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Review Article on Antivenom

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ABSTRACT

Despite its little domain of ~50,000 km2, Costa Rica harbors surprisingly rich biodiversity. Its herpetofauna incorporates 138 types of snakes, of which sixteen pit snakes (family Viperidae, subfamily Crotalinae), five coral snakes (family Elapidae, subfamily Elapinae), and one ocean snake (Family Elapidae, subfamily Hydrophiinae) present possible dangers to human and creature wellbeing. As of late, information on the arrangement of snake toxins has extended drastically because of the advancement of progressively quick and delicate logical methods in mass spectrometry and division science applied to protein portrayal. Among a few insightful procedures to decide the general protein/peptide arrangement of snake toxins, the strategy is known as 'snake venomics' has demonstrated especially well fit and useful, by giving not just an inventory of protein types/families present in a toxin, yet additionally a semi-quantitative assessment of their relative plenitudes. Through a shared exploration activity between Instituto de Biomedicina de Valencia (IBV) and Instituto Clodomiro Picado (ICP), this methodology has been applied to the investigation of toxins of Costa Rican snakes, expecting to acquire more profound information on their synthesis, geographic and ontogenic varieties, connections to scientific categorization, relationship with harmful exercises, and disclosure of novel parts. The proteomic profiles of toxins from sixteen out of the 22 species inside the Viperidae and Elapidae families found in Costa Rica have been accounted for up until now, and an integrative perspective on these investigations is thusly introduced. Following other venomic projects by research bunches zeroing in on a wide assortment of snakes around the planet, these examinations add to a more profound comprehension of the biochemical reason for the assorted harmful profiles advanced by venomous snakes. Furthermore, these examinations give the freedoms to distinguish novel particles of possible pharmacological interest. Besides, the foundation of toxin.

INTRODUCTION:

Snakebite envenoming is a potentially life-threatening disease caused by toxins in the bite of a venomous snake. Envenoming can also be caused by having venom sprayed into the eyes by certain species of snakes that can spit venom as a defense measure. Inadequate past efforts to control snakebite envenoming has produced fragmented, inaccurate epidemiological data. Many victims do not attend health centers or hospitals and instead rely on traditional treatments. However, available data show 4.5–5.4 million people get bitten by snakes annually. Of this, 1.8–2.7 million develop clinical illness and 81 000 to 138 000 die from complications. High-risk groups include rural agricultural workers, herders, fishermen, hunters, working children, people living in poorly constructed houses and those with limited access to education and healthcare. Morbidity and mortality occur most frequently among young people and children suffer higher case fatality. Furthermore, women experience increased barriers to accessing medical care in some cultures and pregnant women are extremely vulnerable (1). The composition of snake venom varies depending on a variety of factors, including the snake family, genus and species; geographical location; typical type of prey; and age and size of the snake. Ninety percent of ophidian accidents in the Americas involve snakes in the Viperidae family, and the lethal dose varies according to the species. The toxicity has been attributed to different components, such as phospholipase A2, which acts as an anticoagulant; a myotoxin that produces hemolysis; thrombin-like polypeptides, which produce low-quality fibrinogen, causing hemorrhage; neurotoxins that block pre and postsynaptic nerve transmission of the neuromuscular plate, producing flaccid paralysis; and other components (2). A paradigm of snake venom evolution is that the venom gland promiscuously secretes proteins that were historically endogenous proteins with a physiological function elsewhere in the body. When such expression in the venom gland confers an advantage, then this trait becomes fixed and amplified in the venom gland, with secondary specific duplication and diversification of the proteins, leading to multigene families with venom-gland specific expression, toxins within such families' neo functionalise through random mutation, with additional evolutionary selection pressures operating upon their evolution. Consequently, within a single toxin family, there may be diverse bioactivities. In snakes, three types of modified venom-delivery systems have been described: (i) enlarged grooved fangs (i.e. 'grooved phenotype') located at the posterior end of the maxilla [5–9], evolved convergent in various lineages of advanced snakes. (ii) fangs with enclosed venom-conducting canals and visible suture line connecting the orifices (i.e. 'closed, non-fused

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phenotype') associated with an anterior fixed position in the maxilla , evolved convergent in elapids including cobras, mambas and sea snakes, and in the atractaspidine Homoroselaps.(iii) fangs with enclosed venom conducting canals and smooth surface between the orifices (i.e. 'closed, fused phenotype') associated with an anterior mobile position in the maxilla, evolved convergently in viperids, including vipers and rattlesnakes, and in the atractaspidine Atractaspis. Anteriorly located fangs develop from the posterior end of the maxilla through the loss of the anterior dental lamina, indicated by a lack in the expression of the sonic hedgehog (shh) gene, and displacement of a developing fang from a posterior to an anterior position. A study on the viperid *Trimeresurus albolabris* showed the venom-conducting canal itself develops through a combination of two mechanisms(3).

General symptoms occur:

Symptom depends upon the type of snake, but may include:

a) Bleeding from wound

b) Blurred vision

c) Excessive sweating

d) Loss of muscle Coordination

e) Nausea and vomiting

f) Numbness and tingling

g) Rapid pulse

h) Severe pain

i) Skin discoloration

j) Swelling at the site of the bite

k) Weakness (4)

Pharmacokinetics of antivenoms

The major issue with the treatment of snake envenoming is that there is a time lag between the snakebite and the administration of antivenom. Some factors contribute to this lag time, the main delay being the time for the patient to get from where the bite occurs to a health care facility that stocks antivenom. There is then an additional delay once the patient arrives in

hospital, between the time of arrival and the time when the decision is made to administer antivenom. The former delay is difficult to reduce and is due to the remoteness of many bites and poor transport being available to patients. In this instance, the focus needs to be on improved first aid to delay venom absorption and distribution. In contrast, the latter delay can be reduced by improved early diagnosis with bedside testing for systemic envenomation and stream-lined treatment protocols. One factor in the patient's favor is that venom takes time to reach the systemic circulation from the bite site, and thence to the target site of the toxin (e.g. neuromuscular junction).

In most cases, snakebites result in subcutaneous/sub-dermal venom injection (short fangs — elapids) or intramuscular venom injection (longer fangs — vipers). However, more recent evidence suggests that for some snakes/toxins, there is intravenous injection. Because snake venoms contain toxins with a range of molecular sizes, the rates of absorption of these toxins into the circulation will vary significantly. Currently, there is little known about the variable absorption processes for snake venoms(5).

Snake antivenoms are almost always administered intravenously and the antibodies reach the systemic circulation almost immediately. Other routes of administration, such as intramuscular and subcutaneous, significantly delay the absorption of antivenom into the circulation and should not be used. However, the distribution of antivenom to toxin target sites is much slower and varies, because of the size of the antibodies. Different types of antivenoms [such as whole IgG, Fab and $F(ab')_2$] have different molecular masses and hence have different pharmacokinetics. The larger IgG molecule has poor distribution to peripheral sites, but a longer elimination half-life. In contrast, the smaller Fab fragments distribute better, but have short elimination half-lives. To date, most information on antivenom pharmacokinetics comes from animal studies, and there is limited information on the pharmacokinetics of antivenom in humans. From the available studies, antivenom concentrations have a biphasic decline after intravenous administration of whole IgG and $F(ab')_2$antivenoms, with an initial rapid distribution phase and a slower elimination phase. Recently, a study based on a population pharmacokinetic approach that incorporates variability of individual patients, demonstrated similar of a $F(ab')_2$ antivenom(6). Kinetics of F(ab0)2 in rams was measured using fluorescent anti-venom labeled with fluorescein or fluorescamine. Labeling with fluorescin was done using 2 ampoules of anti-venom (10 ml) which were mixed with 4.5 ml of 50 mM sodium borate buffer pH 9.6 and 0.5 ml of a solution 4% fluorescin in acetone were added under vigorous agitation in a vortex stirrer. The

tube was then completely covered with aluminum foil and the mixture was left at room temperature (z22 8C) for 6 h and then at 4 8C for 12–36 h. The mixture was then washed by ultrafitration with 150 ml of 50 mM sodium borate buffer pH 8 through an AMICON Millipore membrane (PBDK type, 50 kDa cut-off, 25 mm diameter) under nitrogen pressure (4 bars) at 10 8C in darkness. The labeled material was thus reduced to between 5 and 6 ml and washing buffer was added to make 10 ml(7).

Mechanisms of Venom Toxicity:

These toxins are regularly effective on nerve tissue and synapses, frequently corrupting synapses or depolarizing the axonal layer for significant periods, consequently keeping anxious driving forces from being directed. Cobra cardiotoxin acts likewise to depolarize heart cell films, which prompts systolic capture. Cardiotoxic toxins have a partiality for cardiovascular tissue however seem to work by comparable systems as the neurotoxic elapid toxins. Crotalid neurotoxins are not film depolarizing, but instead are hostile to acetylcholine and go about as a hindering specialist at the neuro-solid intersection. Phospholipases, proteases and lytic factors contained in toxin will in general reason hemolytic impacts and are to a great extent answerable for the rot that follows viperid and crotalid chomps. Cell digestion is hindered by restraint of oxidative phosphorylation, which prompts a lacking stockpile of ATP for the cell. Mitochondrial electron transport is likewise hindered as Q-Cytochrome C, an electron acceptor protein in the Electron Transport Chain, is denatured. For explicit data of components of toxin activity, I should suggest counseling the. There are some awesome books regarding the matter, yet most necessitate that the peruser have a decent understanding of cell science and bio/natural chemistry(8). Pyrogenic responses to neutralizer are brought about by pyrogen tainting during make and may incorporate chills, afflictions, fever, myalgia, cerebral pain, tachycardia and hypotension optional to vasodilation. In kids, febrile seizures might be accelerated. Bacterial lipopolysaccharides are the most well-known pyrogens in serums. Responses commonly happen inside the primary hour of beginning neutralizer implantation. Treatment incorporates decreasing fever by cooling genuinely and antipyretics (paracetamol). Intravenous liquids and adrenaline might be needed in serious cases with hypotension. Counteraction of these responses is by adherence to great assembling practices to maintain a strategic distance from the defilement of counter-agent with microbial products(9), common svPLA2 Inhibitors from Plants, Marine Extracts, and Mammalian Serum. Therapeutic plant extricates as conventional remedies have for quite some time been utilized in nations where urotherapy is ridiculous. What's more,

these conventional and natural medicines are regularly utilized as adjuvant treatments alongside the counter-agent treatment. Most plant antidotal specialists work by killing svPLA2's harmfulness. A functioning glycoprotein (WSG) from Withania somnifera restrains the cytotoxicity, edema, and myotoxicity of NN-XiaPLA2 secluded from Naja atra toxin, however neglects to kill the neurotoxicity. WSG has a comparative design to the α-chain of the PLIs got from Australian elapid serum and was found to collaborate with NN-XIa–PLA2, however the instrument at present remaining parts obscure. The watery concentrate of Casearia sylvestris was discovered to be viable against two snake toxin poisons (Asp49-PLA2 and Lys49-PLA2 confined from toxin of *B. moojeni, B. pirajai, B. neuwiedi, and B. jararacussu*). Undoubtedly, this plant has been found to repress myotoxicity, drain, anticoagulation, and edema . It is likewise ready to forestall myonecrosis started by two Lys49-PLA2 poisons (PrTX-I from B. pirajai and BthTX-I from B. jararacussu toxin) and neuromuscular blockages. As of late exploration has shown that human secretory PLA2 inhibitors (e.g., quercetin, biflavonoid morelloflavone) detached from plant concentrates can likewise hinder svPLA2. Marine organic entities are likewise a supply for neutralizing agents. Manoalide (MLD), a characteristic item from wipe Luffariella variabilis, can irreversibly restrain extracellular PLA2 movement of cobra and poisonous snake toxin with an IC50 estimation of 1.9 and 0.7 μM, respectively]. Its manufactured simple, manoalogue (MLG), is additionally inhibitive to cobra PLA2 action with an IC50 estimation of 7.5 μM . Common svPLA2 inhibitors likewise exist in some mammalian serums. DM64 is an acidic glycoprotein separated from serum of the opossum, Didelphis marsupialis. DM64 can totally forestall myofiber breakdown brought about by myotoxins I (Asp49) and II (Lys49) of B. asper venom(10).

Snake Venom as a Source of Therapeutic Agents:

Although snake toxin is deadly in itself, it is known to have different poison peptides with huge bioactivitie, Tirofiban and eptifibatide are FDA-affirmed antiplatelet drugs, which are disintegrin subordinates from Echis carinatus and Sistrurus miliarus barbouri, individually Captopril is a FDA-endorsed antihypertensive medication, which is a subordinate of bradykinin potentiating peptide acquired from Bothrops jaracusa. Hemocoagulase and batroxobin are advertised medications in certain nations outside of the United States for the treatment of drain and as a defibrinogenating specialist, individually. Hemocoagulases are thrombin-and thromboplastin-like proteins got from Bothrops atrox, while batroxobin is a serine protease got from Bothrops moojeni and B. atrox. Batroxobin can be an expected

instrument in patients on anticoagulant treatment for careful hemostasis. Ximelagatran is a peptide disengaged from cobras, which was at one time a FDA-affirmed drug as an anticoagulant, yet is presently removed from the market. Ancrod was likewise a FDA-endorsed drug as a defibrinogenating specialist, which was subsequently removed and is as of now under stage III clinical investigations. Ancrod is a protein from Agkistrodon rhodostoma. Dendroaspis-NP is presently under stage II clinical examination for the treatment of congestive heart disappointment. It is disengaged from Dendroaspis angusticeps and is a natriuretic peptide. Moreover, α-Cobratoxin and α-cobrotoxin are neurotoxins confined from Naja kaouthia and Naja nivea, separately (11). Venom is a combination of peptides, proteins, and other little particles with changed pharmacological properties. There are numerous bioactive toxin peptides from unfamiliar sources that could bring about possibly new restorative leads. The low endorsement pace of toxin peptides could be because of issues like the low dependability and bioavailability of these peptides. Presentation of peptidomimetics into the natural mixtures could be an answer for conquer the above issues by mirroring the peptide action (12).

Medical Uses of Snake Poison:

Heart attacks/ strokes

- These medication use proteins that dissolve blood clots and skinny the blood.
- Two medications are developed and used since 1998 to stop these diseases.
- The proteins have conjointly been used for those with coronary cardiopathy to breakdown plaque buildup within the arteries.
- Different snakes are found to carry proteins that concentrate on differing types of cancers. One macromolecule referred to as Crotoxin has been found within the South yankee rattler. The macromolecule looks notably drawn to cancer cells and has the flexibility to kill itself. Scientists have an interest in understanding a lot of regarding this macromolecule and the way it is ready to latch on thus tightly to somatic cell receptors and people found on the surface of the brain(8).

Contortrostatin

It is another sort of macromolecule found. It will then work of not attaching and stop those cancer cells from manufacturing signals that prompt new blood vessels to sprout and support

the unfold of cancer, with the National Cancer Institute, this macromolecule has been used and developed into a drug that is presently being tested on animals. If productive, the compounds are used on girls with carcinoma within the next step.

• A macromolecule referred to as Eristostatin has been found within the Asian Sand snake that has been found to assist people's immune systems deflect skin cancer by stopping accomplished cells from colonizing the liver and lungs.

Scaling Brain Disorders

• All brain cells have receptors that pass signals to the body to perform bound functions. Totally different diseases cause these receptors to perform abnormally.

• Proteins in venom utilized in fighting diseases like bronchial asthma, Parkinson's unwellness, Alzheimer's unwellness, and bound pain disorders.

• This medicine would work by sterilization the actions of those receptors so that they are going back to functioning ordinarily(9).

Traditional treatments:

The World Health Organization appraises that 80% of the total populace relies upon conventional medication for their essential medical services needs, techniques for customary medicines of snakebites, despite the fact that of problematic adequacy and maybe even destructive, are in any case pertinent. Plants used to treat snakebites in Trinidad and Tobago are made into colors with liquor or olive oil and kept in rum cups called snake bottles, which contain a few unique plants or potential bugs. The plants utilized incorporate the plant called monkey stepping stool (Bauhinia cumanensis or Bauhinia excisa, Fabaceae), which is beat and put on the nibble. Then again, a color is made with a piece of the plant and kept in a snake bottle. Different plants utilized incorporate tangle root (Aristolochia rugosa), feline's hook (Pithecellobim unguis-cati), tobacco (Nicotiana tabacum), snake hedge (Barleria lupulina), obie seed (Cola nitida), and wild gri root (Acrocomia aculeata). Some snake bottles likewise contain the caterpillars (Battus polydamas, Papilionidae) that eat tree leaves (Aristolochia trilobata). Crisis snake meds are gotten by biting a three-inch piece of the foundation of bois canôt (Cecropia peltata) and overseeing this bit root answer for the nibbled subject (generally a chasing canine). This is a typical local plant of Latin America and the Caribbean, which makes it proper as a crisis cure. Another local plant utilized is mardi gras (Renealmia alpinia) (berries), which are squashed along with the juice of wild stick (Costus

scaber) and given to the chomped. Convenient solutions have included applying bitten tobacco from cigarettes, stogies, or lines. Making cuts around the cut or sucking out the toxin had been thought useful before, however this course of treatment is presently unequivocally debilitating, because of the danger of self-envenomation through blade cuts or cuts in the mouth (pull cups from snake nibble units can be utilized, yet suctioning only sometimes gives any quantifiable advantages (4).

Actual assessment

During the underlying assessment, the nibble site ought to be analyzed for indications of nearby envenomation (edema, petechiae, bullae, overflowing from the injury, and so forth) and for the degree of growth. The chomp site and in any event two other, more proximal, areas ought to be checked and the boundary of the nibbled appendage ought to be estimated each 15 min from there on, until the growing is done advancing. The furthest point ought to be put in a very much cushioned support for at any rate 24 h. Sequential estimation of outline helps in assessing spread of toxin and impact of antibody. Lymph hubs depleting the appendage ought to be touched and the presence of lymphangitic lines noted. Distal heartbeats ought to be checked and observed if there is a presence of gross growth. The presence of a heartbeat doesn't preclude compartment condition in any case, and the compartment pressing factor ought to be estimated straightforwardly if there is worry that a compartment disorder is creating. The conclusion is set up if the compartment pressure, estimated straightforwardly by embeddings a 22G IV cannula and associating it with a manometer, is raised over 55 cm water/saline. Direct estimation is essential before turning to fasciotomy since compartment disorder is uncommon in snakebite casualties and fasciotomy managed without rectification of hemostatic anomaly may make the patient seep to death.

Clues for severe snake envenomation should be sought. They consist of the following:

1. Snake identified is a very venomous one
2. Rapid early extension of local swelling from the site of the bite
3. Early tender enlargement of local lymph nodes, indicating the spread of venom in the lymphatic system
4. Early systemic symptoms
5. Early spontaneous systemic bleeding (especially bleeding from the gums)

6. Passage of dark brown urine(10).

Preparation and storage of snake venom:

Venom preparations are used both to hyper-immunize animals, as part of antivenom production, and to provide reference venom samples for routine and/or preclinical potency assessment of antivenoms. Under GMP for pharmaceutical products, snake venoms are starting materials, and therefore ensuring their quality is critical, and their preparation should follow the principles and recommendations stated below. The essential principles of quality systems should be followed in venom production including traceability, reproducibility, taxonomic accuracy, and hygiene control. Manufacturers of snake venoms used in antivenom production should strive to comply with WHO's Guidelines on GMP for Biological Products and Guidelines for Good Manufacturing Practices for Pharmaceutical Products.

Venoms used for antivenom manufacture should be representative of the snake population living in the area where the antivenom is to be used. To take account of the variability in venom composition within a species. the venom of an adequate number of individual snakes (generally not less than 20 specimens, including males and females) collected from various regions covering the entire geographical distribution of the particular venomous snake species must be collected together. Consideration should also be given to including venom from juvenile or sub-adult snakes in these venom pools as there is strong evidence of age-related venom variation within individual specimens and populations. A similar approach should be used in the preparation of Standard Reference Venoms (national or regional) for use in the validation of antivenom products by reference laboratories and regulatory agencies or in preclinical testing of antivenoms by manufacturers(11).

Venom producers should ensure that they fully document, and can provide evidence of:

- Geographical origin and the length or age (juvenile or adult) of each snake used for venom production;

- Taxonomic details of each snake species used;

- Correct implementation of compliance with local wildlife legislation, and the Convention on International Trade in Endangered Species (CITES) documents in the case of endangered species;

 Application of appropriate withholding rules (e.g.: not collecting venom from animals under quarantine, or which are gravid, injured, sick or in poor condition);

 Individual identification of snake specimens contributing to each venom batch;

 Traceability of each venom batch;

 appropriate handling and stabilization of venoms (e.g.: rapid freezing of the venom after collection and lyophilization for long-term stable storage

 Quality control confirmation of batch-to-batch consistency of venoms of each species/country of origin (e.g.: SDS-PAGE or HPLC profiling of venoms, measurement of residual moisture in lyophilized venom); and

 confirmation of batch-to-batch similarity of venom of the same origin(12).

Quality control of antivenoms:

Quality control of the final product is a key element ensuring the quality assurance of antivenoms. Quality control tests should be performed by the manufacturer or under its responsibility before the product is released. In addition, relevant analyses should be performed on any intermediate steps of the manufacturing protocol as part of the in-process quality control system(13).

The results obtained should meet the specifications approved for each antivenom product or its intermediates, and constitute part of the batch record. For a liquid preparation, some quality control tests, such as the venom- neutralizing efficacy test or the detection of residual reagents used during fractionation, can be performed on the final bulk and may not need to be repeated on the final bottled product if the processing after the bulk preparation has been validated and shown not to have any impact. Quality control assessment of the final antivenom product includes the tests described below.

Standard quality assays

Appearance: The appearance of the product (eg. colour and clarity of the liquid, appearance of the powder) should comply with the description in the marketing dossier.

Solubility (freeze-dried preparations): The time from the addition of solvent to the complete dissolution of freeze-dried antivenom, under gentle mixing, should be determined. Antivenoms should be completely dissolved within 10 minutes at room temperature. The

solution should not be cloudy. Shaking of the container should be avoided to prevent the formation of foam.

Venom-neutralizing efficacy tests: These tests determine the capability of antivenom to neutralize the lethal effect of the snake venom(s) which the antivenom is designed. It is first necessary to determine the lethal potency of the venom, using the median lethal dose (LD50) assay. The exact volume of antivenom required to neutralize venom lethality can then be determined using the antivenom Effective Dose (ED50) assay. The outputs of these tests provide globally-applicable standard metrics of (i) venom lethality and (ii) antivenom efficacy, which enable internal monitoring and external/independent auditing of antivenom efficacy – thereby preventing the distribution of ineffective antivenom. Consistent use of outbred strains of mice, of a defined weight range (e.g. 18–20 g) that receive a defined challenge dose, is recommended for all the assays. Some producers use other test animals, such as guinea-pigs. While weights will vary between animal species, a series of principles, specified for mice, will still apply to these alternative test animals. It should be borne in mind that there are variations in the susceptibility of various strains of mice to the lethal effect of venoms.

Osmolality: Osmolality is used to measure the tonicity of the antivenom solution, and should be at least 240 mosmol/kg. Determination of osmolality is also an indirect means to determine the number of salts or excipients added for formulating the batch.

Identity test: When several types of antivenoms are produced by a single production facility, a system to identify each batch of antivenom should be established for monitoring and auditing purposes. Identity tests may include biological assays as well as physicochemical and immunological tests. Double immunodiffusion assays, confronting the antivenom with the venoms against which the antivenom is designed to act, are often used. In the case of laboratories that use various animal species to raise antivenoms, i.e. horses and sheep, an immunological identity test should be used to identify the mammalian species in which the antivenoms are produced(14).

Purity and integrity of the immunoglobulin: The purity and integrity of the active substance, i.e. intact immunoglobulin or immunoglobulin fragments, should be assessed to identify contaminants and immunoglobulin degradation. Immunoglobulins or their fragments should constitute the great majority of the preparation, ideally greater than 90%. Evidence suggests however that while antivenoms may have physicochemical purity >90% (e.g.:

immunoglobulins or their fragments), immunochemical purity (e.g.: specificity for the snake venoms they are produced from) can be lower than 40%. These findings have emphasized the need to incorporate both physicochemical and immunochemical analyses in the assessment of antivenom purity. Electrophoretic methods in polyacrylamide gels (SDS–PAGE run under reducing or non-reducing conditions) are suitable for this purpose since these techniques allow the detection and monitoring of IgG, F(ab')2, Fab, non-immunoglobulin plasma protein contaminants (in particular albumin), and degradation products. The electrophoretic pattern should be compared to that of a reference preparation, a semi-quantification can be performed by calibration of the procedure. Of particular relevance is the assessment of the albumin content which ideally should not exceed 1% of total protein content.

Molecular-size distribution: The presence of aggregates and other components in antivenoms can be assessed by size-exclusion liquid chromatography (gel filtration) in FPLC or HPLC systems. Densitometric analyses of chromatographic profiles allow the quantification of protein aggregates and of the relative abundances of: intact immunoglobulins, divalent immunoglobulin fragments (F(ab')2), monovalent immunoglobulin fragments (Fab) and dimers, as well as low-molecular-mass enzymatic digestion products. In intact immunoglobulin-based antivenoms, this method allows quantization of albumin as its molecular mass (~66 kDa) can be resolved from the ~160 kDa peak. Antivenoms should comply with the rabbit pyrogen test where required by the local regulations. This test is based on the intravenous injection of antivenoms in the ear vein of rabbits. The dose of antivenom must be calculated by dividing the threshold pyrogenic dose in rabbits by the endotoxin although other doses might be used depending on the Pharmacopeia), followed by the measurement of rectal temperature at various time intervals after injection. The detailed procedures are described in various pharmacopeias. Bacterial lipopolysaccharides can also be detected by the Limulus amoebocyte lysate (LAL) test. The test should be validated for each type of antivenom since there have been reports of false-positive and false-negative reactions when testing antivenoms and other plasma-derived products. The sensitivity of this LAL test should be correlated with the rabbit pyrogen test, and the endotoxin limits established. When regulation allows, a validated LAL test is used in place of the rabbit pyrogen test.

Abnormal toxicity test: The abnormal toxicity test (7day observation of the effects of intraperitoneal injection of 0.2 ml and 0.5 ml antivenom into mice and guinea pigs, respectively), is still required by some pharmacopeias and is performed at the stage of

product development. However, because of the very limited quality control value of this assay, it is increasingly being abandoned by most regulatory authorities. Correct implementation of GMP should provide evidence that the product would comply with the test for abnormal toxicity.

Sterility test: Antivenoms should be free of bacteria and fungi, i.e. they should be sterile. The sterility test is performed following methodologies specified in various pharmacopeias such as the European pharmacopeia. Since antivenoms may contain preservatives in their formulation, it is necessary to “neutralize” the preservatives before the samples are added to culture media. This is usually performed by filtering a volume of antivenom through a 0.45-μm pore membrane, and then filtering through the same membrane a solution that neutralizes the bacteriostatic and fungistatic effects of the preservatives used in antivenom. The membrane is then aseptically removed and cut into two halves. One half is added to trypticase soy broth and the other is added to thioglycolate medium. Control culture flasks are included for each medium. Flasks are incubated at 20–25 ºC (trypticase soy broth) or at 30–35 ºC (thioglycolate) for 14 days. Culture flasks are examined daily for bacterial or fungal growth. The number of vials tested per batch should comply with local regulations.

Determination of pH: The pH of antivenom should be determined using a potentiometer.

The concentration of preservatives: Phenol concentration should not exceed 2.5 g/l and cresols 3.5 g/l. Phenol concentration can be determined spectrophotometrically based on the reactivity of phenol with 4-amino antipyrine, under alkaline conditions (pH 9.0–9.2) in the presence of potassium ferrocyanide as oxidant. Other methods are also available. Phenol and cresols can be determined by HPLC methods.

Chemical agents used in plasma fractionation: The chemical reagents used in the precipitation and purification of antivenoms, such as ammonium sulfate, caprylic acid, and others, should be removed from the final product during diafiltration or dialysis. Limits should be established and their residual amount quantified in the final product. Likewise, the elimination of pepsin or papain from the final preparations should be guaranteed, especially for preparations that are maintained in liquid form, to avoid proteolytic activity that may damage the antivenoms. The determination of the residual amount of agents used in plasma fractionation could be excluded from routine release testing if the process of manufacturing has been validated to eliminate these reagents. The detection of residual reagents can also be performed on the final bulk rather than in the final product(15).

Clinical studies of antivenom:

Although preclinical testing may be valuable in ensuring that antivenoms neutralize the venoms of interest, the complex effects of venoms in humans and the need to consider venom pharmacokinetics mean that, ultimately, the effectiveness and safety of antivenoms for the treatment of human envenoming can only be determined by well-designed clinical studies. Clinical studies of antivenoms primarily address three main issues:

Assessment of the optimal initial dose of antivenom; assessment of the effectiveness of the antivenom; and assessment of the safety of an antivenom, particularly the incidence and severity of early and late reactions. immunoglobulins derived from animal plasma. Antivenom safety and tolerance depend on manufacturing factors (immunoglobulins composition, purification of immunoglobulin fragments, protein concentration, and presence of preservatives). Consequently, the incidence and severity of adverse reactions for similar doses of a given batch of antivenom are unlikely to vary in different geographical locations. Conversely, the effectiveness depends on both manufacturing factors (choice of venoms, immunological title) and also circumstantial factors (quality and quantity of inoculated venom, patient's physical condition, delay of treatment, etc.). However, following initial preclinical testing, both effectiveness and dose-finding studies may need to be repeated for a new geographical location, depending upon the similarity of the snake species in the new place with those where the antivenom was initially tested. If the species are similar, if preclinical testing indicates good neutralization, and if evidence of clinical effectiveness exists in other places, post-marketing surveillance studies may be adequate(16).

Randomized controlled trials

Definitive phase III randomized controlled trials may require large numbers of patients because of considerable individual variation in the clinical manifestation of envenoming (or the great variability in the quantity and quality of venom injected in different patients). The new antivenom is compared with the existing standard antivenom treatment or, if none exists, two different doses of the test antivenom may be compared. Placebo controls are rarely justified unless there is genuine uncertainty about the risk and benefits of antivenom treatment. In this situation, as a safeguard against unnecessary morbidity in either treatment group, a restricted sequential plan might be incorporated [170] which allows evaluation of results as the trial progresses, as in the early trials of therapeutic tetanus antitoxin. To avoid bias, patients should be randomly allocated to the groups and the study should be blinded, at a

minimum to those research personnel who are assessing the clinical response and ideally to both investigators and participants.

There should be a calculation of the number of patients required in each trial arm to give the study sufficient statistical power. These power calculations are based on the expected difference in out between \the treatment groups (if designed to demonstrate the superiority of one treatment over another) or predefined limits of the acceptable performance compared to an existing product (if designed to demonstrate that the new antivenom is not worse than existing products (non-inferiority)). All patients enrolled into a randomized controlled trial and randomly allocated to treatment should be included in the analysis of results according to the principle of "intention to treat", so that deleterious effects of antivenom are not concealed by the recipients' dropping out of the trial.

Effectiveness end-points for antivenom trials

The assessment criteria (end-points) used for antivenom studies should be predefined a-priori and objective. They may be clinical or assessed by laboratory investigations. Common end-points include mortality, development of local tissue effects of envenoming such as necrosis, time is taken to restore blood coagulability (assessed by the 20-minute whole blood clotting test), other laboratory parameters such as the prothrombin time, halting of bleeding or objective clinical improvement in neurotoxicity.

Surrogate markers such as platelet count are less suitable as they may be affected by complement activation resulting from antivenom treatment itself. Patients should be observed for long enough to reveal evidence of recurrent envenoming (seen particularly with short half-life Fab antivenoms). However, due to the high variability of the mode of action of venoms, that of the individual patient's responses and diagnostic capacity of health centers, particularly in developing countries, it is necessary to promote clinical researches to identify appropriate clinical and laboratory criteria.

Safety end-points for antivenom trials

Because antivenoms consist of foreign proteins/fragments that are liable to aggregation, adverse effects are an inevitable risk in therapy. Appropriate manufacturing steps can reduce the rate of adverse reactions. Rates of reaction are correlated with the purity of the antivenom product and the amount of protein-infused Continuous clinical observation at the bedside is

necessary for several hours after treatment to detect acute reactions; late adverse reactions may occur several weeks later. Accurate reaction rates can only be assessed prospectively.

Reaction rates may differ considerably between different antivenoms, but in most cases only a small proportion are life-threatening. Although there is no consensus on classifying or grading Early Adverse Reactions (EAR), studies should aim to detect both early adverse events (anaphylaxis and pyrogenicity) occurring at the time of, or within 24 hours of, antivenom administration (such as urticaria itching, fever, hypotension or bronchospasm) and late reactions such as serum sickness occurring between 5 and 24 days of antivenom administration (e.g. fever, urticaria, arthralgia, lymphadenopathy, proteinuria, or neuropathy)(17).

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