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SPECIAL ARTICLE

Immunodiagnosis of Snake Venom Poisoning

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SUMMARY Uncertainty as to the species diagnosis remains a serious problem in the management of snake venom poisoning. This is particularly so in areas inhabited by numerous species, the venoms of which elicit similar pharmacological effects and clinical symptoms and against which para-specific cross-neutralizing antivenom is not available. Attempts have been made to eliminate some of this ambiguity through the development of various immunodiagnostic tests. Tests based on ELISA are sensitive, specific and even quantitative and adaptable to field application. In the development of diagnostic tests for use in developing countries, however, practical consideration must be given to speed, cost, simplicity in terms of equipment and expertise, and stability to the climate and storage conditions. This may dictate further modification or selection of more suitable alternative methodologies. Furthermore, the test may have to allow more flexibility in accomodating local species distributions and to address probable complications of heterophile antibodies in test samples from rural people.

Snake venom poisoning and problem in management

Snake venom poisoning remains an important health problem in Thailand and many other developing countries in Asia and Africa. Although specific antivenoms are available in most of these countries, a significant fraction of the snakebite victims die and many who survive suffer some degree of permanent injury.

The current scheme of management of snake envenomization includes attempt at identification of snake species involved and the selection of the corresponding antivenom for therapy. The species diagnosis, almost always without catching the culprit snake, is based on configuration of the bite, differentiating clinical manifestations, knowledge of species commonly recognized in a given geographical area and information from the victim or witnesses. None of these provide the clinician with sufficient grounds to arrive at an accurate identification of the source of envenomization and often lead to failure to do so. Differentiating the culprit species on the basis of clinical manifestations is difficult simply because toxins contained in venoms of different species are often physico-chemically and pharmacologically similar. For example, venoms of *Naja naja siamensis, Ophiophagus hannah* and *Bungarus fasciatus* all contain postsynaptic neurotoxins inhibiting neuromuscular transmission leading to similar predominant clinical features, namely, paralysis, respiratory failure and eventually death.[1] The distribution of snakes is not clear cut and each locality is usually inhabited by several species. Higher frequency of inhabitation by a given species does not necessarily indicate higher incidence of bites by that species.

The Thai Ministry of Public Health recently estimated that the culprit species was identified in only around 60 percent of the reported bites. Until recently, however, once an identification was made, there was no way of confirming it. Some indication that a correct identification has been made is obtained when use of the corresponding antivenom effectively and dramatically improves conditions. Unfortunately, for a variety of reasons, this is not often the case; more frequently, correct antivenom merely arrests progressive deterioration without immediate

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confirmation of diagnosis. Thus the actual rate of correct diagnosis is likely to be even lower than estimated.

Accurate identification of the source of envenomization is crucial since there is very little, if any, paraspecific or cross-neutralization by the monovalent horse antivenoms available in Thailand.[2] Thus, only the appropriate specific antivenom will be effective; misdiagnosis is both wasteful of precious antivenom and dangerous to the patient, in that effective treatment may be omitted while the patient is nonetheless subjected to adverse hypersensitivity reactions. This is particularly disconcerning since in antivenom therapy, massive quantities in the range of 30-600 ml may be required in a given case.[3] The incidence of hypersensitivity reactions has been estimated to be as high as 30%-50% according to various reports.[4,5] The inability to achieve a prompt and unequivocal, accurate identification of the source of envenomization remains a major limiting factor in the management of snakebites.

Development of immunoassays and immunodiagnostic tests

As mentioned above, venoms of many snakes are similar physicochemically and pharmacologically yet each of them requires specific antivenom for neutralization. Thus, various assays and diagnostic tests based on the immunochemical differences of these venoms have been developed with different characteristics and degrees of success. The reagents and methods developed were reviewed recently.[6]

Immunodiffusion and immunoelectrophoretic assays for the detection of various venom proteins have been described.[7] Venom proteins were detected in wound aspirates, blister fluids, sera or urine by immunodiffusion assay in 40 out of 101 patients tested. Passive hemagglutination (PHA) was first studied for venom antibody detection by Boche and Russell,[8] who used venom coupled to bis-diazobenzidine treated sheep RBCs. The preparation could be used to detect anti-venom antibody at dilutions as high as 2×10^5, while the venom proteins could be detected by the corresponding inhibition test.

Radioimmunoassay has been developed by several groups of investigators for the detection of venom or anti-venom antibody.[7,9-10] It is by far the most sensitive technique, capable of detecting subnanogram quantities of venom.[9] However, the requirement for routine protein labeling with short-lived ^{125}I, the need for expensive detection equipment and well-trained personnel, the problems of radioactive waste disposal and the time required to complete the assay (24 hrs) preclude its use in diagnosis of venom poisoning especially in rural areas of developing countries.

Theakston *et al.*[11] were the first to use enzyme-linked immunosorbent assays (ELISA) performed in microtiter plates for specific detection of various snake venoms. Using specific antibodies (IgG fraction) immobilized on the plate and an alkaline phosphatase-antibody conjugate for scoring, they could detect 1-5 ng of venom per ml of sera from experimental animals. The time required for the test is about 3 hrs. Coulter *et al.*[12] using similar techniques could detect Australian snake venoms at about 6 ng/ml within 30 min. The improvement was attributed to the use of antibody purified on a protein A-Sepharose column.

Recently, Chandler and Hurrell[13] have developed a new test kit consisting of six glass capillary tubes joined by silicone rubber tubing and connected to a 1-ml syringe. Internally, each glass tube is coated covalently with specific rabbit antibody against one of five poisonous snake venoms. The test sample containing venom is draw up through the tubes resulting in venom attachment to one of the coated tubes. An enzyme conjugate (urease coupled to anti-venom IgG) is drawn up, followed by substrate (urea plus bromocresol purple as pH indicator), resulting in color development on the tube coated with homologous antibody. This test kit, which has a sensitivity of 5-10 ng/ml and a test time of 30-40 min, costs about US$15/test. The cost factor relative to per capita income likely will prevent direct application of this assay methodology or its wide-spread use in developing countries.

Of the various immunodiagnostic tests described, ELISA thus far has been the most widely used. However, Ho *et al.*[14] recently cautioned that various factors influencing the successful application of ELISA in snake venom detection have not yet received due consideration. These factors include nonspecific reactivity, the cross-reactivity and the quality of the reagents. Nonspecificity may be encountered if sera contain heterophile antibodies — especially common in sera of rural people, or rhematoid factors. These immunoglobulins elevate background ELISA values and frequently yield false positives. Cross-reactivity between heterologous venom antigens may give ambiguous ELISA results and thus equivocate the diagnosis. The quality of reagents, *e.g.*, the purity and affinity of the antibody influences the signal to noise ratio and reaction times and hence affect the sensitivity of the test. Ho *et al.*[14] also suggested various technical manipulations to avoid or minimize these problems.

Some possible future improvements

Two extremely simple and useful immunoassay systems, passive

hemagglutination (PHA) and latex agglutination (LA) have received limited or no attention thus far for the purpose of venom detection. PHA has been adapted as a laboratory assay for venom antigen and antibody as reviewed above but has not been adapted for immediate diagnosis. The sensitivity of this assay, calculated from the result of Boche and Russell,[8] should be at the level of detecting 2 ng/ml of antibody, in accord with the observations of Adler and Adler,[15] that PHA and reverse PHA are capable of detecting 1-10 ng/ml of antibody or antigen, respectively. Thus, the sensitivity of this assay should be 2-3 orders of magnitude greater than immunodiffusion or immunoelectrophoresis and comparable to that described by Treakston *et al.*[11] using an ELISA. Venom proteins present in body fluids should thus be detectable by reverse PHA. It should be mentioned that snake venom detection based upon hemagglutination tests may be prone to hemolysis (P. Boquet, personal comm.). However, in our experience, using glutaraldehyde treated sheep RBCs,[16] no hemolysis was observed in the presence of high concentrations of three Elapid venoms, known to contain direct lytic factor and phospholipase A_2. The advantages of PHA and reverse PHA and the corresponding inhibition assays include simplicity, sensitivity and economy.[25] In many cases the sensitized erythrocytes can be lyophilized and reconstituted with distilled water before use.[17,18] It has also been reported that with avian erythrocytes which are nucleated and settle faster, the agglutination result can be read within 30 min.[18]

Latex agglutination assays, although providing additional advantages, have not been adapted as yet for use in venom detection. In addition to enhanced stability and relative insensitivity of the particles to enzymes and toxins, the greatest advantage of this technique is the speed with which the result can be read. It also does not require any special laboratory equipment or expertise. The detection of human choriogonadotropin in the pregnancy test, for example, can be completed within a few minutes, using only medicine droppers and a microscope slide. The sensitivity of the test can be very high, for example, polyribose phosphate can be detected at a level of 0.2-0.5 ng/ml of serum, cerebrospinal fluid, joint fluid or pleural fluid.[19] For protein antigens, for example immunoglobulins, the detection limit is about 35-225 ng/ml.[20]

The successful adaptation of reverse PHA or LA for snake venom detection should offer several advantages over ELISA, in that both are relatively easy to perform and are considerably faster--especially in the case of latex agglutination. Sensitivities may be somewhat less. Preliminary results from our laboratory showed that a reverse PHA using sheep RBC and a reverse LA could detect 5-80 ng/ml and 160-1200 ng/ml, respectively, of various snake venoms (Unpublished observations). Considering the previously reported[21] concentrations of venom in wound aspirates (200 ng/ml) and in serum (100 ng/ml), reverse PHA and possibly, reverse LA with some improvements, should be adequate. They are about 10-20 times less expensive than ELISA to produce in Thailand (B. Petchclai, personal comm.). The stability of the reagents for each test may be an important factor in developing countries. Sensitized latex particles or red cells can usually be stored at 4°C for years[22,23] or in some cases, lyophilized and stored desiccated at room temperature for even longer periods of time.[24] Our preliminary experiment indicated that latex particles coated with rabbit IgG against snake venom are stable to lyophilization and to cycles of freezing and thawing (Unpublished observations). ELISA plates sensitized with anti-venom IgG deteriorated at 4°C after 16 weeks.[12]

Special requirements of immunodiagnostic assays for developing countries

People in the developing countries face a number of serious problems as far as snake venom poisonings are concerned. This is because a far greater variety and number of deadly snakes inhabit the tropics. The occupation and way of life of the people also make them more vulnerable to snakebites. The relatively poorer means of transportation and medical facilities and the resultant delay or shortage of specific antivenoms make treatment of venom poisonings even more difficult as a result.

Against this background, the immunodiagnostic tests for snake venom poisonings also must incorporate certain special features to be useful in developing countries. Not only must the tests be specific, sensitive and rapid enough for diagnostic purposes, but they also must be simple to perform and require minimal equipment or expertise. The tests must be reasonably cheap relative to the per capita income of the people. Lastly, they must be stable to the climate, and be able to withstand transportation and storage conditions which may be far from optimal.

Since the components of the immunodiagnostic tests are dictated by the species of the snakes in the geographic area, each country most likely will have to produce its own tests except where there is an overlap of snake habitats. The ideal test therefore should allow for regional customization to include additional species or to delete those unnecessary within a given area. The low commercial viability provides little incentive for pharmaceutical cor-

porations in developed countries to invest in the research and development of inexpensive venom diagnostic tests tailored to developing countries or regions thereof. Thus, it is essential that researchers in developing countries share whatever methodologies and reagents developed whenever possible.

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