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Full Length Research Paper

Polyvalent horse F(Ab`)$_2$ snake antivenom: Development of process to produce polyvalent horse F(Ab`)$_2$ antibodies anti-african snake venom

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A method to obtain polyvalent anti-*Bitis* and polyvalent-anti-*Naja* antibodies was developed by immunizing horses with *B. arietans*, *B. nasicornis*, *B. rhinoceros*, *N. melanoleuca* and *N. mossambica* crude venoms. Antibody production was followed by the ELISA method during the immunization procedure. Once the desired anti-venom antibody titers were attained, horses were bled and the immunoglobulins were separated from the sera by $(NH_4)_2SO_4$ precipitation, cleaved with pepsin and filtered through a 30 kDa ultrafiltration membrane. F(ab´)$_2$ fragments were further purified by Q-Fast Flow chromatography, concentrated by molecular ultrafiltration and sterilized by filtration through 0.22 µm membranes. The resulting F(ab´)$_2$ preparations were rich in intact L and in pieces of H IgG(T) chains, as demonstrated by electrophoresis and Western blot and exhibited high antibody titers, as assayed by the ELISA method. In addition, the preparations possess a significant capacity to neutralize the lethality of venoms, as estimated by ED_{50} determination in mouse assay and are free of toxic substances, pyrogen and bacterial or fungal contaminations.

Key words: African snake venoms, envenoming, antivenoms, immunoglobulin IgG(T), F(ab`)$_2$ fragments, immunotherapy.

INTRODUCTION

A workshop to discuss progress in the standardization and control of anti-venoms, 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; (Theakston et al., 2003). Among the several important considerations raised by the experts invited from academic institutions, anti-venom manufacturers and national regulatory authorities from 21 countries, was concern regarding the crisis, at that time, of antivenom production and supply in sub-Saharan Africa. Unfortunately, snake bites and scorpion stings continue to be an important public health problem and the crisis in antivenom production persists and antivenom remains scarce in Mozambique. We decided to address this issue, first by adapting current procedures to produce anti-African snake venoms. Since *B. arietans*, *B. nasicornis*, *B. rhinoceros*, *N. melanoleuca* and *N. mossambica* are the main causes of snake bites in Mozambique (Broadley,

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Abbreviations: ELISA, Enzyme-linked immunosorbent assay; **SDS-PAGE,** sodium dodecylsulfate - polyacrylamide gel electrophoresis; **ED_{50}**, half maximum effective dose; **LD_{50}**, half maximum lethal dose.

1968; U.S. Navy, 1991; Manacas, 1981/1982), their venoms were used to immunize horses. We proposed, with a support grant from the Brazilian Research Foundation (CNPq), to develop at the "Instituto Butantan", Sao Paulo, Brazil, two polyvalent anti-venoms, anti-*Bitis* (*B. arietans*, *B. nasicornis*, *B. rhinoceros*) and anti-*Naja* (*N. melanoleuca* and *N. mossambica*) antivenoms. Both antivenoms were obtained from hyperimmunized horses; the immunoglobulins were obtained by ammonium sulfate precipitation and the IgG(T) fraction was submitted to cleavage with pepsin and the resulting $F(ab')_2$ fragments were further purified, concentrated, analyzed with respect to the presence of antivenom specific antibodies by ELISA method. Their efficacy to neutralize the venom lethality was evaluated in *in vitro* - *in vivo* method using mice as animal test and the their potency expressed in terms of ED_{50} as recommended by World Health Organization (1981). Both antivenom preparations were submitted to routine quality control procedures. The results presented herein show that specific, potent polyvalent anti-*Bitis* and anti-*Naja* antivenoms could be obtained on a large scale by using traditional methodology with modifications imposed by the venom diversity. Before being supplied for the treatment of snake bites in Mozambique, these anti-venoms will be submitted to clinical trials under the supervision of physicians the "Hospital Vital Brazil-Instituto Butantan", Sao Paulo, Brazil.

MATERIALS AND METHODS

Animals

Adult horses (400 - 450 kg body mass) maintained in a special animal house at the Sao Joaquim Farm, Instituto Butantan, Sao Paulo, Brazil, were used to produce IgG(T) antivenoms. The horses were vaccinated against the commonest equine infectious diseases. Swiss outbred mice (18-20 g) were provided by the Instituto Butantan Animal Facilities. These male and female mice were included in the protocols routinely used to determine the lethality (LD_{50}) of the venoms and the neutralizing potency (ED_{50}) of the antivenoms. All animals used in this study were maintained and treated under strict ethical conditions, according to the "International Animal Welfare Recommendations (Remfry, 1987) and by Committee Members, International Society on Toxinology (1992).

Antigen preparation and analysis

The *B. arietans*, *B. nasicornis*, B. rhinoceros, *N. melanoleuca* and *N. mossambica* venoms were purchased from Venom Supplies Pty Ltd (59 Murray Street, Tanunda, Australia).

Immunization and bleeding

Anti-African snake polyclonal antibodies were obtained by immunizing horses that were randomly distributed into three groups : Group # 1- (Horses No: 476,494, 495, 496, 497, 498 and 499) - *B. arietans*. Group #2 - (Horses No: 508, 509, 510, 511, 512 and 513) - *B. nasicornis* and *B. rhinoceros*. Group # 3- (Horses No: 501, 502, 503, 504, 505, 506, 507) - *N. melanoleuca* and *N. mossambica*. Horses were s.c. injected in the back, successively two weeks apart with 2.0 ml of incomplete MMT20, or complete MMT20 adjuvant (Marcol-Montanide-Tween -20 adjuvant, Brazil Ltda-Div. SEPPIC/ France/Brazil) mixtures, prepared as described by Herbert (1978) or PBS (phosphate saline, pH 7.5) containing 3.0-4.0 mg of venoms. Two weeks after the last immunization, when the antibodies against the venoms attained an appropriate value, the horses were bled and a volume of blood corresponding to one-twelfth of their body weight was collected in sterile plastic bags containing anticoagulant. Plasma and cells were separated by gravidity sedimentation and the cells were re-infused into the corresponding horse through the jugular vein. Plasmas from the same horse group were pooled and stored at 4°C. Before immunization, blood samples were withdrawn by jugular vein puncture and sera stored at -20°C to be used as negative controls in the anti-venom antibody determinations. Three months after bleeding, boosters with similar doses of venoms in PBS were given and blood was collected and processed as described. This latter procedure will be repeated for the next two years.

$f(ab')_2$ preparation

Serum rich in $F(ab')_2$ fragments was produced essentially as described by Towbin et al (1979) . A saturated solution of $(NH_4)_2SO_4$ was added to the plasma pool obtained from the horses immunized with whole African snake venoms until the 29% concentration was attained. The precipitates containing mostly immunoglobulins were separated by centrifugation, dissolved in distilled water. Pepsin (1.0 g / 80 g protein) was added, the pH was adjusted to 3.0 - 3.2 with 40% citric acid and digestion allowed to occur at room temperature for 40 min. The pH was adjusted to 4.5 - 4.6 also with 40% citric acid and the fibrinogen precipitated by adding 0.01M caprilic acid plus 11.3% $(NH_4)_2SO_4$ and incubated at 55°C for 1h. A saturated solution of $(NH_4)_2SO_4$ was added until attaining a 17.6% final concentration. The mixture was centrifuged and precipitates discarded. The supernatants were passed on a 30kDa exclusion membrane to remove $(NH_4)_2SO_4$, the resultant $F(ab')_2$ - rich supernatants were concentrated by molecular exclusion on a Q - Sepharose Fast Flow chromatography and sterilized by passing through a 0.22 μm filter membrane. The preparations were analyzed for the presence of $F(ab')_2$ fragments by comparing their relative molecular weight with uncleaved IgG(T) on sodium dodecylsulfate - polyacrylamide gel electrophoresis (SDS-PAGE) or Western blot analysis, for the presence of specific antibodies against snake venom antigens using the enzyme-linked immunosorbent assay (ELISA) and for their capacity to neutralize venom lethality. Before release to treat envenomed victims, $F(ab')_2$ fragments are submitted to a quality control in order to certify absence of bacterial contamination, bacterial lipopolysaccharide and toxic substances. The final products are adjusted to contain the desired neutralizing antibody titer in less than 10 mg of protein/ml and labeled as *Bitis* - polyvalent anti-venom or *Naja* - polyvalent antivenom. Each ampoule contains 40 ml of anti-venom. This anti-venom was prepared according to the recommendations of the World Health Organization (1981).

Characterization of horse antivenom IgG antibodies (SDS-PAGE and Western blot analyses)

Western blot analysis was carried out, according to the previously described method. Laemmili (1970). Crude *B. arietans*, *B. nasicornis*, *B. rhinoceros*, *N. melanoleuca* and *N. mossambica*-snake venoms (2mg/ml) were treated with SDS-PAGE sample buffer under reducing

conditions and resolved in 15% polyacrylamide gel before electro-blotting onto PVDF (Immobilon P), after treating membranes with methanol. The membrane, after treating again with methanol at 37°C for 15 min, was blocked with blocking buffer (0.01 M PBS, pH 7.4, with 5% low fat milk) at room temperature for 30 min. After washing with wash buffer (blocking buffer containing 0.1% Tween-20), the membrane was incubated with 5.0 mg of rabbit anti-horse IgG (whole molecule) diluted in the same blocking buffer and incubated for 1.0 h at room temperature on a horizontal shaker. The membrane was washed with blocking buffer and incubated with 1: 2000 dilution of peroxidase-conjugated goat anti-rabbit IgG for 1.0 h at room temperature. After washing, the membrane was placed in peroxidase chromogenic substrate solution (0.1 M citric acid plus 0.2 M sodium di-phosphate, 5.0 mg of DAB (0.05%) and 3 µl of H_2O_2). The reaction was terminated by washing with distilled water.

Detection and evaluation of antibody activity

Detection

Production of anti-venom antibodies was initially monitored along the immunization schedule by a double-immunodiffusion method described by Outchterloni and Nilson (1986). Briefly, 10 µl of undiluted serum samples were deposited in the central well of a 0.8% agarose gel, while 10 µl of different venom concentrations (10 -50µg) were deposited in peripheral wells and the gels allowed to stand overnight in a refrigerator. Developing of precipitin lines by serum indicated the presence of antivenom antibodies.

ELISA

Polystyrene ELISA plate (96 wells) was coated with 1.0 µg of native snake venom in 50 µl coating buffer (0.1 M carbonate bi-carbonate, pH 9.6) kept overnight at 4°C. The wells were washed once with PBS buffer containing 0.05% Tween - 20. The wells were next blocked for 1.0 h at room temperature with 150 µl PBS buffer plus 1.0 % gelatin. The wells were again washed thrice with 300 µl washing buffer. Serial dilutions of horse IgG or F(ab`)$_2$ preparations (1: 1000 to 320000) in PBS buffer plus 1.0% gelatin buffer containing 0.05% Tween - 20 were prepared and 50 µl of each were added to individual wells and the plates were incubated at 37°C for 45 min. The wells were washed five times with the same washing buffer. Rabbit peroxidase-conjugated anti-horse IgG (whole molecule), diluted (1: 800) in PBS buffer plus 1.0 % gelatin buffer containing 0.05% Tween - 20 (50 µl), was added to each well. The plates were incubated for 45 min at 37°C. After five washes with the washing buffer, 50 µl of substrate buffer (0.1 M citric acid, plus 0.2 M sodium di- phosphate, 5.0 ml H_2O, 5.0 mg OPD, 5 µl of H_2O_2) were added and incubated at room temperature for 10 - 15 min. The reaction was terminated with 50 µl of 3N sulfuric acid. Absorbance was recorded at 492 nm using an ELISA plate reader. IgG from horses collected before immunization was always used as a negative control. Wells free of venom were used as blanks. The IgG dilution, giving an optical density of close to 0.2, was used to calculate the U-ELISA per milliliter of undiluted IgG solution. One U-ELISA is defined as the smallest amount of antibody giving an O.D. of 0.2 under conditions of ELISA assay as described by Almeida et al., (2008).

Neutralization of venom lethality by IgY antivenom antibodies

The neutralizing efficacy of horse IgG or F(ab`)$_2$ antivenom antibodies, produced along the immunization procedure, was evaluated by incubating 5 LD_{50} of Bitis or Naja venoms for 1h at 37°C, with different amounts of horse antivenom antibodies. The mixtures were intra-peritoneally (i.p) injected into groups of eight Swiss mice (18-20 g). The deaths were recorded after 72 h and the death/survival ratio recorded (World Health Organization, 1981). The ability of antibodies to neutralize the lethal activity of venoms, expressed in terms of ED_{50}, is the 50% end point of neutralization, as calculated by the Spearman -Karber method Finney (1992).

Quality control of F(ab`)2 anti-venoms Both Polyvalent Bitis-antivenom and Polyvalent Naja-antivenom were standardized, according to World Health Organization (1981). Quality control tests for the presence of bacteria, fungi, toxic substances and pyrogen contami-nations were also performed.

RESULTS

Standardized and certified sterile batches of polyvalent anti-*Bitis* or anti-*Naja* anti-venoms were produced according to the process and flow diagram depicted in Figure 1.

Step # 01: Standardization of the antigen mixtures

SDS-PAGE analysis of venoms. *B. arietans*, *B. nasicornis*, *B. rhinocerus*, *N. melanoleuca* and *N. mossambica* snake venoms were submitted to 15% SDS - PAGE under non-reducing conditions and were resolved into venom components with apparent molecular masses ranging from 14 to 97 kDa, nine protein bands in *B. arietans*; (well # 2), six in *B. nasicornis* (well # 3), six in *B. rhinoceros* (well # 4), five in *N. melanoleuca* (well # 6) and seven in *N. mossambica* (well # 7)). A single protein band with apparent molecular mass ranging from 24 - 36 kDa was observed only for the *B. arietans* venom.

LD_{50} determination of the venoms. LD_{50} values found for the five Africa snakes venoms used in this study were: *B. arietans*, 0.96µg/mouse; *B. nasicornis*, 123.67µg/ mouse; B. rhinoceros, 95.28 µg/mouse *N. melanoleuca*, 13.41 µg/mouse and *N. mossambica*, 22.40 µg/mouse.

Step # 02: Antibody production

With some small individual variations among the horses immunized with African snake venoms, anti-venom antibodies appear early in the plasmas even during the first immunization run, as revealed by a double immune diffusion method (data not shown) and quantified by ELISA. Figure 2 shows that the antibody titers expressed by the number of U-ELISA / ml of serum, varies among the individual horses even when immunized with the same venom or mixtures of venoms.

Step # 03: Refining and standardization of anti-venom F(ab´)$_2$ fragments

Preparation and titration of anti-venom F(ab´)$_2$ fragments.

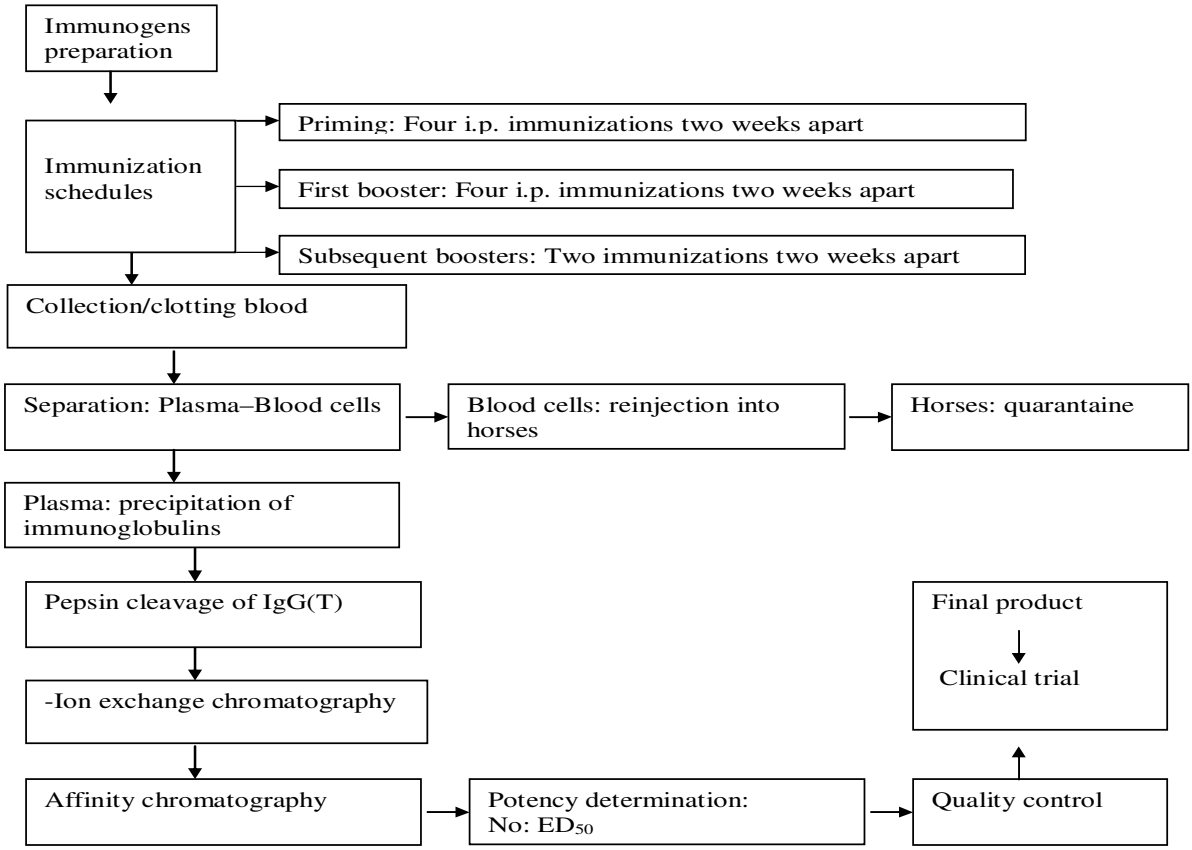

Figure 1. Process and flow diagram: production of polyvalent Anti-*Bitis* or Anti-*Naja* venoms. F(ab´)$_2$ preparation: process and flow diagram.

Plasma from horses immunized with African snake venoms were digested with pepsin and the correspondent F(ab´)$_2$ fragments were prepared according to the scheme (Figure 1). The presence of antibodies against *Bitis* or *Naja* venom antigens was tested by Western blot and by ELISA assays. Western blot analysis indicates that horse antibodies recognize three protein bands in *Bitis* venoms (Figure 3A) only one in *Naja*, venoms (Figure 3B). On ELISA, the antibody titers, were higher in F(ab´)$_2$ fragments as compared with the original IgG preparations (Figure 4). When the antibodies titers were expressed in terms of U-ELISA /ml of each preparations, F(ab´)$_2$ fragments were over 9.0 x 10^5 of U -ELISA, as compared with less than 3.0 x 10^5 for IgG original preparation (Figure 5). These preparations also contain specific antibodies capable of neutralizing the lethality induced by the venoms, as indicated by the ED_{50} / ml values (Table 1). Western blot analysis demonstrates that IgG (T) of both Polyvalent *Bitis*-anti-venom and Polyvalent *Naja*-anti-venom upon cleavage by pepsin under conditions allowing cleavage of the Fc portion of the IgG(T) molecule (Nisonoff, 1973) was converted to F(ab´)$_2$ fragments (Figure 6).

Step 04: Quality control

The final F(ab´)$_2$ preparations of both antivenoms were vandardized according to the WHO recommendations. Quality control tests (bacterial and fungal sterility, abnormal toxicity safety), a pyrogen test, as well as physic-chemical controls (pH 6.7, total protein content (10 -12 mg), total nitrogen content) were carried out. One ml of these anti-venoms was adjusted in order to equal the $5DL_{50}$ of venom. Forty milliliters of antivenoms were packed in 50 ml sterile labeled flasks. Labels included basic information on the packed product regarding the nature and safe use of the anti-venoms.

DISCUSSION

Snake venom poisoning is a disease process that has been successfully treated with polyclonal antibodies. This therapeutic tool has been used since pioneering studies by Sewall (1887) and Calmette (1894). Conventional anti-venoms are prepared by immunizing large animals, usually horse, with an individual venom or a range of

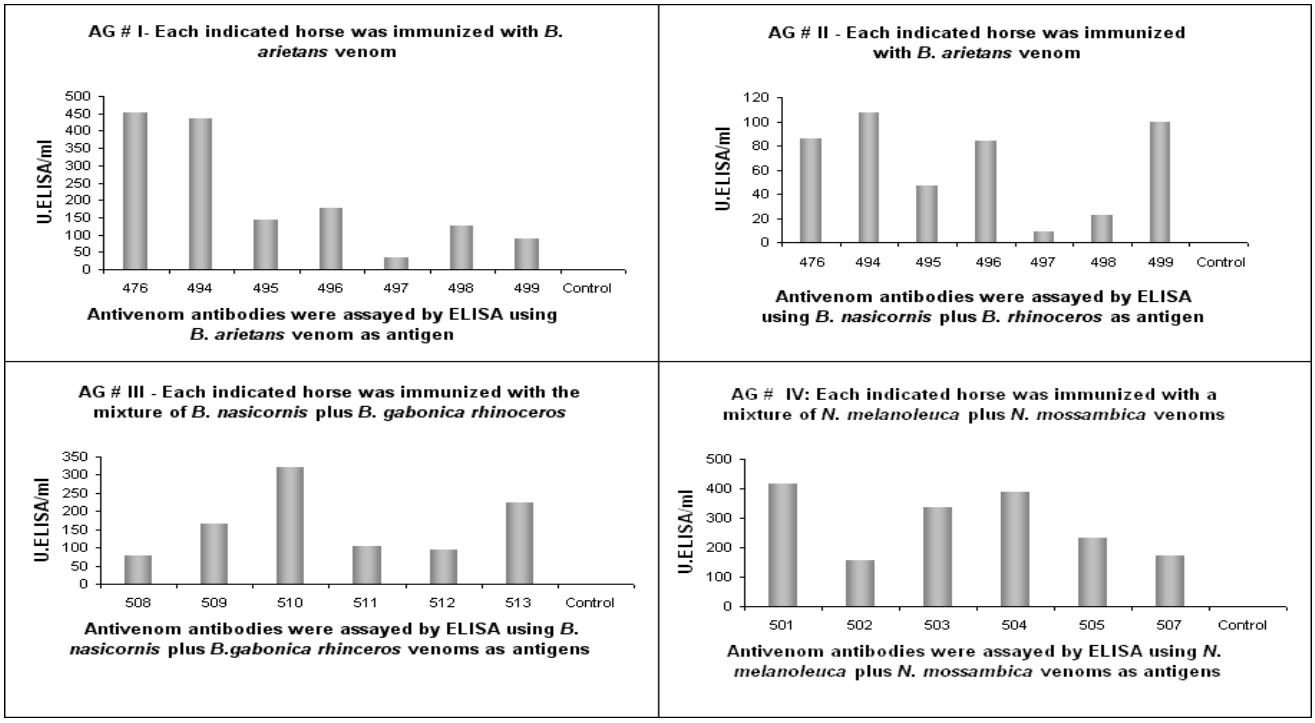

Figure 2. Detection of anti-venoms antibody production in immunized horses by ELISA. Individual horse serum diluted 1:2000 were added on 96-well pre-coated with 1.0 Polyvalent Horse F(ab`)$_2$ snake antivenom µg of snake venom as indicated and results expressed as U-ELISA \ ml of undiluted serum. Data are presented as mean of triplicates. The assays were performed twice and data shown are derived from a single assay.

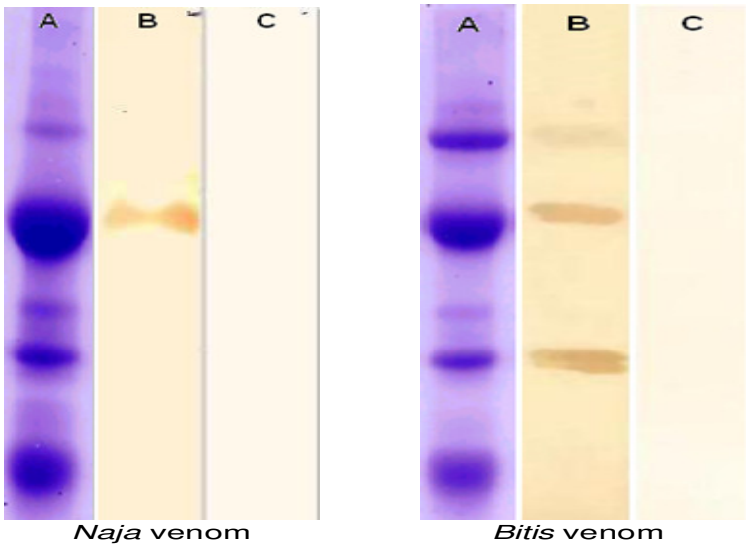

Figure 3. Recognition of some African snake venom components by horse antivenoms (first immunization). Venom components were separated on 15% SDS-PAGE and either stained with Coomassie Blue, or transferred to PVDF (immobilon P membrane). Western blot analysis was performed using predetermined antivenom dilutions as the first antibodies and a peroxidase-conjugated goat anti-horse-IgG as the second antibodies. *Bitis* venom (right); B. *Naja* (left) venom. A) SDS-PAGE; B) Western blot (IgG from immunized Horses); C, Western blot (IgG from non-immunized horses).

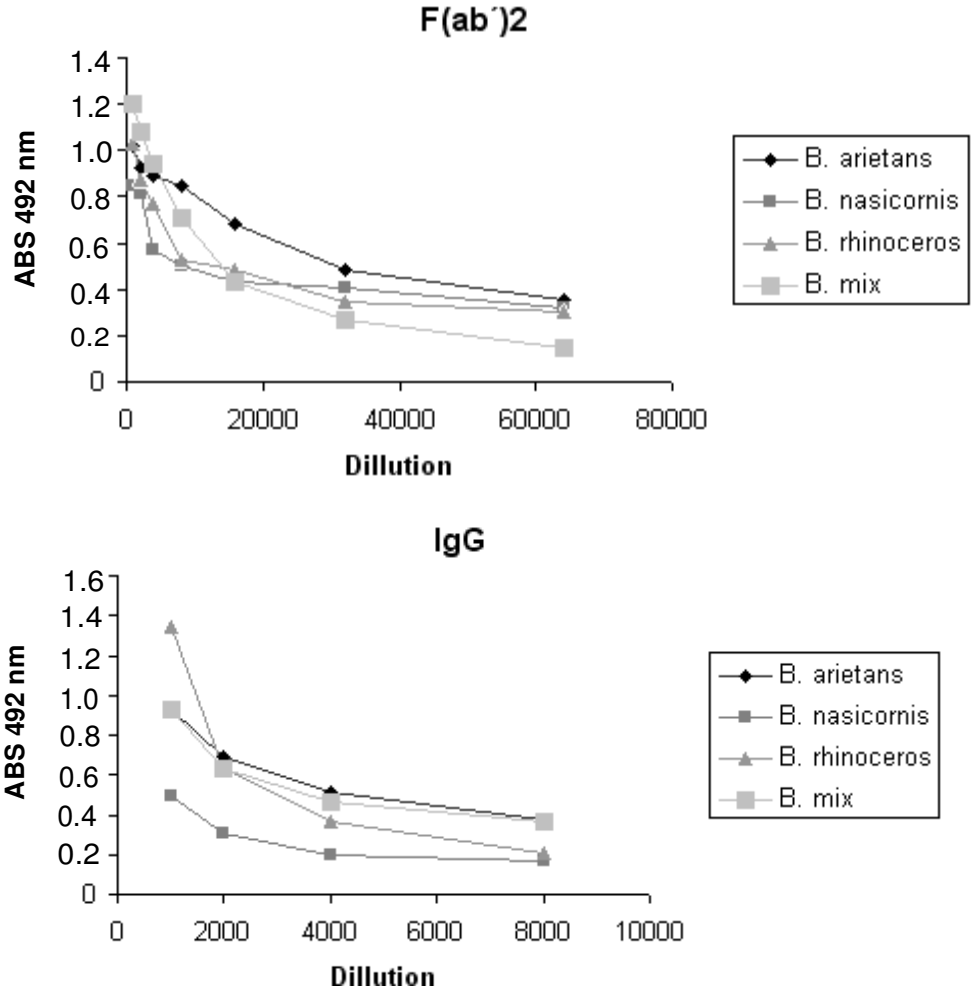

Figure 4. Anti-venom antibodies titers in pooled horse sera and in corresponding F(ab´)$_2$ preparations. Various dilutions of the anti-venom preparations were added on 96-well pre-coated with 1.0 µg of snake venom as indicated and results expressed either as absorbance at 492 nm. Data are presented as mean of triplicates. The assays were performed twice and data shown are derived from a single assay.

different venoms obtained from several snakes in order to eliminate intra-specific variation discussed by Theakston (1996). According to conclusions reached by the participants of the previously-referred-to meeting to discuss progress in the standardization and control of anti-venoms, organized by the Quality Assurance and Safety of Biologicals Unit of WHO, which was held at the National Institute for Biological Standards and Control, Potters Bar, England, 7-9 February 2001 (Theakston et al., 2003), anti-venom immunotherapy mostly uses anti-bodies that are as specific as possible in order to guarantee safety and efficacy. In order to meet these requirements, polyvalent anti-*Bitis* or anti-*Naja* anti-venoms were developed using, as immunogens, snake venom from the snakes considered to be the most dangerous in some Mozambique African regions. Healthy horses were employed as anti-venom producers and were submitted to long immunization schedules to favor production of more efficient and high-affinity antibodies.

Members of the *Bitis* and *Naja* genera are the commonest inflictors of snake bites and envenoming in the African Portuguese-speaking countries of Guine, S. Tome, Angola and Mozambique as described by Broadley (1968), Manacas (1981, 1982) and Warrell et al. (1975). According to unofficial registers, most victims are from rural areas and savanna. Bites are commonly inflicted on on the feet or ankles of farmers, plantation workers, herdsmen and hunters, who tread on snakes in the undergrowth or on bush paths at night (Warrell et al., 1975; Warrell, 1996). In these countries, as well as in

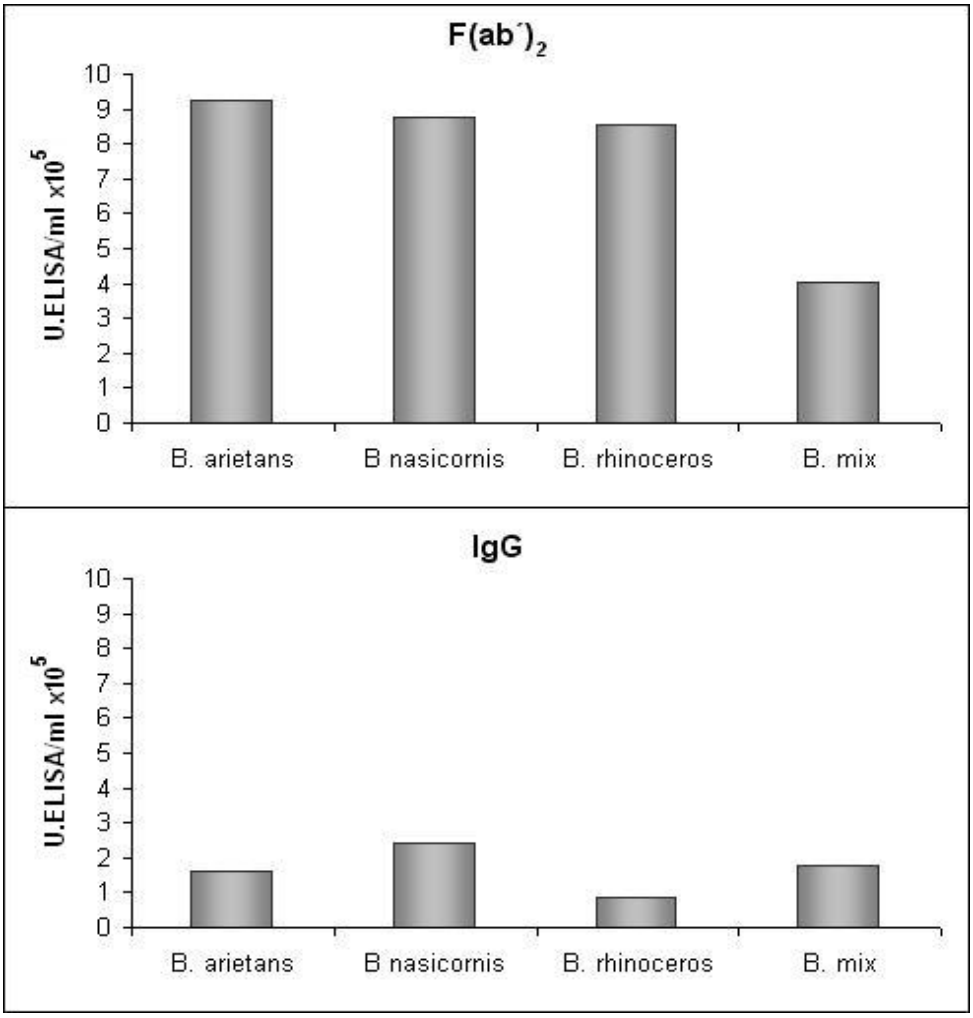

Figure 5. Quantification of anti-venom antibodies in final $F(ab')_2$ preparations by ELISA. Various dilutions of the anti-venom preparations were added on 96-well pre-coated with 20 µg of snake venom as indicated and absorbance at determined at 492 nm. The results were expressed as the number of U – ELISA / ml of preparations. Data are presented as mean of triplicates. The assays were performed twice and data shown are derived from a single assay.

other African countries, mortality due to snake bites is by far underestimated (Warrell and Arnett, 1976). *B. arietans* is responsible for more fatalities than any other African snake. Some factors, such as its wide distribution, common occurrence, large size, potent venom that is produced in large amounts, long fangs that inject it deeply, their reliance on camouflage which makes these snakes reluctant to flee, their habit of basking by footpaths and sitting quietly when approached and their willingness to bite explain the importance of this snake in snake bite envenoming in the African region as discussed by Spawls and Branch (1995), Mallow et al.(2003) and Spaws et al. (2004). The venom is one of the most toxic of any viper (Spawls and Branch, 1995). Its LD_{50} values in mice vary (0.4-2.0 mg/kg i.v., 0.9-3.7 mg/kg, i.p. 4.4-7.7 mg/kg, s.c. (Spawls and Branch, 1995). The average specimen may have enough venom to kill 4 to 5 adult victims (Brown, 1973). *B. arietans* venom is rich in various enzymes, mainly proteinases. Swelling and bruising may develop at the site of the bite within a few minutes or hour. Swelling spreads rapidly and sometimes involves the whole of the bitten limb and trunk. Regional lymphatic vessel and lymph node involvement is common. Blistering and necrosis may develop at the site of the bite. Haemostatic abnormalities, clinically manifested by persistent bleeding from the fang puncture wounds and other partially healed wounds, suggest coagulopathy. The blood clot alterations are sometimes reflected systemically as epistaxis, haematemesis, cutaneous ecchymoses, petechiae or discoid ecchymoses, haemoptisis and

Table 1. Polyvalent Anti-*Bitis* and Anti-*Naja* F(ab´)2 preparations: Final products.

F(ab')2	ED_{50}	Statistical Analysis*
Anti-*Naja* spp. *(melanoleuca* and *mossambica)*	66.5	Potency = 1.346337 mg/ml Precision factor = 0.2257607 Standard deviation = 8.349235E-02 Superior confidence limit (95%) = 80.73936 Inferior confidence limit (95%) = 54.96681
Anti-*Bitis* spp. *(gabonica* and *nasicornis)*	107.1221	Potency = 4.617909 mg/ml Precision factor = 0.3511864 Standard deviation = 0.1193328 Superior confidence limit (95%) = 156.0412 Inferior confidence limit (95%) = 90.06866
Anti-*Bitis arietans*	1.148631	Potency = 3.343111 mg/ml Precision factor = 14.2679 Standard deviation = 0.1439631 Superior confidence limit (95%) = 0.4204936 Inferior confidence limit (95%) = 0.2166872

*Death were recorded over the following 48 h and the ED_{50} estimated by the Spearman–Karber method (World Health Organization, 1981).

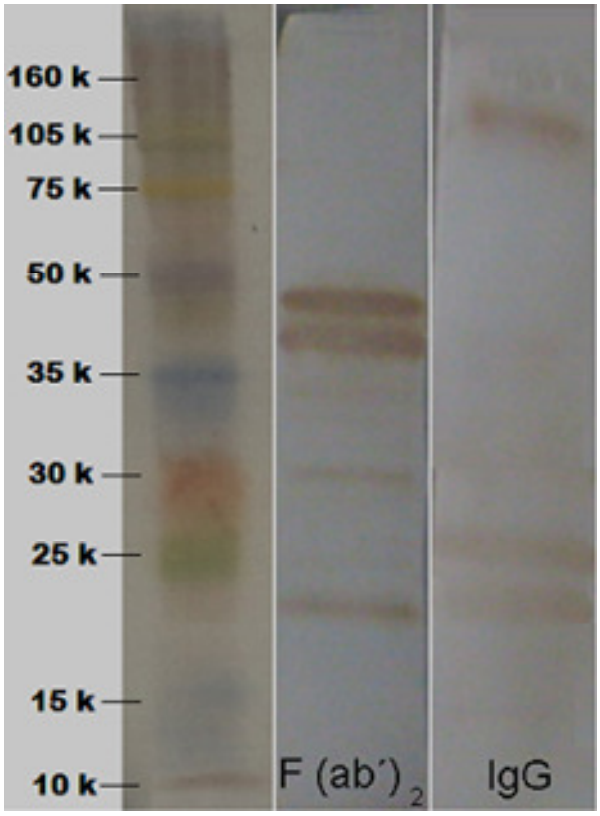

Figure 6. Western blot analysis. Samples names are labeled: Ten micrograms of purified $F(ab´)_2$ and the original IgG(T) anti - venom previously equalized to contain each 1.0 mg of protein/ml and elctrophoresed on 10% SDS-PAGE. The protein bands were revealed by a monospecific goat anti-horse IgG(T).

subconjuntival, retroperitoneal, subaracnoid, intracranial haemorrhages (Warrell 1996). These venom effects can be completely and acutely reversed in response to specific anti-venoms, if the patients or bitten animals are properly treated.

The *Naja* genus comprises several cobra species distributed in Asia (*N. naja, N. kaouthia* and *N. siamensis*) and on the African continent (*N. nigricolis*, *N. mossambica* and N*. melanoleuca)*. Their venoms, essentially neurotoxic, induce minimal local effects, usually restricted to small fang puncture wounds, exceptionally with tender local swelling, blistering and superficial necrosis (Reid, 1964; Viravan et al., 1986). Early systemic envenoming symptoms are vomiting, heaviness of the eyelids, blurred vision, fasciculation, paraesthesiae around the mouth, hyperacusis, headache, dizziness, vertigo, hypersalivation, congested conjunctivae and "gooseflesh". Ptosis and external ophthalmoplegia appear soon after the bite, followed minutes or hours later by paralysis of the face, jaws, tongue, vocal cords, neck muscles and (bulbar) muscles of deglutition with pooling of secretions in the pharynx. The intercostals and diaphragm become paralyzed, leading to abdominal breathing, respiratory distress, with agitation, tachycardia, sweating and central cyanosis (Warrell, 1996). The highest toxicity for venom, 0.96 µg/mouse, found in our determinations of ED_{50} is compatible with previously published data of 0.9-74 µg/mouse. With some individual variations in horses immunized with African snake venoms, anti-venom antibodies first appeared in plasmas one week after starting the immunization schedule, as revealed by the double immune diffusion method (data not shown). The antibody titers, subsequently determined by ELISA, increased until attaining a plateau. Higher antibody titers, observed in plasmas from horses immunized with *B. arietans* as compared with *B. nasicornis* and *B. rhinoceros*, were maintained along the entire immunization period. Such potent unexpected immunogenicity was also reflected both in the complete antibody molecule or in its $F(ab')_2$ fragment with a high capacity for neutralizing the venom-induced lethality. In contrast, horses immunized with *N. melanoleuca* and *N. mossambica* demonstrated anti-venom antibody production, although this production was only detectable during the initial immunization period and increased significantly during the second set of immunizations. These antibodies, as well as anti-*Bitis* antibodies are efficient at neutralizing the *Naja* venom lethality.

A summary of the production of potent sterile batches of polyvalent anti-*Bitis* and anti-*Naja* anti-venoms $F(ab')_2$ is indicated in the Process and Flow Diagram (Figure 1). As is indicated by Western blot analysis using monospecific goat anti-horse IgG(T) the molecular mass of the IgG(T) antibodies present in the polyvalent anti-Africa snake venoms, when submitted to pepsin cleavage at pH 3.0 - 3.2 was reduced to 100 kDa. The one-third reduction in the immunoglobulin molecular mass without noticeable loss in their ability of neutralizing the venoms lethality, indicate that the IgG(T) Fc molecular portion were cleaved leaving intact the $F(ab`)_2$, portion of the most of the antibodies.

Quantities of 40 ml of anti-venom free of bacterial, lipopolysaccharides and other toxic substances were dispensed into sterile flasks. Flasks were labeled with total protein contents and the lethality neutralizing potency was adjusted. These anti-venoms will be submitted to clinical field trials under the supervision of expert medical doctors.

In order to minimize the expected adverse reactions, upon injection into the bitten victims, the IgG immunoglobulins were partially purified, enzymatically cleaved with pepsin enzyme and the resulting $F(ab')_2$ fragments were concentrated and tested for their anti-venom neutralizing properties. Preparations of $F(ab')_2$ fragments resulting from plasma of horses immunized with *B. arietans* (G#1) and *B. nasicornis* plus *B. rhinoceros* (G#2) were mixed together and labeled as Polyvalent Anti-*Bitis* Antivenom. Similarly, preparations of $F(ab')_2$ fragments resulting from the plasma of horses immunized with *N. melanoleuca* plus *N. mossambica* (G#3) was labeled as Polyvalent Anti-*Naja* Antivenom(*nasicornis*), but not against *Naja* sp. The anti-venom was effective, with different potency,of neutralizing the individual toxic activities of the venoms.

The parameters included in Table 1 indicate that both polyvalent antivenoms, developed by the described method, posses high anti-venom antibody titers and, most important, exhibit sufficient lethality-neutralizing activity and are free of toxic substances, as well as of microbial contaminants and are therefore in conditions to be used for the immunotherapy of victims bitten by venomous African snakes. Theses anti-venoms will be submitted to clinical trials. The Polyvalent Anti-*Bitis* Antivenom, Batch IB-BT # 01/07, was recently released for use o treat victims bitten by snakes of the *Bitis* genus.

The "Polyvalent Anti-Bitis antivenom" is capable of neutralizing, *in vivo,* the myotoxicity of three *Bitis* venoms (*B. rhinoceros*, *B. arietans* and *B. nasicornis*). This effect was evaluated by measurement of plasma creatine kinase (CK) activity a well recognized parameter of muscle cells damage a hallmark signal of myotoxic damage induced by several snake venoms (Pontes et al., 2009).

Recent published data indicate that a polyspecific anti-venom native IgG (T) manufactured by caprylic acid fractionation from the plasma of four horses that had immunized with a mixture of the venoms of *E. ocellatus*, *B. arietans* and *N. nigricollis,* are endowed with antivenom neutralizing activities (Segura, et al. 2009). The lethality, hemorrhagic, coagulant and necrotizing neutralizing activities of this IgG horse antibody were analyzed, in a preclinical assessment, against venoms from *Echis* (*E.*

ocellatus, *E. leucogaster* and *E. pyramidum leakeyi*) and *Bitis* (*B. arietans*, *B. gabonica* (Nigeria), *B. gabonica gabonica* , *B. rhinoceros* and *B.*

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