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Research article

APPLICATION OF THE 3R CONCEPT IN THE PRODUCTION OF EUROPEAN ANTIVIPERINUM ON HORSES – MULTISITE, LOW VOLUMES IMMUNIZATION PROTOCOL AND ELISA

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During time, both professionals and general public became aware of the importance of animal welfare. This term not only covers endangered wild animal species, animals used in food industry, pets and experimental animals, but also animals used in production of biologics. The implementation of the 3R concept (Replacement, Reduction and Refinement) is especially important in this type of production. In this article, we describe for the first time the low dose, low volume and multi-site immunization protocol, as well as appropriate ELISA we developed for production of European anti-viper (*V. ammodytes*, long horned) antivenom in horses, which can help to significantly improve the welfare of the used animals.

Key words: adjuvant, animal welfare, antivenom production, European vipers, horses, immunization schedule

INTRODUCTION

Though the exact number of snake bites is unknown, an estimated 5 million people are bitten each year around the world, with up to 2.5 million envenomings. At least 100 000 people die as a result of snake bites each year and around three times as many amputations and other permanent disabilities are caused by snakebites annually [1].

In Europe, snake bites are relatively rare, so rare that there are no new data available. According to available references [2,3] in the European population (ca. 735 million), the annual number of snake-bites could reach 25 000, of which 8 000 involve envenomation of which about 30 deaths could result every year. Such a low number

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of cases, with the death rate of 0.378%, are largely due to wide access to antivenom and health care services.

All venomous snakes found in Europe belong to *Viperidae* family. According to World Health Organization [4], three of them (*V. ammodytes, V. berus* and *V. aspis*) belong to category 1 ("Category of highest medical importance"), which consist of "Highly venomous snakes that are common or widespread and cause numerous snakebites, resulting in high levels of morbidity, disability or mortality". Although European snake venoms have relatively mild potency compared to snake venoms from other continents [5], their bite can still cause significant impacts. The best way to treat snake bites is by use of antivenom, which are usually $F(ab)'_2$ fragments or whole IgG purified from horse antiserum.

The immunization of horses with snake venom(s) need to be done in a way to induce a long-lasting and high titer antibody response against the lethal and other deleterious components in the immunogenic toxins. To achieve this goal, the producers typically immunize horses with maximum amount of venom possible that will not induce the death of the animal. A common consequence of hyperimmunization with venoms achieved this way is the development of local ulcers or abscesses (sterile and infected) in animals at the sites of venom injection. One way to reduce the creation of wounds is the pre-treatment of toxins with agents that neutralize toxicity, such as formaldehyde. The bad side of this approach is that it causes significant loss of antigenic regions and induced antibodies usually fail to neutralize the native toxin [6]. In fact, no detoxification is usually necessary if the inoculation is made with a small dose of venom well-emulsified in an adjuvant such as Freund's complete or incomplete adjuvants (CFA and IFA, respectively). Freund's incomplete adjuvant contains mineral oil and an emulsifier. In addition to that, CFA contains dried mycobacteria. It has been shown in experimental animals that CFA is one of the most potent adjuvants known. However, horses are quite sensitive to CFA which tends to cause granuloma formation. It has been noted that the granuloma caused by CFA is due to injection of a large volume (5–10 ml) of the emulsified immunogen administered at 1 or 2 sites. Thus, the injection of a combination of these very damaging agents proved unfavorable, so some producers prefer to use other adjuvants [7,8].

Aside from Freund's complete and incomplete adjuvants, various types of other immunological adjuvants, for example aluminum salts (hydroxide and phosphate), bentonite and liposomes have been tested in order to enhance the efficacy of immunization [9]. The choice of adjuvant is determined by its effectiveness, side-effects, ease of preparation (especially on large scale) and cost.

In addition to achieving a long-lasting and high titer antibody response after immunogen application, today's obligatory goal is for the immunization regimes used not to seriously affect the health of the animal. All before mentioned goals can be achieved by increasing the number of injection spots, adjusting the volume of immunogen applied at a single site, optimal choice of adjuvant type and optimal

amount of venom used. If the emulsified immunogen is injected subcutaneously in small volumes (50–200 µl/site) at multiple sites of injection, granuloma formation is avoided. Administered in small dose, CFA and IFA induce less tissue damage comparing to the damage observed after application of high dose of snake venom injected without adjuvant. The group of Thailand colleagues published excellent papers about the application of low dose, low volume, and multi-site immunization (hereinafter: low dose immunization) in purpose of anti-venoms production for most venomous snakes of South and Southeast Asia [10-12]. According to these papers, this way of immunization leads to the highly potent monovalent and polyvalent antivenom production, while the administered amount of venom does not affect the health of the animal. This immunization protocol was used to immunize horses with venoms of *Elapidae* (cobras and krait) and recently with Southeast Asia *Viperidae* venoms [13] and other antigens [14], as well as the immunization of people [15].

The goal of our experiments was to establish if low dose immunization of horses can be implemented in the production of antiserum directed against the venom of horned viper, *Vipera ammodytes*. We immunized the horses with *V. ammodytes* venom according to the before mentioned immunization plan and compared the observed efficacy with the one accomplished after a typical high dose venom immunization [21,22]. We showed that the new immunization protocol induces higher yields of antiserum, which results in the reduction of the number of horses needed for production and the cost of their maintenance. In addition, the low dose immunization schedule significantly reduced the amount of venom and number of snakes needed for production, risk for employees and thus reduced the costs of purchase, keeping and milking of snakes. This immunization protocol was shown to be more humane for horses because it did not induce swelling and wounds at the sites of administration, which in turn significantly reduced, and in some cases even completely eliminated the pain and suffering of the animals. We concluded that the described protocol of immunization of horses with horned viper venom is very suitable for antivenom production and at the same time helps to drastically improve the welfare of animals used in production.

The antivenom production process requires the potency test which has been accepted worldwide as standard bench mark [7,16] and includes the use of mice (*Mus musculus*). There are reports [17] that around 400 mice are needed per antivenom batch in order to successfully complete the potency test. In addition, about 50% of mice are not protected against the venom and suffer extreme pain and stress during testing. Moreover, traditionally employed animal assays used in this process are laborious, expensive and time consuming. Therefore, considering the economical and ethical aspects, we developed ELISA for detection of IgG specific for the viper venom. Since we used the same venom used for immunization of horses as antigen in our ELISA, the test we developed is suitable for following the development of the immune response in immunized horses. We showed that in this phase of production, the antivenom ELISA we developed can replace the potency test. That way, the number of used mice is reduced only to the number needed for final batch control.

In this paper, we show for the first time that the low dose immunization protocol can successfully be used for the immunization of horses with venom of the most dangerous species of European viper. The new immunization protocol and ELISA method we developed and we describe in this paper refine, reduce and replace [18] the experimental/production animals and represent the practical example of the use of the 3R concept in welfare of production animals.

MATERIAL AND METHODS

Animals

This study was conducted in accordance with the provisions of revised Appendix A of the European Convention ETS 123, and approved by the Animal Institutional Care and Use Committee at the Institute of Virology, Vaccines and Sera "Torlak" in Belgrade, (016/2011, approved on 20th of February 2011) and by the Veterinary Directorate of Ministry of Agriculture (011-00-00510/2011-05/11), as stated by the Serbian Animal Welfare Law ("Sluzbeni Glasnik RS", no. 41/09, 39/10).

Horses

For immunization process, we used four healthy crossbreed horses (mares), ages 5 - 10 years old and weighing >600 kg. Animals were kept in a stable, housed in separate boxes. All horses were checked for the presence of bacterial and viral diseases e.g. equine infectious anemia (EIA). The regular tetanus vaccination was carried out alongside routine clinical chemistry and hematology, and the horses were treated with preventive anti-parasitic treatment on regular basis. The animals were primarily grass fed, but in addition, alfalfa hay and oats with addition of vitamin and mineral supplements and livestock salt were used. Water was available *ad libitum*. These horses have never before been used for immunization. Prior to the experiment, the horses were kept under quarantine.

Snakes

The venom was obtained by milking *V. ammodytes* adult snakes from the geographical area of Serbia and Montenegro. On arrival, the snakes were examined by a specialized veterinarian and kept in quarantine for two months, in a purpose-built serpentarium at the Institute of Virology, Vaccines and Sera "Torlak". They were housed individually in separate cages with controlled temperature and humidity. Snakes were fed with live mice once per month and after being milked. The venom was collected at 30 day intervals, according to a standard procedure by applying mechanical pressure to the venom gland by hand, i.e. active milking into Parafilm-covered 50 ml Eppendorf tubes. The venom was frozen to -20 °C, before being freeze-dried and stored away from light at -20 °C.

Mice

For potency test, we used outbred Intor:Swiss albino mice of both sexes [19]. Animals were kept in the vivarium, housed in polycarbonate cages, five mice per cage, at a constant room temperature of 22±1 °C with a 12-h light/dark cycle (light on from 7:00 AM till 7:00 PM). Food and water were available *ad libitum*.

All animals were followed up on a daily basis and head veterinarians were contacted in the case of complications. The animals were euthanized if they presented severe pain or suffering, such as food and water intake difficulties (weight loss > 25%), inappetence, abnormal posture or locomotion, weakness, respiratory disorders, vocalization disorders, or severe CNS signs during the follow up period.

Preparation of venom immunogen in CFA, IFA and aluminum salts

Horses were immunized with lyophilized *V. ammodytes* pulled venom VA-7. The lyophilization of the venom was performed at the Institute of Virology, Vaccines and Sera "Torlak", Belgrade, Serbia. Before immunization, the venom was reconstituted in saline to a concentration of 2 mg/ml of proteins. The total protein concentration in the venom sample was evaluated using nitrogen determination. The reconstituted venom was mixed with CFA, IFA or aluminum phosphate ($AlPO_4$), depending of immunization scheme.

The mixture of venom and CFA (or IFA) adjuvant was made by mixing equal parts of venom solution and CFA (or IFA) using two interconnected Luer lock glass syringes at 4 ºC. The final venom concentration in the resulting emulsion was 1 mg/ml. When the immunogen was prepared in $AlPO_4$, a sterile venom solution and a suspension of aluminum salt were mixed to final venom concentration of 1 mg/ml and final $AlPO_4$ concentration of 1%.

All chemicals and adjuvants were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany unless otherwise indicated.

Horse immunization and bleeding schedule

Immunization of four horses was performed as follows. The primary immunization was made with venom mixed with CFA as described. The initial dose of venom was as low as 2 mg/horse with a total combined volume of injection of about 2 ml. About 10 injections in total, 0.2 ml each were made on both sides of the neck of each horse. The immunogen was filled in a 5-ml plastic syringe with an 18G needle for the first dose, and with 20 or 21G needle for second and every other application. The route of injection was subcutaneous. Immunization using CFA was made only once, because repeated use of this adjuvant may in most cases cause serious reactions which can affect the health of the animals. After 2 weeks, the horses received a booster injection (secondary immunization) with the same venom emulsified in IFA. Volume and area of the booster injection was the same as described above. Subsequent three booster immunizations at 2-week intervals were made with same doses of venom mixed with

$AlPO_4$ (Figure 1), until the end of the seventh month. Buster immunizations were repeated until the end of the seventh month.

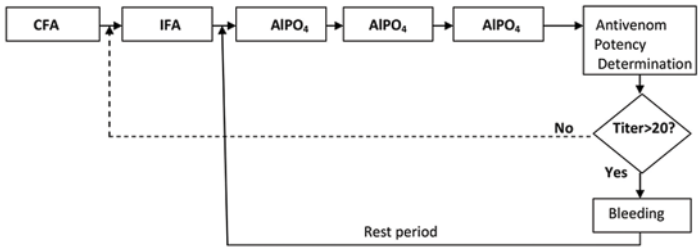

Figure 1. Algorithm of the protocol low dose, low volume, multi-site immunization.

Blood (10–20 ml) was collected by venous puncture after each cycle of immunization for the purpose of potency determination. If the potency assay confirmed that the horses reached the production potency level (neutralizing potency ≥20 AU/ml), they were bled in aseptic conditions using a sterile bottle with 9% potassium oxalate monohydrate solution in normal saline. Considering that all used horses for immunization weighed >600 kg, 8 l of blood was collected from each animal. The volume ratio of anticoagulant to blood was 1:9. The horses were then allowed to rest for about two weeks, depending on their physical condition. After the rest period, a new round of immunization was made as described above, by injecting 3 doses of venom mixed with $AlPO_4$ adjuvant, at one week intervals. In case the potency assay revealed that the potency did not reach the desired level (neutralizing potency <20 AU/ml), the horses were not bled and the immunization was repeated per previous schedule, starting from injection of venom in IFA adjuvant.

Potency test

The potency test was performed on outbred mice Intor:Swiss per European quality standards [16] Briefly, the LD_{50} dose (amount of venom sufficient to induce death in 50% of animals) was determined. After that, the mice were i.v. injected with a mixture of serial dilutions of immunized horse plasma mixed with LD_{50} dose of venom. The potency of the plasma (material for the production of antivenoms) or antivenom (the final product, must be controlled) is defined in terms of the number of LD_{50} of venom that is neutralized and it is expressed in antitoxic units (AU) per ml.

Western Blot (WB) Analysis

V. ammodytes venom was resolved by SDS–PAGE with homogenous 12% polyacrylamide gel and electrophoretically transferred to nitrocellulose membrane (NC 45, Milipore Corporation Serva Electrophoresis GmbH, Heidelberg, Germany). To prevent non-specific binding, membranes were incubated with blocking buffer 3% skim milk / PBS for 2 hr. Horse plasma obtained after immunization was diluted in

1% bovine serum albumin/PBS. Saturated membranes were incubated with prepared dilutions for 1 h at room temperature. The membranes were incubated with anti-horse IgG-B followed by streptavidin–phosphatase. The antibody binding was visualized by exposure to 5-bromo-4-chloro- 3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma-Aldrich, Germany).

Anti-venom IgG ELISA

The detection of venom specific horse IgG by ELISA was as follows. *Vipera ammodytes* venom pool used for coating ELISA plates were milked and lyophilized at the Institute of Virology, Vaccines and Sera – Torlak, Belgrade. Nunc MaxiSorpTM ELISA plates (Nunc, Roskilde, Denmark) were coated (50 ml/well) with venom (2mg/ml in PBS) by overnight adsorption (4°C) in a moist chamber. Blocking (200 ml/well of 1% w/v BSA/PBS 2h at room temperature) and all subsequent steps in the ELISA were followed by washing with 0.1% Tween 20 in PBS (four times, 200 ml/well). Appropriately diluted in 1% BSA/0.1% Tween/PBS sera or plasma samples (depending on the need) were incubated 1 hour at room temperature (50 ml/well). Each sample was assayed in duplicate. Ag-specific sera IgG binding was detected by peroxidase-conjugated anti-horse IgG (whole molecule) or by biotin-conjugated anti-horse IgG (whole molecule), followed by incubation with ExtrAvidin-peroxidase (50 ml/well, 1 h at room temperature). All reagents were used in accordance with the manufacturers` instructions. O-phenylenediamine/H_2O_2 (OPD) system was used to visualize Ag-Ab interactions. Absorbance was monitored at 492 nm and 629 nm ($A_{492/620}$).

Anti-venom IgG ELISA Validation

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. Typical validation characteristics that should be considered are specificity, accuracy, linearity, range, precision, and sensitivity. The validation of our ELISA was performed according to the guidelines of European Medicines Agency [20] and done by determination of the above-mentioned characteristics. The values of all parameters showed that our method is characterized by very high quality. Our ELISA enables quick and easy application in monitoring the immune response in horses after immunization with the venom.

ELISA for High-Affinity Anti-venom IgG Antibodies

Before performing this ELISA, we defined the plasma dilutions that will be used in the test. For this purpose, we generated binding curves using serial dilutions of different plasma prepared in 1% BSA/0.1% Tween 20/PBS and then selected the ones with the same absorbance at the beginning of the linear portion of the curve. Since different plasma contained different amount of antibodies, using dilutions selected this way enabled for comparison of antibody affinities in them.

The ELISA for high-affinity anti-venom IgG antibodies was performed as described previously [21]. The procedure was similar to that given for anti-venom-specific ELISA with the difference of one additional step. After incubation with horse plasma and subsequent washing with 0.1% Tween 20 / PBS, microtiter plate was incubated with potassium thiocyanate (KSCN/PBS) (50 μl/well) for 30 min at room temperature. We used KSCN solutions in increasing concentrations (0–8 M) to detach the low affinity anti-venom Abs. The plates were then washed and subsequent steps were the same as described in the previous section.

The relative affinity of anti-venom Ab binding was defined as the molarity of KSCN that leads to dissociation of 50% of bound Abs, i.e. the decrease of 50% in absorbance in comparison to the one observed for wells incubated with PBS (0 M KSCN).

Determination of the correlation coefficient between the potency assay and ELISA

We examined the correlation of the potency test and ELISA using OriginPro 8 software.

RESULTS

The quantity of venom used for immunization of horses

During immunization of horses using low dose immunization protocol, 22 mg of venom per horse were sufficient to achieve the production potency level of the antivenom in case of good responders, while for the maintenance or increase of the achieved potency, additional 32 mg of antivenom per horse were needed. This is at least 100 times less amounts of venom used in comparison to earlier immunization protocols, which is already a significant improvement. Previous immunization protocols were based on the use of quite large doses [22] of raw venom [23].

Potencies of antivenom produced with low dose, low volume, multi-site immunization

The production level of antivenom potency (>20 AU/mL) using low dose protocol was achieved after 100 days and it continued to rise in all immunized horses after the next four months, and even longer than two years in good responders (Figure 2). The level of neutralizing potency was, with minor fluctuations, maintained according to suggested scheme during the entire time of the experiment. The maximum potency observed in good responders was up to 56 AU/mL, six months after beginning of immunization. In addition, this was the highest potency value achieved after any type of immunization protocol.

The percentage of good responders observed after immunization using this protocol is hard to discuss because we immunized only four horses, which does not represent a statistically relevant sample. Out of four horses used, two achieved the desirable

antivenom potency after the first immunization cycle and they were identified as good responders. The other two horses needed an additional immunization cycle to achieve this potency level. The immune response dynamics induced by low dose immunization protocol is presented in Figure 2.

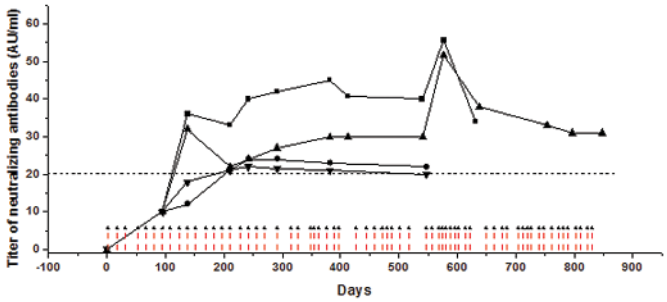

Figure 2. Immune responses (the antivenom neutralization potency) in horses against *V. ammodytes* venom induced by multisite, low dose, immunization protocol. Dashed arrows on the x axis represent time points of venom application. Symbols ▲, ■, ▼ and ● represent each individual horse. ▼ and ● – low responders; ■ and ▲ – good responders. Dashed line represents antivenom potency production level (20 AU/ml).

Horse health

There is a growing concern about the pain and suffering caused to experimental animals and this point has been taken seriously during our study. The horse health (weight, body temperature) and the reactions at the sites of injection were closely monitored during the course of the experiment. The body temperature was measured every day during immunization. An increase in body temperature of 0.5 °C was observed only the first day after immunization, but not after each dose. We concluded that this immunization protocol does not induce a febrile state in the experimental animals and that it did not require any treatment. No changes in weight were observed in any of the horses used in the study.

Since the horses are highly sensitive to CFA, the highest focus was directed to the neck area, where the application of adjuvant was administered. Given that we used CFA in small doses, no granulomas were formed at injection sites in any of the immunized horses and we could only observe a mild edema.

ELISA validation

The critical validation parameters are summarized in Table 1. The specificity of the method was high, and the linearity and scope had correct values. Furthermore, according to the literature data [24], intra- and inter-assay coefficient of variation of 10 % or less is considered to be satisfactory. The values of coefficient of variation obtained in our ELISA were under 5 %, which indicates high-precision assay performance for testing samples once or more times. In addition, given that the value of CV inter-lab was

satisfactorily within 10–15 % [25], our ELISA is characterized by high reproducibility. Based on validation characteristics, we can conclude that the ELISA we developed is reliable and that it can be used for control horse immunization part of antivenom production (Figure 3).

Table 1. The summary of validated characteristics of ELISA for detection of horse IgG antibodies specific for V. ammodytes venom proteins

Validation characteristics	Value
Specificity	High
Linearity	1:1 000-1:250 000 dilution of standard plasma
Range	1:3 000-1:105 000 dilution of standard plasma
Accuracy	High
Precision	
Repeatability (ref. value: ≤ 10%)	3.4%
Intermediate precision (ref. value: ≤ 10%)	3.4%
Reproducibility (ref. value: 10%-15%)	11.1%
Detection Limit	0.02
Robustness	Good

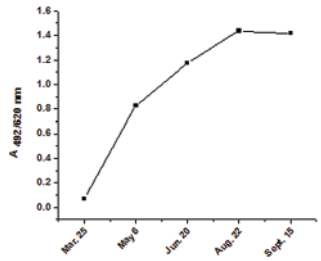

Figure 3. Development of venom specific IgG antibodies induced according to low dose, monitored by ELISA.

We tested what results were obtained depending on whether the sample was plasma or serum and concluded that there is no difference in the results of the ELIZA test.

Correlation between potency test and ELISA

According to results of our experiments (Figure 4), the calculated correlation coefficient between potency test and anti-venom IgG ELISA was 0.682 (68%).

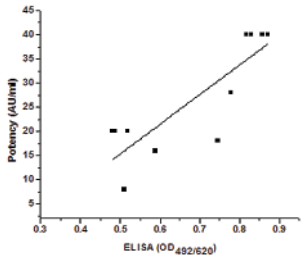

Figure 4. Correlation between ELISA antibody levels (absorbance at 492/620 nm) and *in vivo* neutralizing potency of *V. ammodytes* antivenoms. Microwell plates were coated with the *V. ammodytes* venom. Horse antivenom plasma was used at 1:32 000 dilution. All data points represent means of three experiments.

Titer and affinity of venom-specific horse IgG induced by old and new protocol immunization

On Figure 5 it is easy to notice that immunization using the new protocol leads to significant increase in anti-venom IgG antibody as measured by ELISA. Comparing to the previous protocol, the measured titers were even eight dilution factors higher: for the classic protocol, the titer was 512 x 10^3, and for the new one it was 4 096 x 10^3.

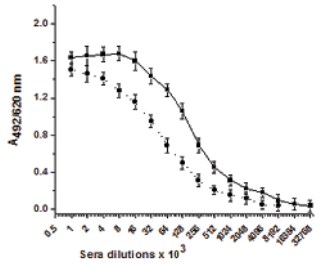

Figure 5. Titer of venom-specific horse IgG induced by the old and the low dose immunization protocol. ■-low dose, ●-old protocol. The figure shows mean values of four horse titers immunized according to the low dose protocol and the mean values of eight horse titers immunized according to the old protocol.

According to that, for affinity determination we used dilution of 1:8 and 1:64 for old and new immunization protocols [21,22], respectively. We determined that 7.5 M KSCN was needed to detach 50% of bound anti-venom IgG antibodies from plasma of both immunization protocols.

This result was confirmed by Western blotting (Figure 6), where membrane staining is much more intense with plasma obtained according to the low-dose protocol. To achieve a similar WB image with both protocols plasma, the one obtained according to the new protocol needed to be diluted at least four times. At the same time, WB confirms the specificity of the induced antibodies for non-reduced venom proteins. No binding in reduced conditions was detected (not shown).

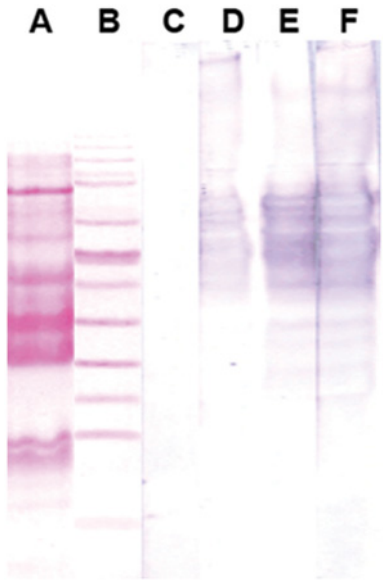

Figure 6. Ponceau S staining and Western blotting of *V. ammodytes* venom protein profile on 12% PAGE in non-reducing conditions. A and B – Ponceau S stained membrane; A - *V. ammodytes* venom, B - protein marker SM 0661 (Fermentas); C-F WB; C – normal horse plasma; D - horse plasma old protocol, E and F –horse plasma low dose protocol, diluted 10 000 and 40 000 times, respectively.

DISCUSSION

Improving antiviperinum production by immunizing horses with horned viper toxin is still important because horned viper is the most venomous and most widespread European venomous snake. In addition, *V. ammodytes* antivenom is cross-reactive with the venom of the common European adder (*V. berus*), as well as with venoms of other European *Viperidae* [26,27]. In this paper, we describe a new immunization protocol used for anti-viper antivenom production. The horses were immunized according to low dose, low volume, multi-site protocol [10,11] which was recommended by the WHO [7,8]. Using this protocol, results both in increase in productivity of antivenom production and in drastic reduction of animals (horses, snakes and mice) used in the process. It is also very useful when a limited amount of antigen is available.

The immunization schedule must lead to the highest titer of antibodies with the lowest antigen consumption. We considered the immunization protocol successful if the achieved antivenom potency is ≥20 AU/mL six months after immunization start, and if it remained at the same level or higher in the next six months. In our case, this potency level was achieved much earlier than 6 months after the immunization protocol was initiated, specifically in 14 weeks. In two immunized horses, the desired titer ≥20 AU/mL was achieved after the first immunization cycle, while in the remaining two horses, additional cycle was needed to hit this mark.

In the end, we established that after reaching the production level, the neutralizing potency could be maintained for at least two years.

In addition to fast manufacture, the quality of antivenom, i.e. antiserum of high antitoxin potency is of utmost importance in the antivenom production process. In classic antivenom production, the antivenom quality was achieved by slow increase in the amount of applied venom. The application of high venom doses used at the end of immunization schedule not only causes the local tissue damage in immunized animals, but also is not immunologically justified because a too high concentration of immunogen can overwhelm the immune system and cause clonal anergy. The induction of high potency anti-viper antivenom by low dose of antigen using the new protocol was achieved by addition of adjuvants to the immunization mixture [28]. The most important result of this approach was the induction of high titer anti-viperin venom (measured by potency test), which allows for high yields of produced antiserum even with smaller amounts of animal blood used, when compared to the classic immunization protocol (Figure 2).

The combination of used adjuvants (CFA, IFA and aluminum phosphate) was shown to be very successful. First, CFA was used because of its high potency, because mycobacterial components within CFA signal T lymphocytes to assume a Th1 profile. Since it was used only in one dose and in low volume, the application of CFA did not affect the immunized horses. Secondly, IFA can trigger depot generation and induction of MHC responses. IFA induces a predominantly Th2-biased response with some Th1 cellular response. And finally, aluminum phosphate adjuvant was used because of its safety and because it has already been used in antivenom production, as well as in human vaccine production for over 70 years. It has been recognized that aluminum adjuvants are strong inducers of antibody-mediated immune responses, but only poorly stimulate cell-mediated immunity. Each of these three adjuvants promote the immune response using different activation mechanisms and using them together, the immune response is modulated in a way that results in maximum activation without causing side effects.

Subcutaneous route of injection recruits a large number of antigen presenting cells which consequently results in high antibody response. The immunization sites were in the areas close to major lymph nodes, preferably on the animals' neck. In our immunization procedure, we used small volume (50–200 μL) injections at as many as 8–12 sites to maximize the total surface area of the immunogen droplets and in turn enhance the interaction with the antigen presenting cells and the immune response. This low amount of venom used in such a small volume, even when administered with CFA and IFA, does not induce any edema, wounds or abscesses.

All of these factors combined caused the use of low dose immunization protocol to result in high antivenom yields achieved by using significantly less venom when compared to the classic immunization protocol. A simple comparison between the two immunization protocols reveals an impressive 100 x – 140 x reduction of the

amount of toxin used (22 mg of toxin used in the new protocol vs 3.16 g used in the classic protocol [21,22]. The preparation of immunogens and the immunization protocol presented in this paper are technically simple and economical, and use a minimal amount of venom, which results in a significant reduction in the number of snakes needed, as well as in reduction of risks and work hours and materials used for maintenance and milking of the venom. This not only improves the financial aspect of the process, but also the ethical aspect of human and animal welfare.

The ELISA described in our paper showed that the new immunization protocol induces antivenom IgG antibody titers of eight times higher dilution factor when compared to the ones observed after using the classical protocol (Figure 5, Figure 6). This result can be caused by the increase in the concentration of produced IgG, or by the increase in the affinity of these antibodies. Since we showed that the antibody affinities in both protocols were on the same level, we can conclude that the increased titer observed after using the new immunization protocol occurs as a result of the increase in the concentration of produced antibodies [29]. Although this is favorable for antivenom production, it is important to remember that the induction of high antibody concentrations can lead to hyperglobulinemia. In horses, hyperglobulinemia can be manifested with cryoglobulinemia, which can lead to glomerulonephritis, swelling and skin ulcers of the limbs in the winter [30]. Using plasmapheresis during the bleeding procedure and by control of the antibody titer can successfully protect the horses from developing these complications.

The efficiency of the antivenom and quality of plasma as a starting material for antivenom production is traditionally assessed *in vivo* by measuring the neutralizing potency in mice [16]. The cost and inconvenience dealing with the animals, the suffering and death of a large number of animals, and the variable irreproducible results obtained are among the usual problems encountered with the *in vivo* assays. Several investigators have correlated the potency of the produced antivenoms to other *in vitro* assays, especially ELISA [31,32].

The correlation between the potency test and ELISA developed in our laboratory was 68%, which was similar to the values reported in similar studies using different venoms [33]. At the first glance, the correlation coefficient of 68% might seem low and ELISA cannot replace the potency test in assessing the plasma quality (efficiency). The observed difference in the two assays can be explained by the fact that ELISA detects IgG antibodies specific to all immunogenic proteins of the viper venom, while the potency assay measures only the functional (neutralizing) capacity of the IgG specific to toxic components of the venom. Since the immune response develops independently of the toxicity of protein components, ELISA can be used to follow the increase in IgG antibody concentrations and to detect the point when the immune response achieves the plateau, after which the potency assay can be used to make necessary decisions during antivenom production. An aliquot of suitable plasma with a known neutralizing potency value can be used as a standard in ELISA and in that way the achieved potency level can be detected even before the plateau phase. Additionally,

ELISA is sensitive enough and can be used during the early immunization phase to differentiate between good and poor responders, which is hard to accomplish using conventional *in vivo* assay at that stage of the process. This is not only financially but also ethically significant because it can help prevent the use of low-responder horses for antivenom production and at the same time prevent the unnecessary immunization during the long period of time. Figure 3 illustrates the course of immunization determined by ELISA.

Bearing in mind that the viper venom consists of 139 protein components, of which only 38 are toxic [34], it can be concluded that the observed correlation coefficient between ELISA and potency assay is actually very high. The most probable reason for this is the fact that the most potent neurotoxic components of the venom, ammodytoxins, are at the same time the strongest immunogens [35]. Mice, which are used for potency assays, are actually the most susceptible to these neurotoxic components. This is the reason why achieving high correlation between ELISA and potency test is not that significant.

In addition to immune response development, ELISA can be used to follow different stages of the plasma fractionating process, but not in the final batch of antivenom for human usage which needs to be tested using classical *in vivo* test in mice (potency test). Namely, contrary to mice, people are more susceptible to hemorrhagic components of the venom [36] so *in vivo* assays show little or no correlation with envenoming and therapy in humans [37]. Although the potency test does not precisely measure the level of protection the produced antivenom provides to humans, currently there is no other, more suitable assay developed for this purpose, which makes it a golden standard until a better method is developed.

The recommended immunization protocol of horses using *V. ammodytes* venom in a small dose, small volume and low concentration and its multi-site application with adjuvants leads to reduced venom burden in immunized horses. At the same time, it results in high quality and high yields of the produced antivenom, accompanied by reduction in costs of antivenom production and the time needed for its completion. This is also the first report of the refined immunization scheme being applied to European *Viperidae* antivenom production.

In the end, it is very important to stress that by the use of the immunization protocol presented in this paper, we significantly reduced the number of horses, snakes and mice needed for the process. We also successfully replaced the potency test, which requires the use of mice, with ELISA for the most part during the course of immunization. And finally, we significantly refined the procedures used on horses. Altogether, our paper represents a good example of successful implementation of the 3R principle in production of biologics.

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Authors` contributions

MV carried out the experiment, made substantial contribution to acquisition, analysis and interpretation of data. MV and DLj conceived and designed the study. KJ and PV have been involved in drafting the manuscript. MR has prepared western blot data. ZI has prepared ELISA data and wrote the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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PRIMENA 3R KONCEPTA U PRODUKCIJI EVROPSKOG ANTIVENOMA NA KONJIMA – IMUNIZACIONI PROTOKOL SA MALIM VOLUMENOM NA VIŠE MESTA I ELISA

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Tokom vremena, stručna i opšta javnost postala je svesna važnosti dobrobiti životinja. Ovaj pojam se ne odnosi samo na divlje životinjske vrste, životinje koje se koriste u prehrambenoj industriji, kućne ljubimce i eksperimentalne životinje, već i na životinje koje se koriste u proizvodnji bioloških preparata. Naročito je važno implementirati 3R koncept (Replacement - zamena, Reduction - smanjenje and Refinement - poboljšanje) u ovaj tip proizvodnje. Ovde prvi put opisujemo primenu novog imunizacionog protokola za konje, malom dozom *V. Ammodytes* venoma, aplikovanog u maloj zapremini i na mnogo mesta, tokom produkcije antivenoma za ujede evropskih vipera. Protokol imunizacije, zajedno sa razvijenom ELISA metodom sa istim antigenom, može značajno da poboljša dobrobit životinja koje se koriste u proizvodnji evropskog antiviperinuma.