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Erratum

“Erratum to Report of WHO workshop on the standardization and control on antivenoms” [Toxicon 41 (5) (2003) 541–557][☆]

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Two errors occurred in the above paper, the corrections are listed below.

On page 546, line 21 from foot of page (righthand column) it mentions that Complete Freund’s adjuvant was used in the ‘intradermal’ injection of horses; it should read ‘subcutaneous’ injection.

On page 544 under the section *Basic Immunology and Pharmacology of Antivenoms*, line 4 and page 546, line 8 (lefthand column) it states pH 2 was used in the pepsin digestion; this should read pH 3.0–3.5.

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Special report

**Report of a WHO workshop on the standardization
and control of antivenoms**R.D.G. Theakston^{a,*}, D.A. Warrell^{a,b}, E. Griffiths^c^a*Alistair Reid Venom Research Unit, WHO Collaborating Centre for the Control of Antivenoms, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK*^b*Nuffield Department of Clinical Medicine, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK*^c*Quality Assurance and Safety, Biologicals, World Health Organisation, 20 Avenue Appia, CH-1211, Geneva 27, Switzerland*

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Abstract

A workshop to discuss progress in the standardization and control of antivenoms, organized by the Quality Assurance and Safety of Biologicals Unit of WHO, was held at the National Institute for Biological Standards and Control, Potters Bar, England, 7–9 February 2001. This was the first meeting convened by the WHO on this subject since 1979 and it brought together experts from academic institutions, antivenom manufacturers and national regulatory authorities from 21 countries. The meeting reviewed antivenom production and quality control measures and special consideration was given to the current crisis in antivenom production and supply in sub-Saharan Africa. The importance of snake bite and scorpion stings as public health issues was re-emphasised. The majority of commercial antivenoms are raised against snake or scorpion venoms.

The review of antivenom production methods indicated that the vast majority of commercial antivenoms were still produced by traditional technology in horses, although some antisera were raised in sheep and rabbits. Methods used for plasma fractionation included salt and heat coagulation, caprylic acid stabilization or ion exchange chromatography, as well as immunoglobulin digestion with pepsin to produce F(ab')₂ or with papain to produce Fab fragments. The meeting agreed that there was much room for improving the production, quality control and safety profile of these products and that lessons could be learnt from the experience gained with the preparation of human immunoglobulins. Many basic assumptions, such as the need to remove Fc fragments by enzyme digestion and to freeze–dry antivenom preparations, required critical re-examination and more attention should be given to clinical trials as a means of assessing efficacy and safety and of defining the average initial dose. The Workshop also discussed concerns about the risks of transmitting infectious agents to humans via animal blood products, especially those posed by viruses or prions and it was agreed that this aspect needed attention. However, there was no documented or even suspected example of this ever having occurred in the case of antivenom treatment.

Current WHO Requirements for the production and control of antivenoms and for immune sera of animal origin date from the late 1960s. The Workshop recommended that these be updated to take account of the progress that had taken place in the production and quality control of biologicals in recent years. In addition, the Workshop discussed the need for better standardization of both the venoms and antivenoms, but concluded that international standards and reference materials were not appropriate in the antivenom field due to the considerable variation in venom characteristics from the same species from region to region. Instead, it was recommended that national or regional standards be prepared and used.

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Keywords: WHO; Antivenom; Envenoming; Standardization; Testing; Quality control; Enzyme refinement; Caprylic acid

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1. Introduction

More than 60 delegates from 21 countries were welcomed to the Workshop on the Standardization Control of Antivenoms by E. Griffiths, Coordinator, Quality Assurance and Safety of Biologicals, WHO, and G. Schild, Director, National Institute for Biological Standards and Control. It was noted that the last meeting held by WHO on this subject was in Zurich in 1979 (WHO, 1981). That meeting had recommended the collection of better data on envenomings and their treatment, improvements to the ICD classification and identification of snake species, the establishment of standard laboratory assays of clinically important venom activities and the development of international standards for venoms and antivenoms. There was also encouragement for clinical trials and for the development of guidelines for treatment and for education and training in this field. Over the next few years, some of these aims were achieved (Theakston and Reid, 1983). However, failure of storage of the archive of candidate reference antivenoms and venoms, and the need to improve the production, quality control and safety profile of antivenoms in line with developments in the biologicals field over this period, as well as an increasing crisis in global antivenom production, had prompted the present meeting.

The main aim of the Workshop was to focus on the standardization and control of medically important venoms, as well as on the production and control of antivenoms for venomous bites and stings. Current WHO Requirements for antivenoms and for immune sera of animal origin date from 1969 to 1971 and required updating in the light of developments in production, standardization, quality control and regulatory oversight for biologicals. Points requiring review included the efficacy of antivenoms, the need for international standards and reference materials and the assurance of consistent quality and safety of the products. However, production, quality control measures and costs needed to be balanced bearing in mind that much antivenom production took place in developing countries and that antivenoms constituted the only effective treatment for snake bites in the rural tropics. In addition, the meeting provided an opportunity to reassess the global availability of antivenoms with special consideration being given to the crisis of antivenom supply in Africa. It was hoped that the Workshop would provide a platform for discussing new actions and initiatives on these topics.

2. Global problem of venomous bites and stings and their management

The overall problem of envenomations worldwide was reviewed by D. A. Warrell.

2.1. Snake bites

Estimates of global mortality from snakebite have been reported to range from 50,000 per year (Swaroop and Grab, 1954) to 100,000 per year (Chippaux, 1998). However, these largely hospital-based figures are likely to be underestimates, as the majority of snake bite victims seek traditional treatment and may die at home unrecorded. The few attempts to carry out properly designed population-based studies have revealed unexpectedly high rates of mortality, ranging from 2 to 16 per 100,000 per year in Nigeria, Kenya, Senegal and West Bengal. To help with the precise reporting of snakebites, there have been repeated calls for snake bite to be made a specific notifiable disease with a distinctive ICD classifier (Warrell, 1999). In ICD10, T63.0 is specific for snake bites. It is likely that for every snake bite death, several survivors are left with chronic disability including physical handicap from necrotic effects of the venom requiring amputations; chronic ulceration, osteomyelitis with malignant transformation; chronic renal failure; chronic pituitary–adrenal insufficiency and neurological sequelae from intracranial haemorrhages and thromboses.

World-wide, most bites are inflicted on the feet and ankles of agricultural workers, hunter gatherers, fishermen and fish farmers and professional handlers of snakes (for food, skins, etc.). Seasonal variations in snakebite incidence reflect rainfall and other climatic cycles but also changes in agricultural activity, such as the paddy harvest in South East Asia.

Clinical grading is of unproven prognostic significance and may obscure useful detail of specific effects of envenoming. Local swelling is a result of permeability enhancing factors in the venom and local necrosis is the result of digestive hydrolases, myotoxins, metalloproteinases, polypeptide cytotoxins, thrombotic factors and secondary infection. Systemic envenoming carries a more urgent and obvious risk of fatal consequences. Anti-haemostatic and thrombotic effects, such as spontaneous haemorrhage into the brain, gastrointestinal tract and elsewhere, incoagulable blood and thrombosis of small or larger blood vessels, is attributable to venom procoagulant enzymes, resulting in disseminated intravascular coagulation and consumption coagulopathy, and to phospholipases, metalloproteinase, haemorrhagins and platelet factors. Shock (hypotension) is usually the result of hypovolaemia (loss of circulating volume into the swollen, bitten limb) but is also caused by venom autopharmacological agents, angiotensin converting enzyme inhibitors and bradykinin potentiating factors, as well as other vasodilators and possibly direct effects on the heart muscle. Progressive paralysis is caused by venom neurotoxins acting pre- and post-synaptically and becomes life-threatening when bulbar and respiratory muscles are involved. Generalised rhabdomyolysis, caused by venom phospholipases A₂ and other myotoxins, contributes to muscle weakness and can cause fatal hyperkalaemia following sea snake bites and renal

complications of myoglobinaemia. Phospholipases A₂ may also cause intravascular haemolysis, while microangiopathic haemolysis is a complication of disseminated intravascular coagulation. Acute renal tubular necrosis can be caused by direct action of nephrotoxins, by pre-renal effects such as profound shock and vasoconstriction or obstruction of renal microvasculature, as well as disseminated intravascular coagulation.

Venomous snakebite without clinically evident envenoming ('dry bites') occurs in about 50% of all venomous snake bites, ranging from 80% in the case of Australian brown snake (*Pseudonaja*) bites to less than 10% with saw-scaled viper (*Echis*) bites. Case fatality of snake bite before the advent of antivenom therapy was reported to be high (more than 50%) in the case of bites by species such as the tropical rattlesnake and Australian elapids (taipan, death adder, tiger snake). With antivenom and modern ancillary treatments it can be reduced to less than 5%.

Most traditional first-aid methods have been proved ineffective and dangerous. These include tight tourniquets, local incisions, suction, snake stones, cryotherapy, inoculation of chemicals and herbal matter, electric shock and vacuum extraction. Modern recommendations emphasize reassurance of the victim, immobilization of the whole patient, especially the bitten limb, while they are transported to medical care by stretcher and the use in patients bitten by neurotoxic elapids (not African spitting cobras) of 'pressure immobilisation' using a long, crepe bandage and splint.

Hospital treatment involves resuscitation, rapid clinical assessment and simple tests such as the 20 min whole blood clotting test (indicating incoagulable blood from consumption coagulopathy). Antivenom remains the only specific remedy. Indications for its use include shock, spontaneous systemic bleeding, incoagulable blood, paralysis, acute renal failure and black urine (myoglobinuria or haemoglobinuria). Extensive or rapidly progressive local swelling is also an indication for antivenom treatment, especially where digits and fascial compartments are involved and the venom is known to be necrotic. Ancillary treatments may be just as important as antivenom in saving the patient's life. Mechanical ventilation, renal dialysis, correction of hypovolaemia and acidosis and the prompt treatment of necrotic tissue (surgical débridement) and local infections is of great importance. The value of fasciotomy is controversial but it should never be attempted in snake bite victims until anti-haemostatic abnormalities have been corrected with antivenom and unless intracompartmental pressure has been measured directly and found to be elevated above recognised danger limits.

2.2. Scorpion stings

D. A. Warrell reported that scorpions are second only to snakes in causing human fatalities from envenoming. Formerly between 1000 and 2000 deaths were reported each year in Mexico but, more recently, there have been an

average of 143,000 scorpion stings each year (77% of all venomous bites and stings in that country (18)) with a few hundred deaths. Species capable of inflicting fatal stings occur in North Africa and the Middle East (genera *Androctonus*, *Buthus* and *Leiurus*); South Africa (*Parabuthus*); India (*Mesobuthus tamulus*); North, Central and Southern America, Trinidad and Tobago (*Tityus* and *Centruroides*). Scorpion venoms, relatively meagre in toxins compared to snake venoms, contain toxins which target sodium, potassium, calcium and chloride channels, causing direct effects and release of neurotransmitters such as acetylcholine and catecholamines which produce signs of autonomic system overactivity or 'autonomic storm', resembling, in some cases, the clinical features of phaeochromocytoma. Intense local pain is a feature of stings by almost all species of scorpion, including those whose venoms have no life-threatening potential in humans. Treatment of pain is with local anaesthetics (emetine is used in India), local nerve block or (disreputably but, it is claimed, effectively) electric shock. Strong systemic analgesics including opiates may be needed, but some have claimed these drugs to be dangerous in victims of *Centruroides* envenoming.

The role of antivenom in the treatment of scorpion stings remains controversial. Reports from North Africa (especially Tunisia), the Middle East, the Americas and South Africa, strongly support the use of antivenom. In Tunisia and Mexico, reduction in childhood mortality from scorpion envenoming has been attributed to the widespread use of antivenom. However, some recent trials have been flawed by inadequate design and insufficient recruitment of severely envenomed patients in whom antivenom treatment is likely to be important (Ghalim et al., 2000; Abroug et al., 1999). Authoritative opinions from Israel and India, on the other hand, strongly contest the role of antivenom, claiming its futility considering the speed of distribution and irreversible effects of scorpion toxins. They prefer to emphasise ancillary treatment with vasodilators. Studies in human patients and in a rat model in Brazil suggest that atropine should not be used except in cases of life-threatening sinus bradycardia and that cardiac glycosides and β -blockers are contraindicated (Freire-Maia et al., 1996). Prophylactic immunisation against scorpion envenoming has also been advocated, especially in Mexico, but acceptable experimental evidence for its value is lacking (Calderon-Aranda et al., 1995). The use of synthetic peptides can be a misleading approach to generate vaccines against scorpion toxins (Calderon-Aranda et al., 1995).

2.3. Other venomous bites and stings

Stings by fish, cnidarians, lepidoptera, centipedes and coneshells and bites by spiders, ticks and one genus of octopus, are responsible for some morbidity and mortality but probably account for not more than a total of about 100 deaths per year. In some countries, venomous Hymenoptera

(bees, wasps, yellowjackets, hornets and ants) cause more deaths through type I hypersensitivity than all other venomous bites and stings together. In England and Wales, about 10 people die each year from Hymenoptera sting anaphylaxis, in Australia 2–3 per year and in the United States 40–50 per year. Mass attacks by Africanised killer bees have caused about 30 deaths each year in the Americas.

3. History of antivenoms

The history of antivenom production dating from 1897 was outlined by RDG Theakston. Following the initial use of crude equine immune serum, salt precipitation and pepsin digestion, which removed the Fc fragment from the IgG molecule resulting in an F(ab')₂ fragment antivenom, was adopted by most producers. This appeared to reduce the incidence of life-threatening antivenom-induced anaphylactic reactions, although there is evidence that whole IgG can be made safe if aggregation is prevented. More recent modifications of this basic technology have included the production of Fab fragment antivenoms, the use of affinity purification to remove non-specific antibodies and caprylic acid stabilisation to precipitate non-IgG proteins from hyperimmune plasma.

4. Basic immunology and pharmacology of antivenoms

A review of the differences between whole IgG, F(ab')₂ and Fab fragments was presented by Kavi Ratanabanangkoon. F(ab')₂ preparations are obtained by pepsin treatment of IgG at pH 2, whereas Fab fragments are produced by papain treatment at pH 7–8. The latter process appears more difficult to standardize than when pepsin is used. About 20% of antigenic binding sites are destroyed during enzymic digestion. Also, in some countries the enzyme quality varies considerably from batch to batch and can lead to variations in product quality. Prolonged pepsin digestion yields Fv fragments but their binding to antigens may be only transient.

Data suggest that F(ab')₂ is better than Fab both in its plasma distribution and neutralization. This is explained by the pharmacokinetic differences between the two fragments. However, experimental data from Brazil showed that IgG protected better than Fab against both *Crotalus* and *Bothrops* venoms. IgG was also more efficient in neutralizing the lethal effect, but IgG, F(ab')₂ and Fab all had the same efficiency against venom-induced haemorrhagic effects (Ismail et al., 1998; Ismail and Abd-Elsalam, 1998; Morais et al., 1994; León et al., 1997).

With regard to adverse effects, reported hypersensitivity reactions indicate the following order of reactions: IgG (30%) > F(ab')₂ (10%) > Fab (0.8%). However, other data

suggest that adverse effects are not generally related to the nature of the fragments used but, more importantly, related to the actual method of production of the antivenom used. It was concluded that it is advantageous to remove the Fc fragment and that F(ab')₂ fragments are easier to produce than Fab because the process can be more readily controlled. It was also generally agreed that aggregates and other contaminants present in antivenom preparations are responsible for the adverse effects of antivenoms. The larger the amount of protein administered, the greater the amount of contaminants given, and this leads to higher incidences of adverse reactions. The amount of total protein administered is also related to the specific activity of the product whether it be composed of whole IgG, Fab or F(ab')₂. Scorpion antivenoms should be less of a problem than anti-snake venom products because less material is needed to neutralise just one major toxin.

The use of monoclonal and polyclonal antibodies were also discussed. Although some monoclonal antibodies seemed to increase survival time in experimental animals, and some had surprising efficacy (e.g. reversal of toxin α (*Naja nigricollis*) binding), there was usually little overall protection because of the limited avidity and duration of immune cover. As a general rule, more monoclonal antibody was needed than a polyclonal preparation to neutralize a given amount of toxin. For this and other reasons, monoclonal antibodies would prove much more expensive in clinical use.

An experimental study was discussed by J-M Gutiérrez in which IgG, Fab and F(ab')₂ fragment antivenoms were compared for their ability to neutralize the local effects of *Bothrops* venom. An important clinical problem in this respect is the often long delay between envenoming and administration of antivenom. Some antivenoms seem poor at neutralizing local effects of envenoming since toxins can rapidly produce irreversible necrosis. IgG may reach the tissue compartment at a similar rate to the antibody fragments because of venom-induced damage to the microvasculature which increases vascular permeability. In fact, experimental results showed no differences between the efficacy of IgG and F(ab')₂ and indeed, for myonecrotizing activity, IgG was found to be better than Fab initially. The overall conclusion was that, experimentally, Fab, F(ab')₂ and IgG did not vary much in their ability to neutralize local venom effects. It was stressed that the reported results were for *Bothrops* antivenom and that the situation may be different for other venoms, such as those from scorpions which may act differently.

5. Current methods of antivenom production

Representatives of the 21 antivenom production laboratories from around the world, both public and private, presented the methodologies used for manufacturing

Table 1
Aspects of antivenom production

| |
|-------------------------------------------------------------------------------------------------------|
| Screening production animals for adventitious agents |
| IgG concentrations [(NH ₄) ₂ SO ₄ /NaSO ₄ precipitation] |
| Enzyme digestion (pepsin → F(ab') ₂ ; papain → Fab) |
| Caprylic acid stabilization |
| Ion exchange (removes Fc) |
| Affinity purification (concentrates venom-specific IgG) |
| Pasteurisation (10 h at 60 °C) |
| Endotoxin exclusion (to not more than 0.5 u/kg/dose) |
| Lyophilisation |

antivenoms (Table 1). Both monospecific and polyspecific antivenoms are produced in the majority of laboratories.

5.1. Animals used for immunization

The vast majority of producers used horses, immunized with crude venoms, for antivenom production. Only one producer used sheep. In general, Freund's complete and incomplete adjuvants were used to promote immune responses, although in some cases aluminium hydroxide or alginate was used as an adjuvant. The meeting agreed that the health of animals used for venom production is of great importance. Endemic diseases, such as Trypanosomiasis ('durina'), limited the use of horses in some geographical regions. Diseases such as African horse sickness, viral equine encephalitides and glanders ('muermo') and equine infectious anaemia, caused problems in other countries (see below).

5.2. Fractionation protocols

The majority of manufacturers used the traditional method for obtaining F(ab')₂ fragments. Plasma is treated with pepsin at acidic pH to remove the Fc fragment, and then F(ab')₂ fragments purified by ammonium sulphate or sodium sulphate precipitation. The salt is then removed by dialysis or ultrafiltration. Two laboratories used an ion-exchange chromatography step to further purify F(ab')₂ fragments. One laboratory produced Fab antivenoms using papain digestion (pH 7–8), followed by ion-exchange chromatography and affinity chromatography. Some laboratories produced antivenoms consisting of whole IgG molecules purified by ammonium sulphate precipitation, and one laboratory produced a whole IgG antivenom by using caprylic acid precipitation of non-IgG proteins (dos Santos et al., 1989), followed by ultrafiltration. Only one laboratory reported still manufacturing a crude, non-refined whole equine serum antivenom. Phenol, cresol and thiomersal were used as preservatives by some producers, whereas other antivenom preparations did not contain any preservative. Most antivenoms were in liquid presentation, with a shelf life of 3 years, but some manufacturers also

produced lyophilized antivenoms, with a shelf life of 5 years.

5.3. Type of antibody fragments

There was considerable discussion of the advantages and disadvantages of antivenoms made of whole IgG molecule, F(ab')₂ fragments and Fab fragments. Despite strikingly different pharmacokinetic profiles of these products, neutralisation studies had not shown substantial differences in the ability of these antivenom preparations to neutralise the toxic effects of some venoms. Owing to the considerable variation in the biochemistry, pharmacology and immunology of venoms, it was agreed that no generalizations could be made as to whether one type of antibody or antibody fragment is better than another in the neutralisation of venoms. Instead, detailed and well-designed studies were required for each venom–antivenom system in order to decide the best choice in each particular case.

5.4. Safety of antivenoms

Conflicting results have been reported concerning the adverse effects of antivenoms. Incidences of early adverse reactions range from 5 to 80% for products made of the same type of molecule or fragment. This again points to the quality of the preparation being more important than the type of antibody molecule or fragment in the induction of early adverse reactions. The Workshop agreed that every effort should be made to improve production protocols with a view to improving the purity of the IgG, F(ab')₂ or Fab fragments and minimising contaminating proteins or protein aggregates. The aim should be a product with a total protein concentration as low as possible. Also, strict limits should be set for the presence of pyrogenic materials (e.g. endotoxin). It was generally accepted that the removal of Fc fragments from IgG prevented complement activation and so reduced the risk of reactions. However, although Fc is not required for venom neutralisation, it was suggested that it might be involved in Ab–Ag complex elimination. More controlled clinical trials were necessary to characterise the efficacy and safety of these products.

6. Problems associated with conventional antivenom production

Several problems associated with much of present day antivenom production were raised by I Raw and S Jadhav. In developing countries, the cost of manufacture and distribution of antivenoms was a major factor. Ideally this cost should be covered by the public sector (Health Service) and antivenoms should be affordable by and available to anyone who had been bitten or stung and needed treatment. The possibility of arranging for the content of one vial to be

sufficient to treat even the most severe case of envenoming was raised. This would theoretically absolve the treating physician from having to decide how many vials should be given, but it would involve the expensive over-treatment of all but the most severe cases.

It was reported that many antivenom producers frequently did not adequately control the pH conditions for pepsin digestion (pH 2). Also, inadequately refined pepsin was sometimes used for digestion. Batch to batch variation in the enzyme quality can result in inadequate digestion of the IgG molecule leading to poor quality F(ab')₂ antivenoms with a high incidence of early reactions.

The need for preservatives, such as phenol and cresol, in the final product was also discussed. Their use was considered necessary to prevent microbial growth in crude, non-aseptically manufactured antivenoms. Although the use of preservatives for this purpose should, ideally, be phased out, it was acknowledged that if their use were to cease, there would need to be an expensive improvement in manufacturing facilities.

Venom variation within the same species both locally and from region to region was also noted and had to be taken into consideration in the production of effective antivenoms. Reference preparations needed to be developed for countries or regions. It was reported that in India, cooperation between the various antivenom producers had resulted in the production of reference venom preparations for *Vipera* (= *Daboia*) *russeii*, *Naja naja*, *Echis carinatus* and *Bungarus caeruleus*. These standards are held and distributed by the Indian National Control Laboratory. As a result all producers in India can now manufacture antivenom to a uniform standard by testing their products against these reference venom preparations. It was also strongly recommended that the system of mouse lethality testing should be standardised and that this could be achieved most easily by following WHO methodology.

The need to produce lyophilised antivenoms was questioned by I Raw, pointing out the added costs that this incurred. Lyophilization was sometimes insisted upon in some countries to ensure that antivenom preparations remained effective at ambient tropical temperatures for long periods.

7. New approaches to production

C Bon presented several ideas which might lead to possible improvement of antivenom therapy. These included: improvements to the production processes to increase the purity of antivenoms and to render treatment safer and more effective; the use of clinically important toxic fractions of venoms for raising antibodies so as to enhance immunoreactivity, thus leading to the administration of reduced amounts of therapeutic material; education of physicians and nurses to improve clinical

treatment of patients; the use of the same distribution routes for vaccines and antivenoms: use of cost-effective technological improvements. B. Bissumbhar proposed that improvements in antivenom purity could be achieved by the introduction of a specific caprylic acid step to improve product purity. B. Golding pointed out the claimed viral-inactivation properties of caprylic acid but emphasised the need for validation data to substantiate such claims.

J. Landon reviewed the advantages and disadvantages of the horse as an animal for use in antivenom production. High levels of heavily glycosylated IgG(T) antibodies are present in equine serum. IgG(T) (M_w 160 kDa) is powerfully immunogenic when injected into other species, including humans, and may contribute to antivenom reactions. Sheep serum lacks IgG(T) and is therefore, inherently, safer. However, the production of large volumes of antisera from horses is clearly an advantage. Cost, in Europe at least, is reduced if sheep are used. For small volume immunotherapy, such as is used to treat envenoming by, for example, black widow spider (*Latrodectus*), large animals are unnecessary. Sheep have the advantages of good tolerance to effective adjuvants, ease of handling and maintenance, and availability in large numbers to provide batch-to-batch consistency and lower immunoreactivity. The enviable safety record of the ovine-derived antibody preparations was cited as an example of this. Experimental production of antivenom in eggs by immunising chickens has the advantages of lower costs, simpler downstream processing (IgY present in the yolk only) and a rapid immunological response. However, there are problems of egg allergies to be considered. Where horses or any other animal is used for raising antibodies, the question of promoting immune responses with appropriate adjuvant arises. Kavi Ratanabanangkoon reported that immunological responses in horses are improved by using multi-site intradermal injection of immunogen in Freund's Complete adjuvant (Pratanaphon et al., 1997). He reported that the poor quality of the existing Thai Red Cross *Naja kaouthia* antivenom was found to be due not to the use of native versus attenuated toxin as immunogen, but to the use of an inefficient bentonite adjuvant. When this was replaced with Freund's adjuvant, high quality antivenom was produced thus eliminating supply problems (Chotwiwatthanakun et al., 2001).

L Sjöström presented a brief summary of the advantages and disadvantages of immunisation of animals using whole venom or venom fractions. Caution was expressed about the use of fractions as little is known about the interactions between different venom components. J-M Renjifo emphasised the importance of immunisation using a pool of venom from the clinically-important species to produce an effective polyspecific antivenom. Lei Dianliang presented two cases of envenoming by *Bungarus multicinctus* (Chinese krait) in China which did not respond to standard antivenom therapy. Physicochemical investigations suggested recent changes in the venom used to manufacture the antivenom. It was

thought possible either that a new venom component had evolved recently in the snake population or that the antivenom prepared with the old venom no longer provided protection. The potential for use of DNA immunisation in antivenom production was discussed by RA Harrison. Certain toxins such as the pathogenic venom haemorrhagins can be sequenced and targeted areas inserted into an expression plasmid. This results in expression of the foreign peptide possibly resulting in potent antibody stimulation. Comparison of peptide sequence homology with haemorrhagins of other species was proposed as a means of identifying regions that might induce cross-reactive antibodies. It was proposed that this method using the gene gun could be an effective technique for production of antibodies to venom toxins although little was known of the effectiveness of DNA vaccines in potential production animals.

8. Stability of immunoglobulins

The merits of liquid versus lyophilised products with respect to stability were considered at some length. R Thorpe noted that purified immunoglobulin products are usually quite stable and lyophilised preparations normally had longer shelf lives than liquid presentations if appropriately stored, an obvious advantage where distribution and storage conditions cannot be well controlled. Fc is the most fragile part of the molecule. Experience with human immunoglobulin preparations had demonstrated that instability problems can arise due to denaturation of IgG during production and/or proteolytic cleavage during or after purification, resulting in the formation of aggregates. Instability is not easy to predict, and subtle changes in the production process or quality of the raw material have been shown to have adverse effects on the product.

The solubility of lyophilised IgG depends critically on how well it was freeze–dried. Whole IgG is less soluble than fragments lacking Fc. Difficulty in re-dissolving commercial lyophilised antivenoms is a commonly reported experience and may reflect denaturing of the product during freeze–drying, implying loss of stability and activity. The main techniques for stability monitoring are size exclusion HPLC, SDS PAGE under reducing conditions, potency assay and other methods including measurement of anticomplementary activity, Fc function and binding activity.

The use of excipients to prolong the stability and shelf life of liquid products was raised as a possible alternative approach to freeze–drying. There was a strong warning, however, against the use of sucrose as this had been implicated in impairment of renal function. Such effects could exacerbate the renal effects caused by some venoms. An important suggestion was that the shelf life of most antivenoms could be safely extended, and that this would avoid premature disposal of material, reduce costs and

increase availability in developing countries. The generation of real time stability data for submission to national regulatory authority would of course be needed to support any extension of shelf life.

9. Polyspecific versus monospecific antivenoms

The advantages and disadvantages of antivenoms raised to one particular venom (monospecific) or to two or more venoms (polyspecific) were discussed by DA Warrell (Table 2).

Monospecific antivenoms can show considerable potency for neutralising venom, but are limited in use to a single species of venomous animal or a few closely related species whose venoms show cross reactivity with the serum. Polyspecific antivenoms can be produced using venom from a range of species of venomous animal, which clearly broadens their usefulness and makes identification of the biting species less important. However, there is some controversy over the potency of polyspecific antivenoms, most reports suggesting lower potency than monospecific preparations, whereas a few indicate a ‘synergistic’ effect when a mixture of venoms is used as immunogen. It is important to maximise the possibility of correctly identifying the biting species and procedures have been established for testing patients’ serum for identification of specific venom in some cases. Use of monospecific antivenom requires a species identification, in some cases by examination of the snake involved, but usually based on the history and evolving symptoms of envenoming. This could be simplified by use of algorithms (Warrell, 1999). When the snake responsible can be confidently identified, monospecific antivenom should be used. However, it is not always possible to differentiate from the clinical symptoms (e.g. in the central rice-growing area of Thailand there are three species of vipers capable of causing local swelling, bleeding and incoagulable blood). Polyspecific antivenom is preferable in these circumstances. The simplicity of

Table 2
Advantages and disadvantages of monospecific vs polyspecific antivenoms

Monospecific antivenoms

Raised against venom of a single species
Maximal neutralising activity per dose of IgG/fraction
Smaller dose necessary (decreased risk of reaction)
Specific diagnosis of the snake responsible is necessary

Polyspecific antivenoms

Raised against venom of two or more species
Covers the most important species in a particular geographical area
Less neutralising activity per dose of IgG/fragment
Larger dose necessary (entailing an increased risk of reactions)
Specific diagnosis of the snake responsible is less important

the 20 min whole blood clotting test for specific diagnosis in certain areas (e.g. *Echis* spp. in the northern third of Africa) and for assessing the effectiveness of antivenom treatment was discussed. The use of a snake venom detection kit in Australia has permitted the use of a lower dose monospecific antivenom.

10. Standardization, quality control and safety

10.1. Lessons from production and quality control of human immunoglobulins

The considerable experience of production and quality control gained with human immunoglobulin preparations, some of which may be relevant for antivenoms was discussed by R Thorpe. Human immunoglobulin products are available for intramuscular or intravenous administration and are used for a range of clinical indications. Most are produced by cold ethanol fractionation and those intended for intravenous use are further treated using a range of procedures which include chromatographic methods and incubation with trace amounts of liquid or solid phase protease. These additional steps are required to avoid adverse reactions related to intravenous use of immunoglobulin. Chemical modification has been used in the past, but these methods normally adversely effect the Fc part of the IgG molecule and are not recommended. The Fc component is important for opsonization of pathogens by IgG given to patients with hypo γ globulinaemia.

Quality control of immunoglobulin products needs to assess their efficacy and safety. Efficacy testing should address batch-to-batch consistency and stability. Safety testing includes procedures designed to ensure freedom from infectious agents and also safety in respect to causing adverse reactions in recipients. WHO have produced recommendations for desirable characteristics of human immunoglobulin preparations as well as for viral inactivation and removal procedures (WHO, 2003).

Laboratory characterisation of batches of intravenous immunoglobulin can be conducted to assess quality and safety. Normally, a range of complimentary procedures need to be carried out to show purity, identity, IgG content, levels of aggregates and fragments and levels of contaminants such as PKA and IgA. Immunoassays can be used to show that the preparations contain antibodies directed at appropriate micro-organisms or antigens. Immunoglobulin products which are to be used for clinical indications which require immunobiological activity should be assessed for appropriate Fc function. Anti-complimentary activity has been associated with adverse reactions in some cases and this can be assessed using appropriate methodologies. It is important to validate all methods for use with immunoglobulin preparations. In some cases, methods which perform adequately with single donor serum or plasma samples do

not produce valid results when applied to immunoglobulin products. Methods such as immunoassays often require adaptation if they are to be used with immunoglobulin products.

10.2. Reducing risks of transmitting transmissible agents

Minor discussed general principles associated with the viral safety of biological medicines with particular reference to antivenoms. A range of lethal viral infections of humans are known or believed to originate in animals, including HIV from chimpanzee and mangabey, Marburg and possibly Ebola from monkeys, Hantaan and Lassa from rodents, Nipah and other paramyxoviruses via pigs, the Australian bat Lyssavirus from fruit bats and equine morbilli virus (Hendra) from horses. Several have caused epidemics. In addition, live SV40 virus of monkeys (rhesus) is known to have been a contaminant of early polio vaccines and inadvertently given to humans through vaccination. There is recent evidence that SV40 is associated with some human tumours although it is not known if the virus is the causative agent, nor whether the presence of the virus in the human population is the result of the early polio vaccination. The risk of viral contamination of biological products is, therefore, a subject of great concern to regulatory authorities.

As a general principle, it is desirable to exclude all viruses and this is approached, when possible, by using specific pathogen-free colonies of animals. In the case of murine monoclonal antibodies this has involved screening for up to fourteen viruses in colonies of animals kept under strictly contained conditions. This is not readily applicable to large animals such as horses where containment is impractical and the virology has not been exhaustively studied. Moreover, the tests used for mouse colonies are widely available as a result of commercial demand so that although they are fairly sophisticated in nature, they are accessible. This would not be true of viruses infecting animals used in the production of antivenoms, such as horses or sheep. Good husbandry and some virological screening could reduce the concern, but confidence would be increased by demonstrating that the process used to produce the antivenoms is able to remove or inactivate likely categories of virus contaminant. In fact, the widely used processes involved in antivenom production seem to have several promising steps, including a low pH pepsin treatment or the use of agents such as caprylic acid, which could act as a detergent to inactivate lipid-containing viruses. In the past, regulatory authorities have insisted on specific data for the production process for specific products because of variations observed between the ability of processes, which are nominally the same, to remove virus infectivity. This may not be practical in the case of antivenoms and it is possible that satisfactory generic data might be generated if the production process could be harmonised to an acceptable degree.

10.3. Monitoring systems for animals used in antivenom production

D Galbraith and E J Ruitenbergh discussed risk assessment and reduction of possible contamination of antivenoms by transmissible agents at a more practical level. First, the production process should be evaluated and assessed in order to establish what risks are likely. If a particular pathogen is a cause for concern, it should be established whether it is likely to be present in the animals and immune plasma and, if so, what steps could be taken to minimise its presence, before evaluating the residual risk and establishing a final specification. In practice, a virus could be actively excluded from the animal herd or colony by a process of screening and suitable precautions, or simply excluded without the need for screening because of the way in which the animals are kept. For example, animals could be kept free of a particular arthropod-borne virus if they are maintained in an area free of the particular arthropod vector. Alternatively, a virus may be unavoidably present in the animals and may be eliminated by the production process. With antivenoms, the favoured animal is the horse, and information on potential horse virus contaminants is incomplete. Viruses of concern might include Equine Infectious Anaemia Virus, a lentivirus of the same family as HIV, influenza and Japanese Encephalitis or Venezuelan Equine Encephalitis virus, but there are others some of which are classified as pathogenic for humans. Novel viruses, such as equine morbilli virus (Hendra), can also arise unexpectedly. In principle, product safety can be enhanced by surveillance of the source animals, by screening, health monitoring, including routine blood chemistry and haematology and post-mortem examination. Also, by preventive measures such as good husbandry or vaccination, where appropriate, and by remedial action where required, including removal of infected animals and sanitization procedures. Vaccination may not be a desirable strategy in all circumstances, however, as an animal may be rendered partially immune and carry the virus without signs of disease. The second element is to examine the ability of the process to remove or inactivate viruses, including lipid-enveloped viruses such as HIV, Bovine Viral Diarrhoea Virus and Pseudorabies virus as well as non-enveloped viruses such as SV40 and Encephalomyocarditis virus.

J L Southern discussed bacterial contamination of antivenoms and the possibility that bacterial toxins might survive the production procedure, even where the antivenom was subjected to sterile filtration at the end of the process. It was generally accepted that bacterial contamination was clearly undesirable and relatively easily avoided. However, there was less whole-hearted acceptance of the possible hazards of viral contamination by some of the participants. In view of the conditions under which many of these products are likely to be produced, the regulatory view was that potential viral contamination constituted a real issue. The possibility of generating generic data on viral removal

or inactivation using harmonized processes was discussed and promising steps in the production process were identified. Whilst a generic approach is unlikely to be acceptable in general for a product license, each manufacturer being expected to validate his own process, it was considered that, in the case of antivenoms produced on a small scale by developing country manufacturers, such an approach could contribute greatly to the reassurance of the virological safety of the products.

10.4. Potency assays and preclinical assessment

An important initial step in the development of an antivenom is pre-clinical testing using both *in vivo* and *in vitro* methods to assess the neutralising potential of the antivenom against a range of different venom effects; for example, the ability of an antivenom to eliminate the lethal, haemorrhagic, defibrinogenating, necrotising and neurotoxic activities of a particular venom. G Laing described the routine methods recommended by WHO for the assessment of venom potency, LD₅₀ (*in vivo* lethality test in mice), Minimum Haemorrhagic Dose (MHD), an assessment of venom-induced haemorrhagic activity, Minimum Coagulant Dose, an assessment of the procoagulant effect of venom on fibrinogen and plasma (MCD-F, MCD-P), respectively, Minimum Necrotizing Dose (MND), an assessment of local necrotising effect and Minimum Defibrinogenating Dose (MDD), an assessment of the *in vivo* defibrinogenating effect of venom and the neutralisation of these activities by antivenom (Theakston and Reid, 1983).

A review of the potency assays used in various countries indicated that most accepted the standard murine lethality assay (LD₅₀ of venom and ED₅₀ of antivenom) as the yardstick for assessing antivenom preparations. Mice (5–6) of a particular weight per dose, challenged intravenously or occasionally intraperitoneally with venom preincubated with different doses of antivenom were used in this assay. There was considerable variation in the details of how the test was carried out, with different volumes injected, different numbers of LD₅₀ and different routes used for injecting venom/antivenom mixtures, weight range and strain of mice used. The additional tests for neutralisation of specific venom activities (e.g. haemorrhage, oedema, procoagulation, defibrinogenation, necrosis) were not carried out routinely. It was recommended that, when used, the procedures for *in vivo* assessment of antivenoms published in WHO Offset Publication No 58 (1981) (Warrell, 1999) should be followed closely. Local venom reference preparations and standard antivenom preparations are essential to standardize these assays and to allow batch to batch comparisons as well as comparisons between different laboratories. Ideally antivenom activities should be expressed in toxin neutralizing units based on national or regional standards.

There was general agreement that the currently used *in vivo* murine assays cause considerable suffering, are

expensive and show little or no correlation with envenoming and therapy in humans. Indeed, in recent years the use of the mouse lethality assay (LD₅₀ and ED₅₀) and the other *in vivo* tests have been questioned in the Europe and the USA, and the UK Home Office, for example, had requested that alternative assays be developed to replace such tests. The Workshop agreed that research efforts should be directed to developing alternative assays to replace rodent assays for the potency of antivenoms. One possible alternative is provided by fertile hens' eggs (Sells et al., 2001). A hen's egg is poured into a cling film hammock and for the antivenom efficacy test, five times the median lethal dose mixed with different amounts of the antivenom under test applied on a filter-paper disc over the vitelline vein and death or survival of the embryo is then recorded. It was reported that death or survival of embryos correlated well with the murine lethality and neutralising tests of venoms and antivenoms. The same test can be applied to measure haemorrhagic activity. In this case, the diameter of the haemorrhage-induced halo is measured. A good correlation between haemorrhagic activities measured using eggs and rodent assays was also reported. Advantages of such a method include the fact that in the UK no special licence is required from the Home Office, there is no pain involved as the tests are carried out before the development of neural arcs and the test is cheap to perform. However, the egg method cannot assess neurotoxic effects of venoms. Recent progress in developing molecular, non-animal tests for neurotoxins and antitoxins was described by Sesardic. The measurement of the activity of botulinum toxins and therapeutic antitoxins was used as an example where *in vitro* toxin neutralization assay provides for the detection of serotype specific antibodies. It was pointed out that although an enzyme immunoassay is useful for measuring antibody titres following immunisation, such titres do not necessarily reflect actual antibody potency in an animal model.

The use of pre-clinical testing, combined with clinical studies, including pharmacokinetic evaluation, can be used for satisfactorily testing antivenoms. The point was also made that the choice of immunoglobulin fragment should be made on a case-by-case basis and based both on its pharmacokinetic properties and also on the pharmacodynamic properties of the venom being neutralised. For example, a single dose of a Fab fragment antivenom with a short half life (8 h) may be suitable for the short pharmacodynamic action of a scorpion venom, but not for neutralisation of most snake venoms with an elimination half time about 30 h.

10.5. Regulatory issues

From a regulatory perspective, both the venom(s) used for antivenom production and the antivenom itself should be standardised and subjected to strict quality controls to ensure the consistent production of a safe, effective and stable product. B Golding emphasized the need to

characterise and standardize venoms both biochemically and functionally using validated quantitative assays. Disease-free animals should be used for immunisation although, as already discussed above, issues of testing horses and other large animals for potential pathogens, in particular viral pathogens, was an issue. Scrapie or other transmissible spongiform encephalopathy agents (TSEs) were a potential problem for sheep, and production animals should be from well-monitored herds from TSE-free countries. Except for viruses and prions, pathogens could be removed from hyperimmune plasma by filtering through a 0.22 µm filter. Again it was emphasized that there should also be a commitment to achieving viral safety of antivenoms. Low pH pepsin digestion, caprylic acid and pasteurization could inactivate some viruses and the potential for viral inactivation should be explored further. The resulting antivenom should also be well characterized and standardized for potency and specifications set for process contaminants. Routine testing for lot release should also be established and standardized again using validated assays. During improvement of production methods, an 'interim plan' might be allowed until the new methods had been fully established.

10.6. International standards and other reference materials

E Griffiths described the long-standing activity of WHO in developing and establishing International Standards and Reference Reagents for biological substances used in medicine or in the quality control of such substances. It was regretted that a group of candidate reference venom and antivenom preparations generated as a result of the 1979 meeting (WHO, 1981) had been lost during the transfer of reference material stocks from the WHO Laboratory for Biological Standards, Staten Serum Institute, Copenhagen to the WHO Laboratory for Biological Standards, National Institute of Biological Standards and Control, Potters Bar. The development of these materials had involved many years work by 13 different laboratories world-wide collecting and assessing a series of eight medically important venoms, as well as the collection and ampouling to WHO standards. However, none of these materials, produced in the 1980s, had ever been developed into an International Standard and had been rarely used. Only one International Standard for snake antivenom exists, the 1st International Standard for (*Naja* and *Hemachatus* species) antivenin, equine, and this had been established in 1964. In view of the improved understanding of venoms and the recognition that there could be considerable variation between venom preparations from the same snake species from different regions, it was clear that International Standards were not appropriate in this field and that in future only national or regional standards should be developed (see also above). Lei Diangling reported that China already had a bank of Chinese National Antivenom Standard preparations and S Jadhav reported the availability

of a series of standard venom preparations for use in India held by the National Control Laboratory (see above).

10.7. Clinical testing of antivenoms

Although conventional clinical studies using healthy volunteers (Phase 1) are not justified in the case of antivenoms, D A Warell emphasized the great importance of an initial dose-finding pilot study in envenomed patients (Table 3). This involves only a small number of patients treated under careful clinical supervision to provide evidence suggestive of efficacy and safety and to indicate the dose of antivenom required for a definitive randomised controlled trial. A definitive (Phase 3) study, discussed by DG Laloo, requires large patient numbers because of wide individual variability in the amount of venom introduced at the bite site and the wide time variations between the bite involving envenoming and the start of treatment. To avoid bias, the study must be blinded (or at least semi-blinded) and should involve a randomised comparison with the existing standard antivenom treatment (if one exists) or between two different doses of the test antivenom. The assessment criteria used in the study must be predefined and objective, such as reversal of clinical effects of envenoming, time to extinction of venom antigenaemia, time to restoration of coagulable blood, incidence of side-effects and even mortality. Laboratory procedures, such as the 20 min whole blood clotting test, provide excellent objective criteria but surrogate markers such as platelet count are less suitable as they may be affected by complement activation resulting from antivenom treatment. Early antivenom reactions should be recorded. Finally, patients must be observed carefully for long enough to reveal evidence of recurrent envenoming (seen particularly with short half-life Fab antivenoms) and to detect late serum sickness.

Problems with clinical testing include its expense, identification of the species responsible for envenoming, inaccessibility of areas where snake bite is common and

the need for large numbers of patients; clinical trials may therefore need to be multicentre studies which always involve additional complexity and expertise. Placebo-controlled trials are unlikely to be ethical unless there is genuine uncertainty about the risk-benefit of a newly introduced antivenom. The need for post-marketing surveillance (Phase 4) was also emphasised.

10.8. Major factors influencing antivenom costs and supply

The cost of antivenom production was discussed by J Landon. Application of new technologies to antivenom production almost always increase costs, making the exercise impracticable for the majority of developing countries. In developed regions of the world, such as Europe, Australia and USA, antivenom treatment costing thousands of pounds may be feasible, but in developing countries where snake bite takes its greatest toll, even treatment costing as little as £3 (US\$4.5) per vial may be neither affordable nor available. For this reason, it was suggested that ways of reducing costs and increasing antivenom availability should be explored, such as extending the shelf life of the product or, where appropriate, eliminating the need for freeze-drying. Antivenom use should also be rationalized by training doctors, nurses and dispensers and applying rigorous indications for antivenom use and dosage regimens.

The critical problems associated with supply and distribution of antivenoms in sub-Saharan Africa were reviewed by A Nasidi. In Africa, snakebites cause more than one thousand deaths each year and thousands of cases of permanent physical disability. *Echis* (saw-scaled viper), *Naja* (cobras) and *Bitis arietans* (puff adders) are the most important species. In the past, two large European companies produced polyspecific antivenoms suitable for Africa, supplementing the production of antivenoms from the South Africa Institute of Medical Research (SAIMR). Even so, insufficient antivenom was distributed to treat more than a fraction of African snakebite victims. In recent years, the situation has become more acute since one of the European manufacturers has stopped production entirely, whilst the other has only very recently resumed limited production after revising its production method; the SAIMR has been privatised. Other antivenom manufacturers have raised concerns about costs. As a result, the burden of human suffering from snakebite in Africa, including mortality, is increasing. The choice is now between imported, ineffective, nonspecific antivenoms manufactured using geographically inappropriate venoms, and unproven and frequently dangerous traditional treatments.

Established antivenom producers, especially those with unused production capacity, were encouraged to consider manufacturing antivenoms for use in Africa. However, it is essential that these antivenoms should be raised using venoms from African snakes.

Table 3
Antivenom: clinical testing

Pilot study

Test clinical effect in a relatively small group of patients
(Suggests efficacy/safety, indicates dose)

Definitive study

Randomised comparative

Blinded or semi-blinded *not* open label

Compare two different antivenoms or two different doses

Large degree of variation in pre-treatment severity
demands large numbers of patients

Objective assessment criteria

Consider paired-sequential design if case fatality is high

Ethical issues: 'double standards' 'sustainability'

11. General discussion, conclusions and recommendations

On the characteristics of antivenoms

- Experimental and clinical evidence indicates that generalisations about the preferred type of antibody or antibody fragment of antivenoms should be avoided. Instead, for each particular country/region and venom, preclinical and clinical studies should determine which is the most appropriate type of antivenom (either whole IgG, F(ab')₂ or Fab). The pharmacokinetic and pharmacodynamic characteristics of each antivenom–venom system should be taken into consideration in making such a decision.
- Whatever kind of antivenom is produced (whole IgG, F(ab')₂ or Fab), it is essential to assure its safety by using fractionation protocols that will yield an appropriately purified product. The absence of protein aggregates and of contaminating, non-IgG proteins, should be ensured by key quality control procedures. Production protocols might be based on the following procedures, which can be adapted or combined to give the best results
 - pepsin digestion and ammonium sulphate precipitation, to obtain F(ab')₂,
 - pepsin digestion and caprylic acid precipitation, to obtain F(ab')₂,
 - caprylic acid precipitation, to obtain whole IgG,
 - ammonium sulphate precipitation, to obtain whole IgG,
 - papain digestion to obtain Fab.

Additional steps, such as ion-exchange chromatography to remove Fc and affinity chromatography to concentrate venom-specific fragments, could be added to improve quality and safety.

- The decision to produce polyspecific or monospecific antivenoms should be made on a case-by-case basis and based on the feasibility of correct clinical diagnosis by physicians. Whenever a rapid and correct clinical diagnosis cannot be made to identify the snake species involved in envenoming, polyspecific antivenoms should be used. On the other hand, monospecific antivenoms are recommended whenever the snake can be examined and identified or when the diagnosis can be made clinically or by the use of rapid laboratory tests. In each country, antivenom should be distributed according to the known distribution of venomous species.
- If an adequate cold chain is in place, antivenoms could be prepared in the liquid form, since this reduces production costs and avoids potential adverse physico-chemical alterations to the product sometimes incurred by lyophilization. On the other hand, if the integrity of the cold chain cannot be guaranteed, antivenoms

should be lyophilized to maintain stability. The proportion of liquid and lyophilized antivenom produced by a manufacturer should be based on knowledge of the conditions of transport and storage to which the product will be subjected.

On antivenom quality and standards

- National or regional, as opposed to International standards of venoms and antivenoms are required. Venoms should be collected from the most important national or regional species from a medical point of view. These venom standards should be used both by manufacturers and National Control Laboratories in the assessment of antivenom potency. The National Institute for Biological Standards and Control, UK, offered to assist in the preparation of these reference materials on a case by case basis but it was likely that they would be held and distributed from regional or national centres. Such venom standards should have the following characteristics
 - They should be pooled venoms from more than 50 different snakes of the same species and of various ages (sizes), collected in different geographical regions representing the distribution of that species in the country/region.
 - Venoms should be lyophilized, distributed in aliquots in sealed vessels and stored at –20 °C. Reference laboratories in each country should apply accepted basic methodologies to characterize the venoms. The median lethal dose (LD₅₀) of each venom should be determined, in addition to the most relevant pathophysiological activities of each venom (Theakston and Reid, 1983).
- Each country should establish regulatory requirements for antivenoms on the basis of WHO recommendations. WHO recommendations for antivenoms need to be updated in line with developments in biologicals over the past few years. Despite the revolution in molecular biology and biotechnology, production of antivenoms will, for the foreseeable future, be based on the immunisation of large animals, mainly horses. Therefore, major efforts should be undertaken to assure the potency and safety of these products. The quality control of antivenoms should be improved and measures needed to ensure the viral safety of the products strengthened. This should involve the development of new laboratory tests as well as improving the currently available assay systems. Viral validation studies involving spiking studies with lipid and non-lipid enveloped viruses are recommended to evaluate the ability of the production process to remove or inactivate any adventitious viral agents and thus contribute to viral safety. Quality controls should also include measurement of the ability of the antivenom to neutralize the lethal activity and other clinically relevant pharmacological activities of

the corresponding venoms. Currently, different protocols for potency assessment are being used in different countries. Attempts should be made to develop uniform standard protocols at least within the region and national or regional reference materials used to calibrate assays. Activities should be expressed in units related to the national or regional standard. It was suggested that vials could be filled with enough antivenom to treat a 'normal case of envenoming' and the neutralising capacity of one vial be expressed in toxin neutralizing units based on the regional/national standard, rather than by volume or weight.

- Laboratory assessments of antivenom potency should be used as a guide to the use of an antivenom in a particular country or region. However, the limitations of these data in establishing dosage regimes for the treatment of human patients must be clearly recognised. Clinical studies should be performed to determine the average initial dose and incidence of reactions and all antivenom preparations should fulfil national regulatory requirements.

Future developments

- Research into the development of alternative methods to animal testing in the evaluation of venoms and antivenoms should be encouraged. When live animals are indispensable, anaesthesia or analgesia should be used whenever possible.
- Well-designed, randomised controlled clinical trials should be performed in different countries to provide reliable data on the clinical features of envenoming and

the therapeutic response to antivenoms. This neglected field should be given a much higher priority in the future. Only very few randomised controlled trials have been carried out on antivenoms in the past.

- The crisis in antivenom supply in various regions of the world demands an international effort to facilitate the transfer of technology by established antivenom manufacturers to other countries or regions, or the production of antivenom in such established centers to be used in regions lacking adequate supply. This international effort should be promoted by the WHO, governments and manufacturers around the world.
- The epidemiology of bites and stings by venomous animals (especially those by snakes and scorpions) has been greatly neglected. As a result it is usually impossible to quote accurate information as to the morbidity and mortality caused by venomous animals throughout the world. The following approaches should be followed to resolve this problem in the future:
 - WHO should arrange to collect more information on the global impact of the problem.
 - National statistics should include information on the extent of the problem by organizing well-designed population studies.

12. Summary recommendations

Table 4 summarizes the main recommendations of the Workshop and below are indicated some of the proposed actions.

Table 4
Summary recommendations

| | |
|---------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Production | Demonstrate proof of principle of a generic virus inactivation and removal protocol, including feasibility of scale-up (viral validation), and critically re-examine traditional production processes in a pilot study at Instituto Butantan, Brazil |
| Supply | Publicize crisis in Africa—recruit outside manufacturers to produce appropriate antivenoms for Africa |
| Pre-clinical and Clinical testing | Promote and develop clinically relevant new quality control assays; encourage randomized controlled clinical trials |
| Information | Encourage statutory reporting using a precise ICD classifier; encourage population-based epidemiological studies |
| Training | Generate national guidelines and teaching materials and arrange training courses in endemic countries |
| Reference materials and WHO recommendations | Develop national/regional reference venoms and antivenoms |
| Advocacy/funding | Update WHO requirements for antivenoms and for immune sera of animal origin. These should include appropriate guidance on the level of testing expected to ensure exclusion of potential virus contaminants, including viral validation studies Explore WHO, European Commission, Gates Foundation and other potential donors, including national donors |

12.1. Reassessment of production protocols

The Instituto Butantan in São Paulo, Brazil offered to undertake pilot studies to

- Investigate the design of a possible standard (generic) method for antivenom production which included validated viral removal or inactivation steps and which could then be used by other antivenom manufacturers. Such a process might include $(\text{NH}_4)_2\text{SO}_4$ precipitation and caprylic acid purification of the IgG molecule, the use of chromatography, heat sterilisation (pasteurization) and pH control during manufacture.
- Evaluate proposed 'generic' production processes, or critical production steps, for their ability to remove or inactivate potential virus contaminants. Viral validation studies should be undertaken using both lipid-enveloped and non-lipid enveloped model viruses.
- Following proof of principle studies, investigate scale-up of the proposed production process and confirm appropriate viral removal or inactivation at scaled-up level.
- Once an acceptable production process has been developed and validated for virus removal/inactivation, provide appropriate training in these methodologies to other manufacturers.
- Develop illustrated WHO guidelines providing details of the recommended step-by-step production and quality control procedures.
- Standardise in vivo and/or in vitro methods for quantitative measurement of potency.

The Instituto Butantan offered to provide 50% of the total cost of the project including laboratory and small scale production facilities and housing for future trainees. The additional 50% funding would need to be found from outside sources. It was proposed that an international steering committee be established to monitor the project.

12.2. Urgent need for antivenom for Africa

Increased efforts should be made to improve the supply of antivenoms through inter-regional collaboration. Some of the manufacturers represented at the Workshop with excess plant availability expressed their willingness to produce antivenom for use in Africa. These included the Instituto Nacional de Salud, Bogotá, Colombia; Instituto Butantan, São Paulo, Brazil; Instituto Clodomiro Picado, San José, Costa Rica and Instituto Bioclon, Mexico City, Mexico. Appropriate African venoms would need to be provided for the immunization of animals. These might be sourced from the Liverpool School of Tropical Medicine, UK. Some funding would be required for this work.

12.3. Training

There is much ignorance about treating envenomed patients in many countries where bites and stings are common. WHO urgently needs to support the production of regional Guidelines for treatment in countries (or continents) where there is a real problem. This has already been achieved for Southeast Asia (Warrell, 1999) but the Guidelines were not widely distributed. Guidelines for Africa are an urgent priority.

12.4. International standards and WHO requirements

National, as opposed to International, reference venoms and standard antivenoms are required. The only existing International Standard for an antivenom, the First International Standard for snake antivenin (*Naja* and *Hemachatus* species) should be disestablished in order not to cause confusion in the field. The WHO Requirements for Snake Antivenins (1971) and the WHO Requirements for Immune Sera of Animal Origin (1969) are very old and need considerable updating to reflect current developments in the quality control of biologicals.

Appendix A

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Postscript

Since this workshop was held in 2001, there has been progress towards the implementation of two of its recommendations. Two national antivenom producers in Latin America have raised new polyspecific antivenoms against the venoms of medically important snake species of Sub-Saharan Africa. These await clinical testing (Laing et al., 2003).

Plans are advanced for the development of a model antivenom production pilot study at the Instituto Butantan, São Paulo, Brazil.

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