg.

The enzymatic activities of copperhead venoms have been reported by many researchers. Tu et al. (1965) showed that the venoms from copperheads possess considerable proteolytic activity on synthetic substrates such as TAME and BAEE. They also found that proteolytic activity was widely distributed in many venoms from snakes in the subfamilies Crotalinae and Viperinae but not in the family Elapidae. Kocholaty et al. (1971) in a comprehensive study of some of the enzymatic properties of venoms from crotaline, viperine, and elapid snakes also showed considerable proteolytic activity in copperhead venoms. In addition they found that copperhead venoms had the highest L-amino acid oxidase activity of venoms from 29 snake species tested with the exception of C. v. viridis venom. They also showed that copperhead venoms did not possess any phosphodiesterase activity but did have moderate phospholipase A_2 activity. In isolating the phospholipase A_2 component from A. c. contortrix venom, Welches et al. (1985) demonstrated that most activity was found in a monomeric protein with a molecular weight of 8,000-10,000. The monomeric phospholipase A_2 from members of the genus Agkistrodon was different from those found in species of the genus Crotalus which are usually dimeric molecules.

The venoms from copperheads have also been shown to have pronounced effects on the vascular system. Bajwa et al. (1982) showed that crude A. c. contortrix venom exhibited fibrinolytic activity using the fibrin

plate assay but did not show any thrombin-like activity. However, after fractionation on Sephadex G-100 both fibrinolytic and thrombin-like activities were found in fractions with molecular weights of 73,500 and 25,000, respectively. Herzig et al. (1970) using column chromatography also separated the venom of A. c. contortrix into fibrinolytic and procoagulant fractions. The procoagulant fraction clotted fibrinogen directly and had proteolytic, esterolytic, and amidase activities. The fraction released fibrinopeptide B at a much faster rate than fibrinopeptide A, however, a visible clot did not form until considerable amounts of fibrinopeptide A were released. Markland et al. (1985) isolated a fibrinolytic enzyme from A. c. contortrix venom using CM-cellulose, Sephadex G-100, benzamidine-Sepharose, and isoelectric focusing that was a single chain glycoprotein that had a molecular weight of 25,000 using SDS-PAGE. The fraction was free of thrombin-like activity and acted both directly on fibrin formed by thrombin clotting of human fibrinogen and on a fibrin clot using the fibrin plate assay.

Distribution of Venoms in Envenomated Animals

The distribution of radiolabelled snake venoms in experimental animals has been well documented. Lee and Tseng (1966) showed that in rabbits injected intravenously with iodinated venom from the banded krait (Bungarus multicinctus), the highest concentration of venom was distributed in the kidney followed in decreasing order by the lungs, spleen, heart, stomach, intestine, diaphram and skeletal muscle. The distribution of the venom in the kidney was also considerably higher than in blood samples taken from the carotid artery. Lebez et al. (1973) in a

study using *Vipera ammodytes* venom also showed a high concentration of Se-75-labeled fractions in the kidney of artificially injected mice.

High concentrations of venom have also been reported in the urine of animals injected with snake venoms. In a study using rabbits, Shu *et al.* (1968) showed that 70% of cobrotoxin labeled with I-131 is excreted in the urine within 5 hours of a single intravenous injection.

Detection of Venom Using Immunoassays

Clinical immunoassays that have detected a specific venom in snakebite victims where the snake was not identified at the time of the bite have been described. Sutherland and Coulter (1977) using a radioimmunoassay with I-125-labeled rabbit IgG against several Australian elapid venoms were able to detect the presence of tiger snake (*Notechis scutatus*) venom in urine and extracts from clothing in two of three unusual snakebite cases in which the patients were found unconscious. Post-mortem examination of one of the patients where death occured before antivenom could be administered revealed traces of *N. scutatus* venom in skin from the possible bite site as well as in blood, liver, and urine samples.

The use of an enzyme linked immunosorbent assay (ELISA) for the identification of snake venoms has also been described. Theakston *et al.* (1977) used an ELISA for the detection of snake venoms in a test system as well as in experimental animals. Using 14 venoms from different species diluted in normal human serum and normal rat serum they were able to detect the venoms in concentrations of 1-5 ng/ml. Antisera against venoms from *Naja haje*, *N. nigricollis*, *Bitis arietans*, *Causus maculatus*,

and Echis carinatus did not cross react with any of the 14 venoms with the exception of the venom from N. naja reacting against the N. haje antiserum. Blood samples taken from mice injected i.v. (0.4 mg/kg) with E. carinatus venom showed high venom concentrations up to 2 hr, however, levels were just detectable after 24 hr. After subcutaneous injection (6.0 mg/kg), venom levels in the blood were low after 24 hr, reached a maximum after 48 hr, and then remained relatively constant for 3 weeks. The authors also showed similar results using rats.

Coulter et al. (1980) developed a clinical ELISA for the rapid identification of the snake species involved in snakebite in Australia. By using horseradish peroxidase-labeled rabbit IgG against several crotaline, viperine, and elapid snake venoms, they were able to identify the snake responsible for the bite by detecting the presence of specific venoms in swab eluates of the bite site. Later, Chandler and Hurrell (1982) described a modification of an ELISA which allowed for the identification of snake venom in blood, urine, and bite site washings that does not require the use of sophisticated laboratory equipment and is suitable for field use. The assay utilizes capillary tubes coated with monospecific antisera against a variety of different snake venoms. The test sample is drawn into each capillary tube and after washing, a secondary antibody conjugated to urease is added to the tubes. Urea is used as substrate and the amount of ammonia released is determined by a decrease in pH and is measured by a visible color change of Bromocresol purple. In a review of the assay after two years of clinical use in Australia, Hurrell and Chandler (1982) showed that in 38 patients showing definite signs of venom poisoning, the identity of the snake was

determined in 31 of the cases. However, 6 patients showed definite signs of envenomation but were negative when tested using five monospecific antisera against venoms from the snakes responsible for the majority of snakebite in Australia.

Interaction of Serum Components and Snake Venom

The interaction of snake venoms and serum components from a variety of animals has been reported. Venomous snakes are one group of animals that have been shown to possess serum components that interact with both their own venom and the venoms from other snake species. Omori-Satoh *et al*. (1972) isolated an antihemorrhagic factor from the serum of the habu snake (*Trimeresurus flavoviridis*) that effectively neutralized the hemorrhagic activity of its venom. The purified component had a molecular weight of 70,000, an isoelectric point of about 4.0, and migrated similarly to albumin and alpha-1 globulin using immunoelectrophoresis. They also showed that the crude serum from *T. flavoviridis* inhibited hemorrhagic activity of a variety of crotaline, viperine, and elapid snake venoms. Ovadia (1978) isolated a factor from the serum of the snake *Vipera palaestinae* that neutralized all the hemorrhagic activity of its venom. It had a molecular weight of about 80,000, an isoelectric point of 4.7, and did not form precipitin bands against the venom using immunodiffusion. These results suggested that it was not an immunoglobulin but was probably an albumin or alpha-globulin.

Sera from non-venomous snakes have also been shown to contain factors that interact with venom components. The serum of the florida kingsnake (*Lampropeltis getulus floridana*) was fractionated into 3

components that effectively inhibited the proteolytic activity of water moccasin (A. piscivorus) venom (Bonnett and Guttman, 1971). All three fractions migrated similarly to the gamma-globulins using electrophoresis, and the inhibition was thought to result from the combination of gamma-globulin antibodies with antigenic venom proteases.

The inhibition of snake venom components by the sera from mammals has also been reported. Pichyangkul and Perez (1981) isolated a component from the serum of the hispid cotton rat (Sigmodon hispidus) that neutralized the hemorrhagic activity of Crotalus atrox venom. The molecular weight of the factor was about 90,000 and it had an isoelectric point of 5.4. Neither the crude serum or purified component formed a precipitin line against C. atrox venom using immunodiffusion. These results suggested that the factor was probably an alpha-globulin which had characteristics similar to the antihemorrhagic factors isolated from some snake sera. An antihemorrhagic factor isolated from the serum of the opossum (Didelphis virginiana) was shown to have a molecular weight of 68,000 and an isoelectric point of 4.1 (Menchaca and Perez, 1981). They suggested that the antihemorrhagic factor from opossum serum is probably an albumin or closely associated with albumin.

Human alpha-2 macroglobulin or its equivalent in animals is another serum component that has been shown to interact with certain snake venom proteases. Kress and Catanese (1981) demonstrated that the addition of 0.1 mg human alpha-2 macroglobulin to 14 snake venoms resulted in 40-80% inhibition of the proteolytic activity of the venom. However, Crotalus adamanteus venom retained approximately 50% of its proteolytic activity even after addition of 1.5 mg alpha-2 macroglobulin. C. adamanteus peak

I proteinase, which is responsible for most of the proteolytic activity of C. adamanteus venom, also retained 75-80% of its original activity in the presence of human alpha-2 macroglobulin. Since alpha-2 macroglobulin was in excess, the residual proteolytic activity of peak I proteinase suggests that some venom proteinases are only slightly inactivated by complex formation with alpha-2 macroglobulin. Pitney and Regoeczi (1970) also showed a short-lived complex of human alpha-2 macroglobulin with the thrombin-like esterase from A. rhodostoma venom.

In a study using non-human macroglobulins, DeWit and Westrom (1987) isolated 3 fractions from serum of the European hedgehog that were able to neutralize all hemorrhagic activity of European viper (Vipera berus) venom. The proteins migrated in the alpha-2, alpha-2-beta, and beta region on agarose electrophoresis and showed inhibiting activity against trypsin, chymotrpsin, elastase, collagenase, papain, and plasmin. Polyacrylamide gel electrophoresis showed one band corresponding to a molecular weight of 780,000 and three weak bands with molecular weights of 670,000, 550,000, and 539,000. Using immunoelectrophoresis, the alpha-2 protein cross-reacted with antisera against human alpha-2 macroglobulin, swine alpha-2 macroglobulin, and rat alpha-2 acute phase globulin while the alpha-2-beta and beta proteins cross-reacted with swine alpha-2 macroglobulin.

Cross-reaction between Snake Venoms

Chemical differences in the venoms of taxonomically distinct snake species have been shown by many researchers. However, since the turn of the century, many common components in snake venoms have been reported.

In an early study on the antigenic composition of snake venoms, Lamb (1902) showed precipitin reactions between venoms of Russell's viper (V. russelli), saw-scaled viper (E. carinatus), banded krait (B. fasciatus), and tiger snake (N. scutatus) and antiserum prepared in rabbits against venom of the Indian cobra (N. naja naja). Minton (1968) using immunodiffusion showed that the venom from Wagler's pit viper (Trimeresurus wagleri) has at least six venom components common with other members of the genus. Also, the venoms from T. okinavenis, T. albolabris, and T. wagleri all cross-reacted with antiserum to T. flavoviridis. Venoms of three American and two Asian species of Agkistrodon were studied by Tu and Adams (1968) who found that immunologic differences were roughly proportional to geographic distance between species. However, the venom of A. halys blomhoffii of Japan was antigenically as close to A. contortrix and A. piscivorus of North America as to A. rhodostoma of Southeast Asia. Among Asian Agkistrodon, venoms from A. h. blomhoffii, A. h. brevicaudus, A. caliginosus, and A. h. caucasius gave similar immunodiffusion patterns while those of A. acutus and A. rhodostoma differed markedly from this group.

Purified components isolated from specific snake venoms have also been shown to be distributed among both taxonomically related and distinct snakes. Weinstein et al. (1985) isolated Mojave toxin from the venom of C. s. scutulatus and produced an antiserum against it in rabbits. Using an ELISA, they found high amounts present in 4 of 6 C. s. scutulatus venoms, 2 of 3 C. durissus venoms, and in venom samples from C. viridis concolor and C. tigris. Low amounts were present in one C. s. scutulatus venom sample, 2 of 12 C. atrox venoms, and one T. flavoviridis

venom sample. The toxin was absent in the venoms tested from species of *Agkistrodon*, *Sistrurus*, and *Vipera*.

CHAPTER III

CASE HISTORIES

In this investigation serum and urine from three snakebite victims from Oklahoma were screened for the presence of copperhead venom using an enzyme-linked immunosorbent assay. All three patients developed clinical signs consistent with bites by copperheads, however, in all cases the identity of the snake was not accurately determined. Case histories for these patients are presented below.

Patient #1

Locale- Pottawatomie County, Oklahoma; Subject- Caucasian female; Age- 26. Circumstance and etiology. On the night of 3 June, 1986 the subject was walking through a pasture near her home when she was bitten on the left foot by an un-identified snake. Within a few minutes a burning sensation developed in the foot which soon spread up to the calf and thigh. The following morning the pain was still present and the patient was transported to the Springdale Medical Clinic in Oklahoma City, Oklahoma. Upon arrival at the clinic, vital signs were normal and the foot was swollen but not vesiculated. The toes were strutted and tender to palpation. Areas of discoloration were present around the bite site and on the dorsum of the foot proximal to the toes. On 5 June, 1986 the foot was still swollen and the edema had spread to the calf. Areas

of ecchymosis were present over much of the foot, calf, and thigh and left inguinal adenopathy was present. The patient was treated conservatively with corticosteroids and allowed to go home. On 18 June, 1986 the swelling and ecchymosis were decreased and the foot was normal size. No eschar or tissue loss resulted and the patient recovered without complication.

Patient #2

Locale- Logan County, Oklahoma; Subject- Caucasian female; Age- 20. Circumstance and etiology. On the night of 14 July, 1984 the subject was walking near a pond when she was bitten on top of the right foot by an un-identified snake. Considerable pain and edema developed in the foot and the patient was admitted to the Mercy Hospital in Oklahoma City, Oklahoma at 3:45 a.m. At this time swelling extended to the knee and right inguinal adenopathy was present. The foot and calf were tender to palpation but were not vesiculated. Ecchymosis extended from the bite site to the ankle and the calf. Antivenom was withheld because of clinical findings and the risk of sensitization.

On 16 July, 1984 the discoloration of the foot was slightly increased, however, there was good movement of all digits and sensory functions were intact. Excellent micro-circulation in the toes was demonstrated using a doppler recording. The swelling, adenopathy, and ecchymosis gradually decreased and the patient was dicharged on 18 July, 1984. The patient recovered without complication and retained complete movement of all digits.

Patient #3

Locale- Caddo County, Oklahoma; Subject- American Indian male; Age- 13. Circumstance and etiology. On the night of 16 June, 1985 the patient was home working in his garage when he reached back for a tool and was bitten on the second digit of the first finger of the left hand by a snake believed to be a copperhead. He was admitted to Deaconess Hospital in Oklahoma City, Oklahoma early 17 June, 1985. He appeared to be in some distress, principally by anxiety over his condition. Examination of the left extremity revealed a slight draining of serosanguous material from two puncture wounds of the volar aspect of the second digit of the first finger. Considerable discoloration extended from the digit into the hand and the entire area was markedly tender to palpation. Edema continued to develop in the hand, and the patient was skin tested for antivenin. This was negative and the patient was given two vials (20 cc) of Wyeth's Polyvalent (Crotalidae) antivenin. He responded well to treatment and demonstrated no allergic reaction to the antivenin. The patient was discharged from the hospital on 20 June, 1985 at which time the swelling had decreased and he had good joint movement in the digit, wrist, and elbow. An eschar developed at the bite site which was later replaced by scar tissue.

CHAPTER IV

MATERIALS AND METHODS

Venoms

Lyophilized southern copperhead (A. c. contortrix) venom was purchased from Biotoxins Inc., St. Cloud, Fl, U.S.A (Lot# AC/85A). Venom from the northern copperhead (A. c. mokeson) was purchased in lyophilized form from Miami Serpentarium Laboratories, Salt Lake City, Utah, U.S.A (Lot# AM7SZ). For cross-reaction studies, venoms from twenty four species of Crotalus inhabiting the U.S., Mexico, and Central and South America were kindly donated by James Glenn, Venomous Animals Research Laboratory, Veterans Administration Hospital, Salt Lake City, Utah, U.S.A.

Production of Antisera

Antisera against both A. c. contortrix and A. c. mokeson venoms were prepared in New Zealand white rabbits using the technique of Ownby et al. (1979). Venoms were first diluted in 0.85% saline to a concentration of 1.0 mg/ml. One milliliter of the venom solution was mixed with an equal volume of Freund's complete adjuvant (DIFCO Laboratories, Detroit, MI) and injected intramuscularly between the shoulder blades. A booster injection containing 1.0 ml of the venom solution mixed with an equal volume of Freund's incomplete adjuvant was given one week after the

initial injection and then again approximately every two months. The rabbits were bled from the marginal ear vein two weeks after the first booster injection and again approximately every month. Sera were collected and centrifuged for 10 minutes at a setting of 7 on a IEC clinical centrifuge to remove cellular debris. Aliquots of the sera were stored at -20°C until needed.

Detection of Antibodies

Sera from the bleedings were tested for the presence of antibodies to either *A. c. contortrix* or *A. c. mokeson* venoms using Ouchterlony double immunodiffusion in 1% agarose gels (Ownby *et al*., 1979). Diffusion plates were prepared by adding 5.0 ml of agarose to small Petri dishes. Six wells were then cut into the gel; the diameter of the wells was 0.5 cm and the distance from center to center of wells was 1.0 cm. The wells were filled twice with ca. 20 microliters of either homologous venom or antivenom and the plates allowed to develop at room temperature for 48 hours.

Enzyme-Linked Immunosorbent Assay

The indirect and double-antibody methods of an enzyme-linked immunosorbent assay (ELISA) were both used to test serum and urine samples from three snakebite patients seen at the Springdale Medical Clinic, Oklahoma City, Oklahoma, for the presence of copperhead venom using monospecific antisera against venoms from either *A. c. contortrix* or *A. c. mokeson*. In addition, both assays were used to test for possible interference of the ELISA by human serum components while the

indirect ELISA was used to test the degree of cross-reaction between copperhead antiserum and other crotaline snake venoms.

Indirect ELISA. Two hundred microliters of test sample or controls were added to wells of polystyrene microtiter plates (Flow Laboratories, McLean, VA.) and coated overnight at 4°C. The plates were washed 3 times in washing buffer containing NaCl and Tween 20 allowing a 5 minute soaking between each wash step. Two hundred microliters of a 1.0% bovine serum albumin (aqueous) were then added to each well and incubated for 1 hour at 37°C as a blocking step. The plates were again washed three times and dilutions (1:500, 1:750, 1:1000,) of crude antisera against either venoms from *A. c. contortrix* or *A. c. mokeson* in incubation buffer containing PBS, Tween 20, and NaN_3 were added to the wells and the plates incubated at 37°C for 2 hours. After washing, 0.2 ml of a 1:1000 dilution of a goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma Chemical Co., Inc.) in incubation buffer was added and the plates incubated for 2 hours at 37°C. The plates were washed a final time and 0.2 ml of a 1.0 mg/ml solution of p-nitrophenyl phosphate (Sigma, Inc.) in diethanolamine buffer added to each well as substrate. The substrate was incubated for either 20, 30 or 60 minutes and the reaction stopped by the addition of 0.05 ml of 3 M NaOH to the wells. The absorbance of each well was determined at 405 nm using a Bio-Tek EL 307 ELISA plate reader. Incubation buffer and serum and urine purchased from Ciba Corning Diagnostic Corporation (Irvine, CA.) were used as negative controls. A standard curve of known amounts of venom ranging from 1000 to 1.97 ng/ml were made in incubation buffer, control serum, and control urine and run on each plate.

Double-Antibody ELISA. The double-antibody ELISA method was the same as described for the indirect method with the exception that 0.2 ml of a 1:1000 dilution of the crude antiserum in coating buffer (pH 9.6) were added to each well of polystyrene microtiter plates and coated overnight at 4°C. The plates were washed and 0.2 ml of test samples or controls added to the wells and incubated for 2 hours at 37°C. The remainder of the assay including the addition of antibody, conjugate, and substrate was run as described for the indirect ELISA.

Determination of Interference of ELISA by Serum Components

Possible interference of the ELISA by certain human serum components was investigated using both the indirect and double-antibody ELISA methods. Human alpha-2 macroglobulin and human IgG purchased from Sigma Chemical, Inc. were diluted in incubation buffer (pH 7.4) to concentrations of 5.0 mg/ml and 10.0 mg/ml, respectively and added to separate standards of *A. c. contortrix* venom. The standards ranged from 250 to 15.8 ng/ml and were prepared by making serial dilutions of the venom in incubation buffer and adding the appropriate amount of the alpha-2 macroglobulin or IgG stock so that each dilution contained either 0.5 mg/ml alpha-2 macroglobulin or 5.0 mg/ml human IgG. A third standard was prepared by making serial dilutions of *A. c. contortrix* venom in incubation buffer containing 20.0 mg/ml human serum albumin (Sigma, Inc.). A fourth standard consisted of only *A. c. contortrix* venom in incubation buffer.

Determination of Cross-Reaction

Cross-reactions between antisera against venoms from *A. c. contortrix* or *A. c. mokeson* and venoms from 24 crotaline snakes were investigated using the indirect ELISA method. Lyophilized venoms were reconstituted in incubation buffer to a concentration of 1.0 mg/ml. Wells on polystyrene microtiter plates were loaded with 0.2 ml of the venom solutions and coated overnight at 4 C. The assay was then performed as described for the indirect ELISA method. Venoms that gave an absorbance greater than 2.0 at 405 nm were diluted 1:1000 in incubation buffer and the assay repeated. Venoms that cross-reacted with either antisera were scored semi-quantitatively based on their absorbances at 405 nm compared to the homologous venoms.

Statistical Analysis

The Student's t-test (p less than or equal to 0.05) was used to determine the statistical significance between test samples and negative controls.

CHAPTER V

RESULTS

Detection of Antibodies

Ouchterlony immunodiffusion showed the presence of antibodies in both antisera to *A. c. contortrix* and *A. c. mokeson* venoms. Four precipitin bands formed between homologous venom and antiserum to *A. c. contortrix* venom whereas 3 precipitin bands formed between antiserum to *A. c. mokeson* venom and homologous venom.

Double Antibody ELISA.

Determination of Parameters. Preliminary studies were done to determine both the substrate incubation time and concentration of conjugate that would provide maximum sensitivity and minimum non-specific binding of conjugate in each well. Table I shows the results of a 1:500, 1:1000, and 1:2000 dilution of conjugate and a 30 minute substrate incubation using a 1:1000 dilution of antiserum to *A. c. contortrix* venom as primary antibody. The highest sensitivity and lowest non-specific binding was obtained with a 1:1000 dilution of conjuate. At this concentration, the assay detected venom at approximately 30 ng/ml. Although a slightly higher sensitivity was obtained using a 1:500 dilution, high absorbance values were present in the negative controls and suggested increased non-specific binding of the conjugate.

TABLE I

STANDARD CURVES OF *A. C. CONTORTRIX* VENOM USING THE DOUBLE-ANTIBODY ELISA AND THREE DILUTIONS OF CONJUGATE

Venom (ng/ml)	1/500	1/1000	1/2000
1000	-	-	-
500	-	-	1.81
250	-	1.99	1.15
125	1.72	1.04	0.73
62.5	0.72	0.46	0.35
31.3	0.48	0.29	0.19
15.6	0.27	0.21	0.14
7.8	0.22	0.18	0.12
3.9	0.18	0.15	0.12
1.9	0.16	0.13	0.13
IB	0.19	0.16	0.13
	r=.99	r=.99	r=.99

Absorbance values are expressed as the mean of duplicate wells; -, off scale, absorbance greater than 2.0

Substrate incubation = 30 minutes, IB = Incubation buffer, r = correlation coefficient

All standard errors were less than 0.17

At 60 minutes substrate incubation, the highest sensitivity and lowest background were again obtained with a 1:1000 dilution of conjugate. At 60 minutes, a 1:1000 dilution had a slightly higher sensitivity compared to 30 minutes, detecting venom at about 15 ng/ml, however, the absorbance of control blanks was about 50% higher. Because of the lower non-specific binding with a 1:1000 dilution at 30 minute substrate incubation, these parameters were chosen for the remainder of the assays.

Human Serum and Urine Samples. The results from screening serum and urine samples from possible snakebite patients for the presence of copperhead venom using the double-antibody ELISA are shown in Table II. Because of high absorbance values in negative serum and urine controls, the only samples giving significant positive results using the antiserum to *A. c. contortrix* venom were urine samples from patient #2 on both the day of the bite and on the day following the bite. Using the antiserum against *A. c. mokeson* venom, a positive reaction occured only in the urine from patient #2 on the second day following the bite (results not shown).

Interference by Human Serum Components

Figures 1-3 show the results of the addition of human alpha-2 macroglobulin, human IgG, and human serum albumin to standards of *A. c. contortrix* venom. Absorbance values of the standards made with alpha-2 macroglobulin were approximately 30-35% lower compared to the standards made in incubation buffer or containing human serum albumin. However, standards made with human IgG gave approximately 40% higher absorbance

TABLE II

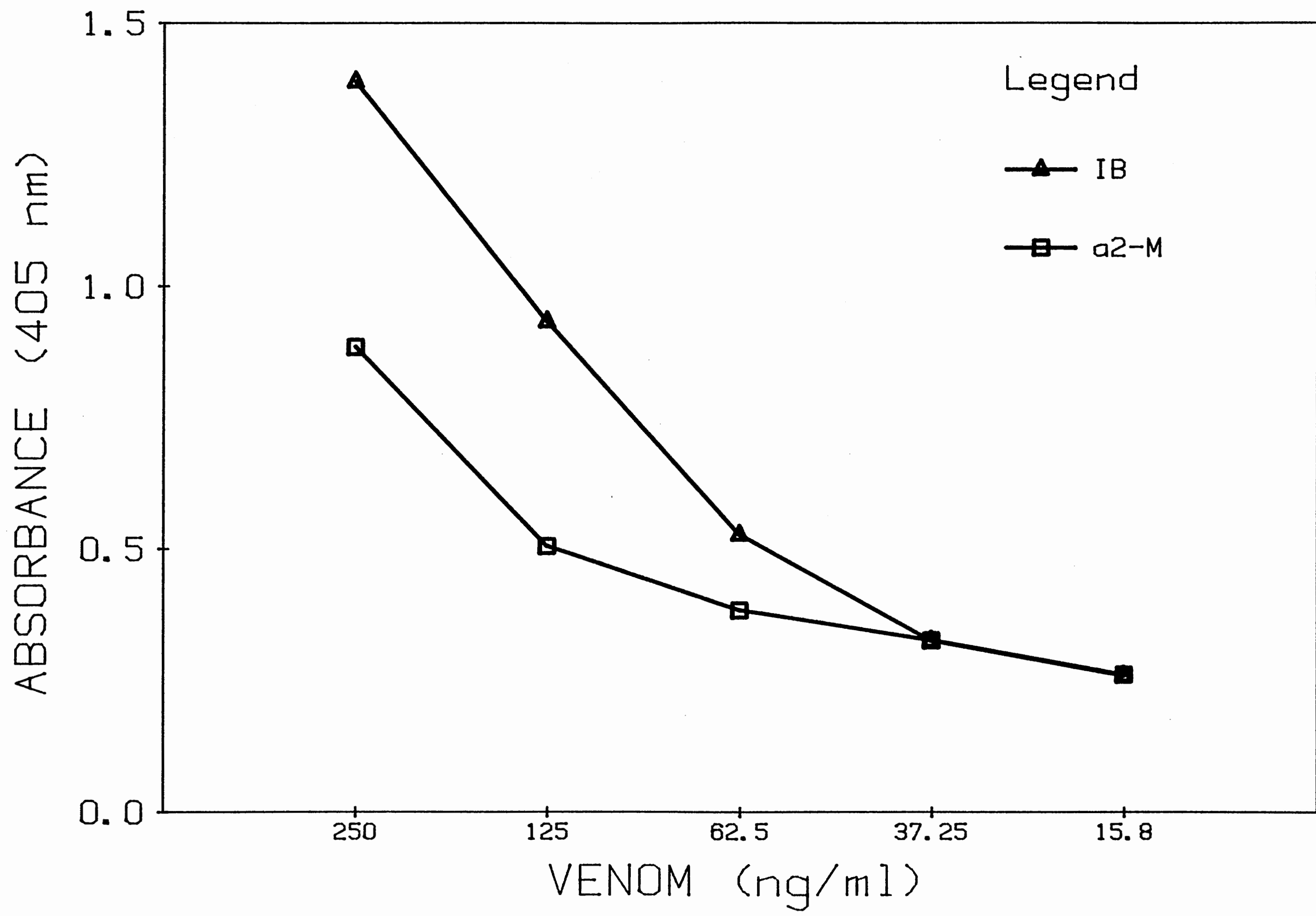
SCREENING SERUM AND URINE FROM POSSIBLE SNAKEBITE PATIENTS FOR THE PRESENCE OF COPPERHEAD VENOM USING THE DOUBLE-ANTIBODY ELISA AND ANTISERUM TO A. C. CONTORTRIX VENOM

	Date of Sample	Absorbance at 405 nm	Significant
Patient #1			
Serum	6/04/86	0.14	-
	6/05/86	0.17	-
	6/09/86	0.16	-
	6/11/86	0.15	-
Urine	6/05/86	0.34	-
	6/09/86	0.30	-
	6/11/86	0.31	-
Patient #2			
Serum	7/15/85	0.23	-
	7/16/85	0.20	-
	7/18/85	0.18	-
Urine	7/15/85	0.38	+
	7/16/85	0.47	+
	7/17/85	0.27	-
	7/18/85	0.29	-

Absorbance values are expressed as the mean of duplicate well

All standard errors were less than 0.09

Figure 1. Inhibition of *A. c. contortrix* venom standards by human alpha-2 macroglobulin using the double-antibody ELISA. IB= standards made in incubation buffer only, a2-M = standards made in incubation buffer containing 0.5 mg/ml human alpha-2 macroglobulin. Absorbance values are expressed as the mean of duplicate wells, standard errors ranged from 0.02-0.007.



ABSORBANCE (405 nm)
1.5
1.0
0.5
0.0
Legend
IB
a2-M
250
125
62.5
37.25
15.8
VENOM (ng/ml)

Figure 2. Effect of human IgG on the reactivity of standards of *A. c. contortrix* venom using the double-antibody ELISA. IB = standards made in incubation buffer only, IgG = standards made in incubation buffer containing 5.0 mg/ml human IgG. Absorbance values are expressed as the mean of duplicate wells, standard errors ranged from 0.01-0.009.

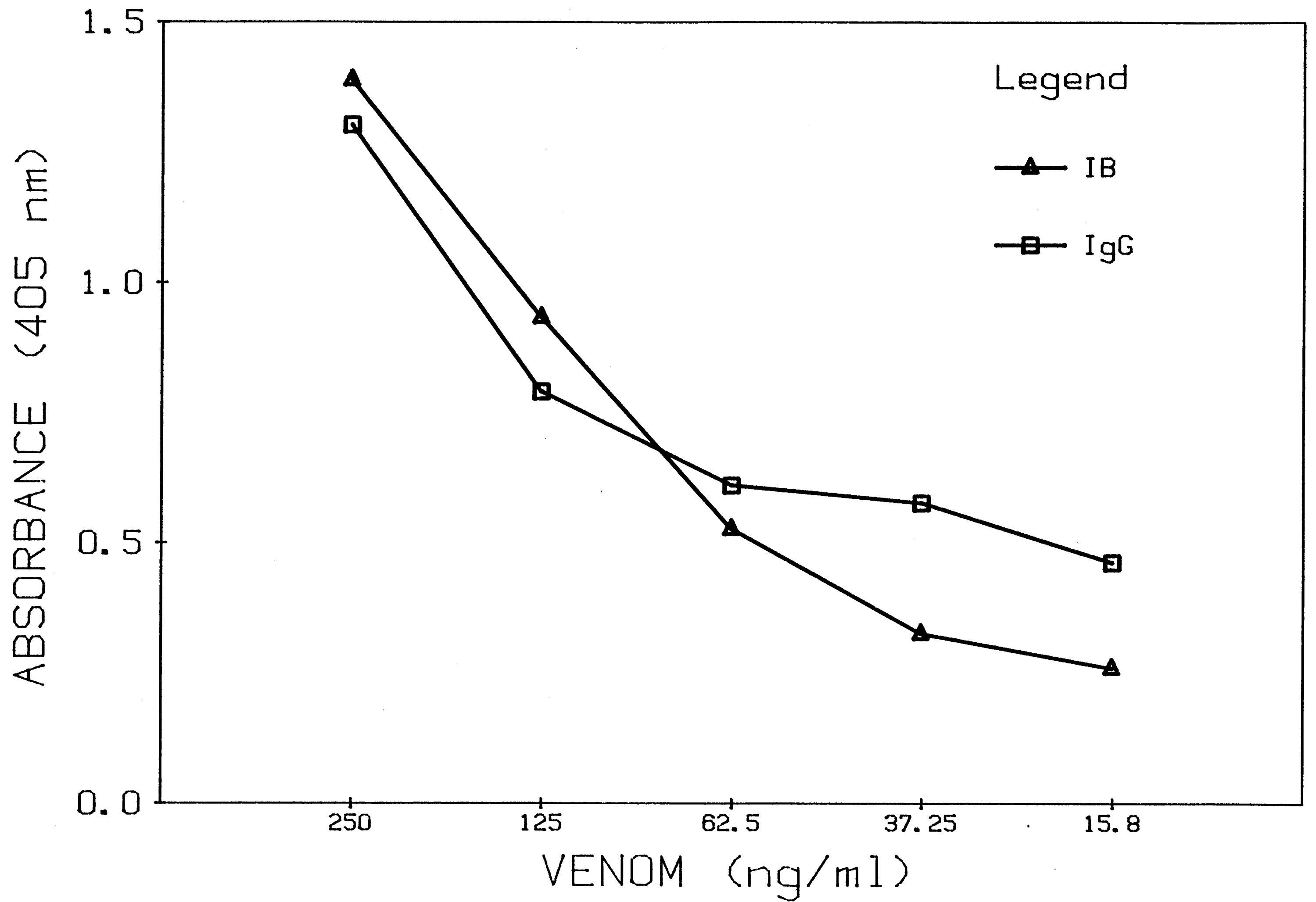
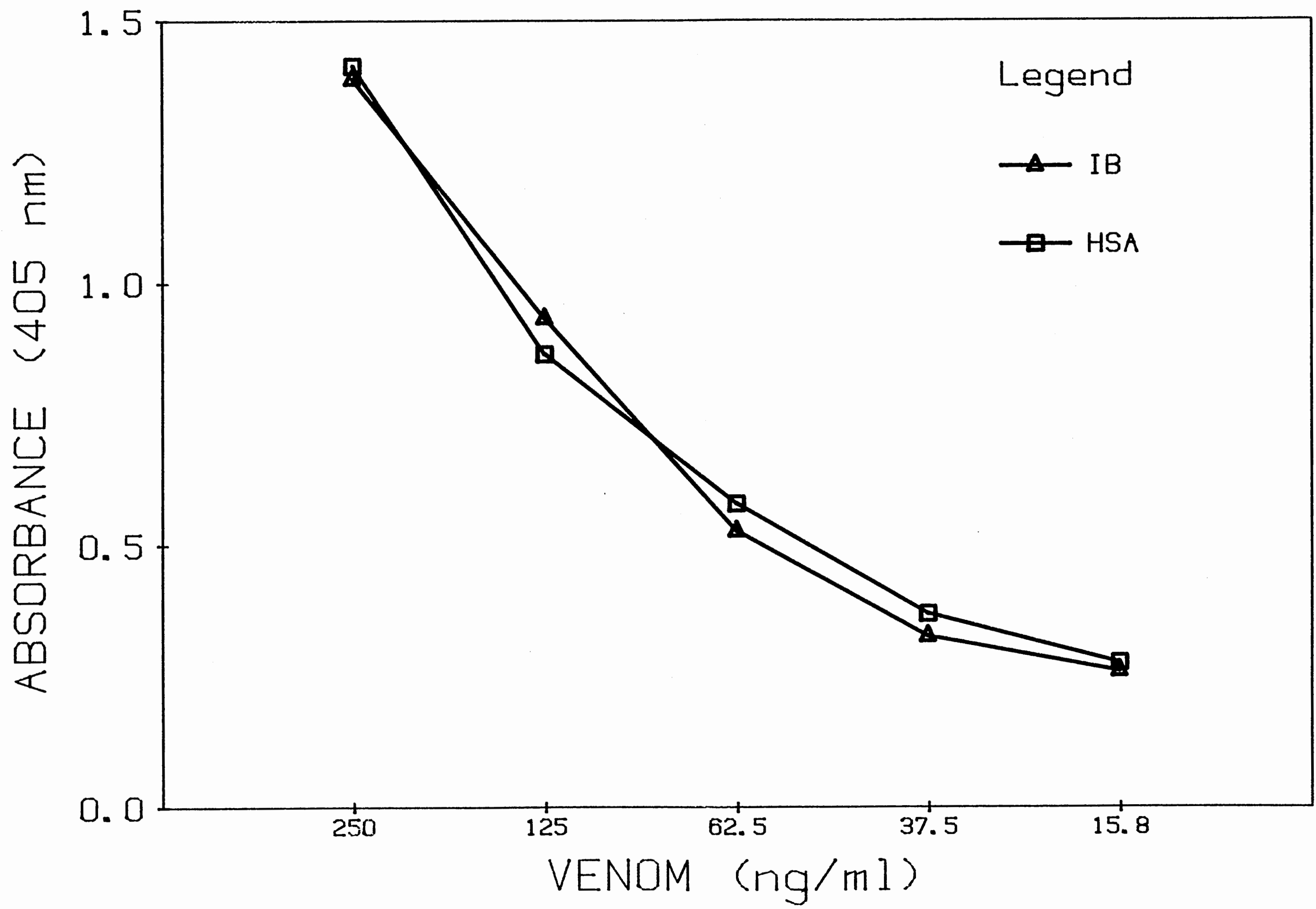

Legend
IB
IgG
ABSORBANCE (405 nm)
1.5
1.0
0.5
0.0
250
125
62.5
37.25
15.8
VENOM (ng/ml)

Figure 3. Effect of human serum albumin on the reactivity of standards of *A. c. contortrix* venom using the double-antibody ELISA. IB = standards made in incubation buffer only, HSA = standards made in incubation buffer containing 20 mg/ml human serum albumin. Absorbance values are expressed as means of duplicate wells, standard errors ranged from 0.01-0.007.



Legend
IB
HSA
1.5
1.0
0.5
0.0
ABSORBANCE (405 nm)
250
125
62.5
37.5
15.8
VENOM (ng/ml)

values at venom concentrations of 15.8 and 31.7 ng/ml compared to standards made with human albumin or in incubation buffer.

Indirect ELISA

Determination of Parameters. Parameters such as primary antibody concentration, conjugate concentration, and substrate incubation times giving the highest sensitivity and lowest non-specific binding were determined in a manner similar to that described for the double antibody ELISA. The results of standards (1000 to 15.8 ng/ml) using a 1:1000 dilution of conjugate, 60 minutes substrate incubation, and two different dilutions of antiserum to *A. c. contortrix* venom (1:500 and 1:1000) are shown in Table III. The sensitivity of the assay using the indirect method was slightly higher than with the double antibody method, and it was able to detect venom at a concentration of approximately 15 ng/ml. A 1:750 dilution of primary antibody gave lower blank values yet had the same sensitivity as a 1:500 dilution. Also, reducing the substrate incubation time from 60 minutes to as low as 20 minutes did not affect the sensitivity of the assay yet reduced the absorbance values of control blanks. Therefore, a 1:750 dilution of primary antibody, a 1:1000 dilution of conjugate, and a 20 minute substrate incubation gave the highest sensitivity and lowest non-specific binding and were thus used for the remainder of the indirect ELISA assays.

Human Serum and Urine Samples. Table IV shows the results of screening serum and urine samples from snakebite patients using the indirect ELISA method and antiserum against *A. c. contortrix* venom. The indirect ELISA resulted in lower absorbance values in control blanks and

TABLE III

STANDARD CURVES OF *A. C. CONTORTRIX* VENOM USING THE INDIRECT ELISA AND TWO DILUTIONS OF PRIMARY ANTIBODY

Venom (ng/ml)	1/500	1/1000
1000	–	–
500	–	–
250	1.97	1.89
125	0.81	0.64
62.5	0.73	0.56
37.5	0.43	0.36
15.8	0.29	0.27
7.8	0.20	0.16
3.9	0.23	0.17
1.9	0.21	0.17
IB	0.20	0.17
	r=.99	r=.98

Absorbance values are expressed as the mean of duplicate wells; –, off scale, absorbance greater than 2.0

Substrate incubation = 60 minutes, conjugate = 1:1000
r = correlation coefficient

All standard errors were less than 0.07

TABLE IV

SCREENING SERUM AND URINE FROM POSSIBLE SNAKEBITE PATIENTS FOR THE PRESENCE OF COPPERHEAD VENOM USING THE INDIRECT ELISA AND ANTISERUM TO A. C. CONTORTRIX VENOM

	Date of Sample	Absorbance at 405 nm	Significant
Patient #1			
Serum	6/04/86	0.21	−
	6/05/86	0.26	−
	6/09/86	0.26	−
	6/11/86	0.23	−
Urine	6/05/86	0.26	−
	6/09/86	0.29	−
	6/11/86	0.41	+
Patient #2			
Serum	7/15/85	0.28	−
	7/16/85	0.29	−
	7/18/85	0.35	+
Urine	7/15/85	0.34	+
	7/16/85	0.41	+
	7/17/85	0.49	+
	7/18/85	0.27	−
Patient #3			
Serum	6/17/86	0.48	+
	6/19/86	0.19	−
	6/24/86	0.21	−
Urine	6/17/86	0.38	+
	6/19/86	0.35	+
	6/24/86	0.28	−

Absorbance values are expressed as the mean of duplicate wells

All standard errors were less than 0.05

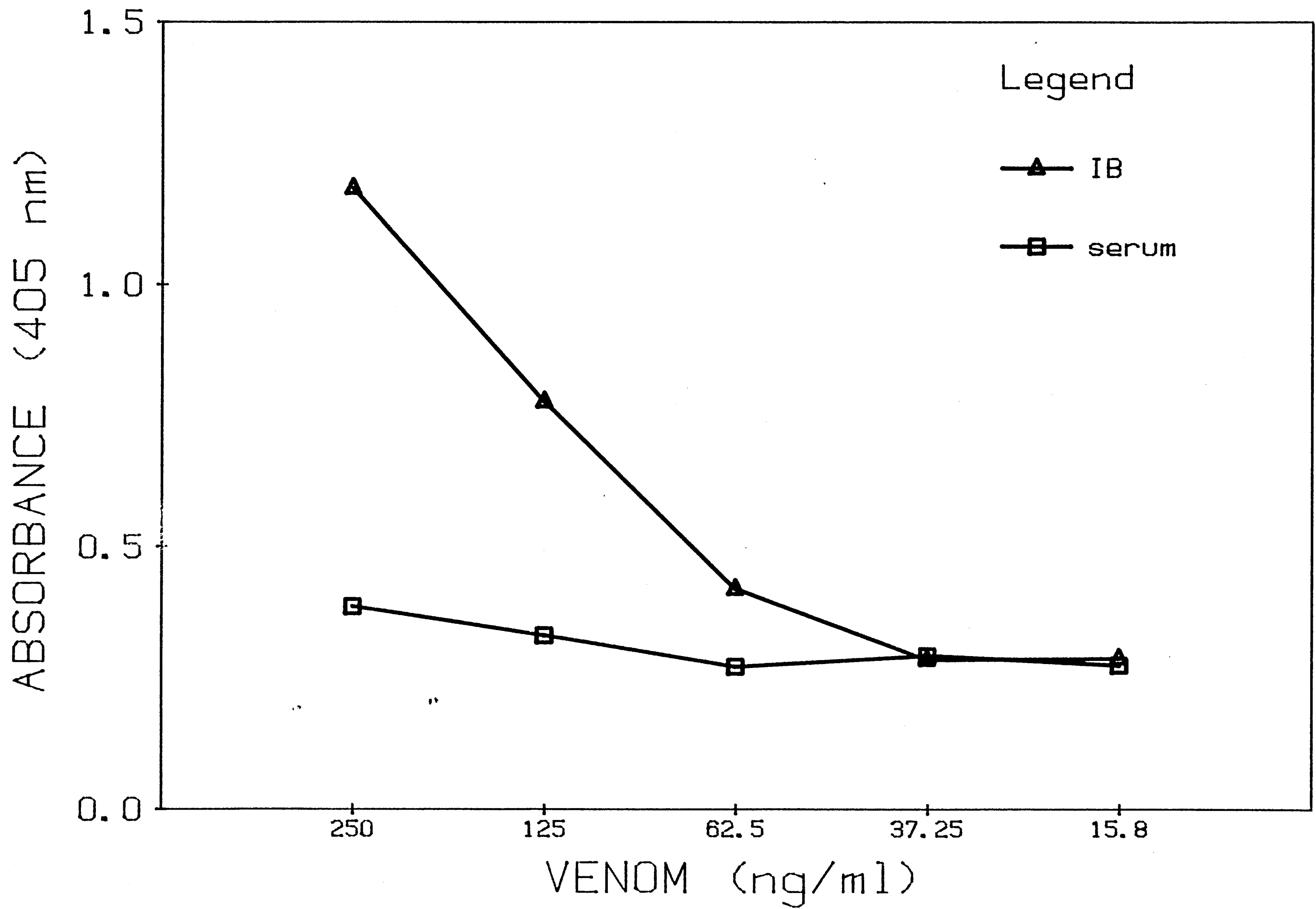
an increase in the number of samples with significant positive reactions compared to the double-antibody method. In general, urine samples from all three patients were more likely to give positive results than were serum samples. The only sample from patient #1 that gave a positive reaction was urine from the second day after the bite. Serum from patient #2 gave a positive reaction on the third day following the bite while urine samples from patient #2 were positive the same day of the bite and on the second and third day following the bite. The serum from patient #3 tested positive on same day as the bite but not on subsequent days. The urine from patient #3 gave positive results on both the same day of the bite and the second day following the bite.

As a control, normal serum and urine from several different individuals and human serum and urine purchased from a commercial source were assayed using the indirect ELISA and antiserum to *A. c. contortrix* venom. The results showed a considerable individual variation in the absorbance of serum and urine samples. The absorbance of these serum samples ranged from 0.24 to 0.49 whereas urine samples varied from 0.22 to 0.45. The serum and urine taken from one of the control individuals over a seven day period, however, showed very little day to day variation in absorbance. The absorbance of serum samples varied by only 0.01 absorbance units between the first and seventh day while the urine varied by 0.04 units.

Interference by Human Serum Components

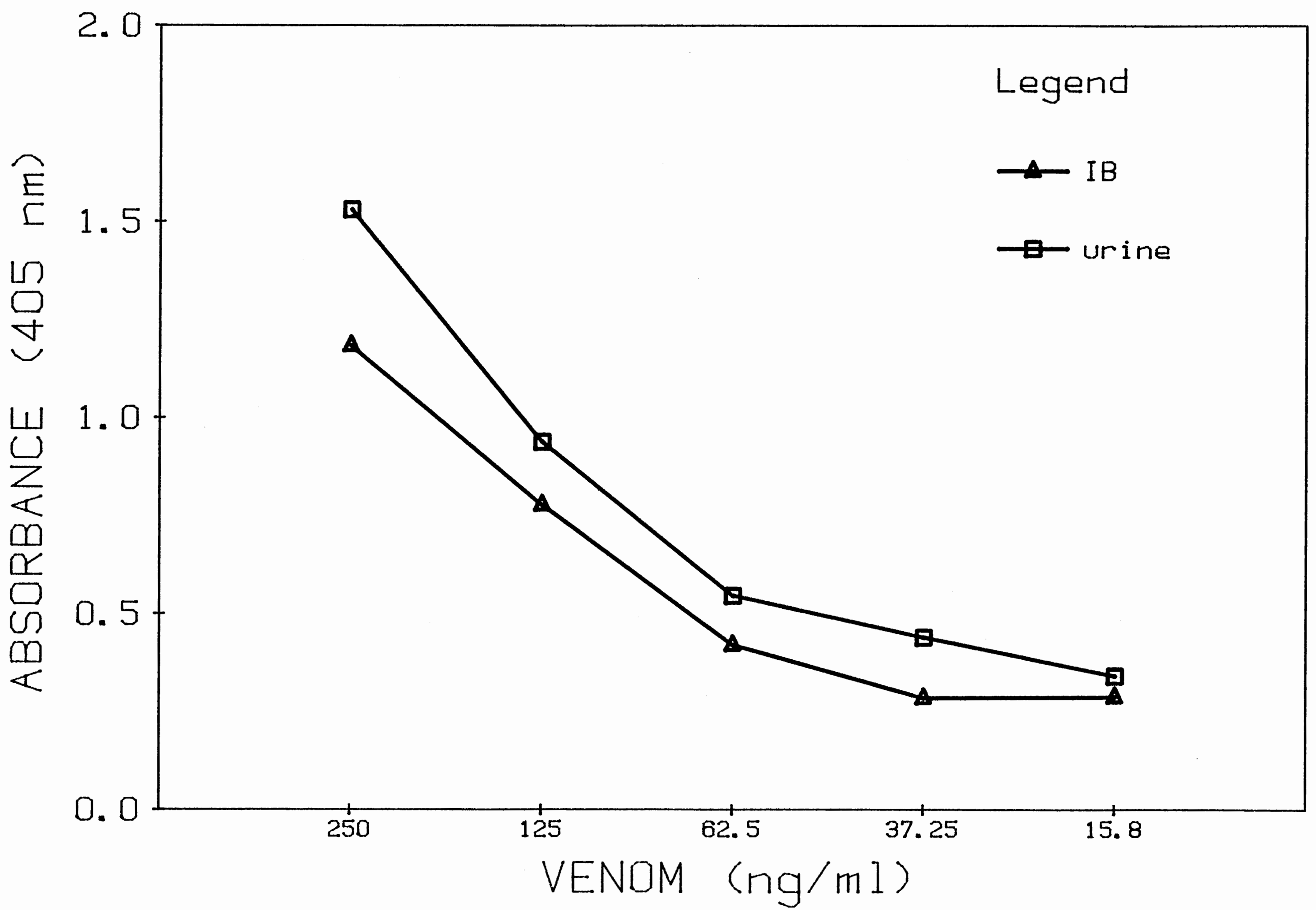
Figures 4 and 5 show the result of an indirect ELISA using *A. c. contortrix* venom standards made in either normal human serum or urine.

Figure 4. Results of an indirect ELISA using standards of *A. c. contortrix* venom diluted in either incubation buffer or control human serum.



Legend
IB
serum
1.5
1.0
0.5
0.0
ABSORBANCE (405 nm)
250
125
62.5
37.25
15.8
VENOM (ng/ml)

Figure 5. Results of an indirect ELISA using standards of *A. c. contortrix* venom diluted in either incubation buffer or control human urine.


Legend
IB
urine
ABSORBANCE (405 nm)
2.0
1.5
1.0
0.5
0.0
250
125
62.5
37.25
15.8
VENOM (ng/ml)

The standards made in human serum resulted in absorbance values approximately 60% less than standards made in incubation buffer at venom concentrations ranging from 250 to 62.5 ng/ml. Human urine, however, did not inhibit the ELISA and standards made in urine had absorbance values slightly higher than standards made in incubation buffer.

The standards of A. c. contortrix venom containing 0.5 mg/ml human alpha-2 macroglobulin showed a 25% reduction in absorbance at a venom concentration of 250.0 ng/ml compared to standards made in incubation buffer or with human serum albumin. However, venom concentrations from 125 to 15.8 ng/ml were not significantly different. Similar to the double-antibody ELISA, standards made with human IgG gave approximately 60% higher absorbance values at 62.5 ng/ml, 31.7 ng.ml, and 15.8 ng/ml compared to the standards in incubation buffer or containing human serum albumin.

Determination of Cross-reaction

All twenty-four crotaline venoms tested cross-reacted with both antisera against A. c. contortrix venom and A. c. mokeson venom (Table V). Venoms from the canebrake rattlesnake (C. horridis atricaudatus), Central American rattlesnake (C. durissus durissus), banded-rock rattlesnake (C. lepidus klauberi), midget-faded rattlesnake (C. v. concolor), and prairie rattlesnake (C. v. viridis) showed the least amount of cross-reaction with the antiserum to A. c. contortrix venom whereas Cedros Island rattlesnake (C. exsul) venom had the highest cross-reaction. Using the antiserum against A. c. mokeson venom, C. h. atricaudatus and C. exsul venoms had the least and greatest cross-reaction, respectively.

TABLE V

CROSS-REACTIONS BETWEEN CROTALINE SNAKE VENOMS AND ANTISERUM TO *A. C. CONTORTRIX* VENOM

Venom Sample	Degree of Cross-Reactivity
A. c. contortrix (Southern copperhead)	+++++
A. c. mokeson (Northern copperhead)	+++++
C. basilicus basilicus (Mexican West-Coast rattlesnake)	+++
C. catalensis (Santa Catalina Island rattlesnake)	++
C. cerastes cerastes (Mojave Desert sidewinder)	++
C. durissus culminatus (Northwestern Neotropical rattlesnake)	+++
C. durissus durissus (Central American rattlesnake)	+
C. durissus totanacus (Totonacan rattlesnake)	++
C. enyo enyo (Lower Californian rattlesnake)	+++
C. exsul (Cedros Island rattlesnake)	++++
C. horridus atricaudatus (Canebrake rattlesnake)	+
C. horridus horridus (Timber rattlesnake)	++
C. lepidus klauberi (Banded-Rock rattlesnake)	+

TABLE V (Continued)

Venom Sample	Degree of Cross-Reactivity
_C._ _mitchellii_ _pyrhuss_ (Southwestern speckled rattlesnake)	+++
_C._ _mitchellii_ _stephensi_ (Panamint rattlesnake)	++
_C._ _molossus_ _nigrescens_ (Mexican black-tailed rattlesnake)	++
_C._ _ruber_ _ruber_ (Red diamond rattlesnake)	++
_C._ _vergrandis_ (Urocoan rattlesnake)	++
_C._ _viridis_ _cerberus_ (Arizon black rattlesnake)	++
_C._ _viridis_ _concolor_ (Midget-faded rattlesnake)	+
_C._ _viridis_ _helleri_ (Southern Pacific rattlesnake)	+++
_C._ _viridis_ _oreganus_ (Northern Pacific rattlesnake)	++
_C._ _viridis_ _viridis_ (Prairie rattlesnake)	+

C. = _Crotalus_, A. = _Agkistrodon_

Concentration of venom samples was 0.001 mg/ml

Cross-reactivity is expressed as the absorbance at 405 nm as follows:

+: 0.0-0.50 absorbance units
++: 0.51-1.0 absorbance units
+++: 1.01-1.5 absorbance units
++++: 1.51-2.0 absorbance units
+++++: >2.0 absorbance units

CHAPTER VI

DISCUSSION

The use of an enzyme-linked immunosorbent assay (ELISA) for the detection of copperhead (_Agkistrodon contortrix_) venom in serum and urine from snakebite victims and its possible role as a clinical tool for the diagnosis of venomous snakebite in the United States was investigated. In this study, two different ELISA methods were used; the indirect ELISA in which the antigen was adsorbed directly to wells of polystyrene microtiter plates, and the double-antibody ELISA in which antigen was bound to immunoglobulins that were adsorbed to wells of the microtiter plates. Using standards of _A. c. contortrix_ venom made in incubation buffer, the double-antibody ELISA was able to detect venom at a concentration of about 30 ng/ml. However, high absorbance values in negative controls using serum and urine made evaluation of the human samples difficult. The indirect ELISA resulted in lower absorbance values in negative controls when using human serum and urine and had a slightly higher sensitivity compared to the double-antibody method. For this reason, the indirect ELISA method appears superior in screening possible snakebite patients for the presence of venom in their serum and urine.

Using the indirect ELISA with _A. c. contortrix_ antiserum, the assay gave positive results in at least some of the serum and urine samples

from three snakebite victims seen at the Springdale Medical Clinic in Oklahoma City, Oklahoma during 1984-1986. In general, urine samples were far more likely to give positive results than were serum samples. In some cases, serum samples failed to give positive results whereas urine samples taken on the same day from the same individual tested positive. Since this was a clinical situation, serum and urine samples from each patient were not taken at identical times following the bite. Therefore, it is only possible to make generalized statements about the appearance and disappearance of the venom from the serum and urine. Usually the venom first appeared in the urine on the same day of the bite and was still present the following day. On subsequent days, urine samples from some patients gave positive results whereas others did not. In one patient, the urine gave a positive reaction six days after the bite. These differences between patients could be explained by physiological differences in the clearance by the kidney and/or by variations in the amount of venom injected by the snakes.

A consistent result using the ELISA in this investigation was the high absorbance values in human serum and urine negative controls compared to incubation buffer blanks. The higher absorbance values in the serum and urine controls made interpretation of results difficult and could be caused by several factors. Crude copperhead antisera were used in this investigation and it is possible that natural antibodies in the antiserum were cross-reacting with components in the human serum and urine. Also, endogenous alkaline phosphatase present in the serum and urine could be binding to the microtiter plates and reacting with the substrate added to the wells. Since results of screening negative

control serum and urine from several people show that the absorbance values differ considerably between individuals, incubation buffer or normal pooled serum and urine purchased from commercial sources are not good negative controls for the assay. The results of this investigation suggest that in a clinical situation where control serum and urine obtained before the snakebite are usually not available, serum and urine should be taken from an individual over a period of time and the sample giving the lowest absorbance value used as a negative control. However, in treating snakebite in the clinic, the diagnosis of the bite and the identity of the offending snake must be determined within a few hours if antivenom is to be effectively administered. Because the length of time required to obtain several samples from a patient in order to get a negative control would limit the effectiveness of the assay in the diagnosis of snakebite, many clinicians may be inclined to use pooled serum and urine as negative controls. Caution must be made, however, in interpreting positive ELISA results using these controls because of the individual variation in human serum and urine samples.

The _A. c. contortrix_ venom standards made in normal human serum at concentrations ranging from 250 to 61.5 ng/ml constistently gave absorbance values 60-70% lower than standards made in incubation buffer. The standards made in normal human urine were not inhibited and gave absorbance values that were similar to standards made in incubation buffer. The addition of 0.5 mg/ml human alpha-2 macroglobulin to standards made in incubation buffer also resulted in about 30% reduction in the aborbance values; this is approximately half the reduction seen with normal serum, however, alpha-2 macroglobulin is normally present in

the serum at concentrations of 2-3 mg/ml. Human alpha-2 macroglobulin is a non-specific protease inhibitor with a molecular weight of 750,000 and has been shown to form complexes with certain snake venom proteases (Kress and Catanese, 1981). Copperhead venoms possess considerable proteolytic activity on substrates such as TAME and BAEE (Tu *et al.*, 1965) and may contain more than one protease. If the proteases in copperhead venoms are also immunogenic, then a large number of antibodies in a copperhead antiserum will be specific for these components. It is possible that alpha-2 macroglobulin can form complexes with these copperhead venom proteases and thus inhibit antibodies from binding. The inhibition could result from either an alteration of the epitope on the protease or by steric hindrence of the alpha-2 macroglobulin. Since the binding of alpha-2 macroglobulin to proteases is irreversible (Swenson and Howard, 1979), the presence of the molecule in serum would effectively inhibit the ELISA assay. This could explain the reduced capability of the assay to detect the venom in the serum in this and other investigations (Hurrell and Chandler, 1982) and suggests that samples, such as bite site washings, that do not come in contact with this particular serum component would be more likely to give positive ELISA results following a snakebite.

The standards made with human serum albumin were not significantly different from those made in incubtion buffer. Therefore, human serum albumin does not appear to interfere with the ELISA in detecting copperhead venom. Human serum albumin, unlike certain other mammalian albumins, apparently does not interact with snake venom components, at least to the extent of inhibiting their reactivity in the ELISA.

The results of the addition of human IgG to *A. c. contortrix* venom standards are interesting in that they consistently gave higher absorbance values at low venom concentrations compared to standards made in incubation buffer or those containing human serum albumin. This was seen using both the double-antibody and indirect ELISA methods. One possible explanation for this is that human IgG molecules were cross-reacting with the goat anti-rabbit IgG antibodies of the conjugate. This could also explain the high blank values in negative control serum and urine compared to incubation buffer blanks.

The cross-reaction studies indicated that a large number of crotaline snake venoms cross-react with copperhead antisera. The cross-reaction varied between snake species and did not appear to follow any phylogenetic pattern. Therefore, based on the results of this investigation it does not appear possible to accurately determine the identity of a snake as a copperhead or other crotaline snake using an enzyme-linked immunosorbent assay in the clinic. However, a crude antiserum against copperhead venom was used, and it is possible an antiserum produced against an individual component of copperhead venom which is not present in other crotaline venoms may be used to overcome this problem of cross-reaction.

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