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Prospects of Obtaining Antitoxins and immunoglobulin preparations from animal blood, their Purification and Application

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ABSTRACT

Animal hyperimmune sera containing specific protective antibodies against bacterial and viral antigens, snake and insect venoms have been used for more than 120 years. Currently, antitoxins against bacterial toxins (diphtheria, tetanus, botulism, etc.), viruses (rabies, Ebola, SARS-CoV-2, etc.), venoms of snakes (viper, gyurza, efa, cobra, etc.), spiders (black widow spider, red-backed spider, etc.), and scorpions have been developed and are successfully used. The review is devoted to modern technologies for producing antitoxins and immunoglobulin preparations, as well as the main areas of their application, such as the prevention or treatment of bacterial and viral diseases, bites of venomous snakes and spiders. The main stages of the production of antitoxins and immunoglobulin preparations are considered. An important step in the production of these drugs is the proteolytic digestion of native immunoglobulin molecules using enzymes to obtain F(ab')2-fragments and remove Fc-fragments, that leads to the elimination of undesirable interactions with Fc-receptors in tissue, which reduces the frequency of side effects. Today, antitoxins and immunoglobulin preparations are widely used in practical medicine. In addition to the application of well-known antitoxins, purification methods are being improved and new drugs are being developed to fight with actual threats such as antitoxin against COVID-19.

1. Introduction

The production of antitoxic hyperimmune sera (antitoxins – ANTs) was started at the end of the 19th century, and since that time and up to today this area has been actively developed. As we know from the work of Louis Pasteur on vaccine development, animals and humans become immune to subsequent infection with the same pathogen after illness or vaccination, since specific protective antibodies (ABs) appear in the body[1]. In 1890, German bacteriologist Emil Adolf Behring grew cultures of diphtheria and tetanus bacteria, filtered the cultures, and injected the sterile filtrate of the culture fluid into laboratory animals. As a result, he discovered that neutralizing ABs against toxins secreted by living bacteria appeared in the blood of animals. In collaboration with Japanese microbiologist Shibasaburo Kitasato, he discovered that serum injection into another animal can protect it from toxins. A year later, the sick child was injected with serum of animals immunized with diphtheriae toxin, and it was the first case of diphtheria treatment. The use of anti-diphtheria ANT allowed to decrease the incidence of diphtheria from 35 to 5%. In 1901, E. A. Bering became the first Nobel Prize laureate in physiology or medicine. Thus, animal immune sera containing ANTs against bacterial and viral antigens (AGs), snake and insect venoms have been used for more than 120 years.

These studies accelerated the development of immunology and in particular preventive and therapeutic ANTs[2, 3]. Bering`s research marked the beginning of the biotechnological production of ANTs, aimed at preventing dozens of infectious and other diseases. Horses, domesticated by humans in ancient times, are most commonly used for ANTs as highly effective animal producers. The study of ABs composition, started in the 1970s, showed that there are eleven subclasses of ABs in horse serum (IgM, IgD, IgA, IgE, and IgG isotypes), which are determined by different genotypes of heavy chain constant region, that makes it possible to evaluate the horse as a mammal with the largest number of IgG constant region variants. Furthermore, IgGa, IgGb, IgGc, IgG(T), and IgG(B) subclasses were identified among IgG[4, 5]. Currently, seven IgG subclasses that protect animals from various infections have been identified.

In the 20th century, Russian Empire, and later the USSR, was one of the countries where the development and production of ANTs was successfully developed. In the USSR, the production of ANTs was implemented in several enterprises, particularly in Kharkiv, Ufa, Perm, Stavropol[6, 7]. In 1898 in Kharkiv (city in modern Ukraine), it was established an enterprise for the production of immunobiological preparations, later called "Biolik", which has produced ANTs for more than 90 years[8]. During this period, anti-diphtheria, anti-tetanus, anti-scarlet fever, anti-dysenteric, anti-pneumococcal ANTs were produced by the enterprise. In subsequent years, the manufacturing expanded to ANTs against diphtheria, tetanus, influenza, and polyvalent ANTs against gangrenous and botulinum[9].

Over the years, new methods for ANT production, standardization and control have been proposed. Particular attention was paid to the isolation and purification of AGs for immunization of horses, immunization schemes, methods of purification and control of ANTs against gas gangrene[10, 11] and botulism[12].

2. Antitoxins from Animal Blood

2.1 Antitoxin against Diphtheria

Considering that the first ANT used for the treatment of patients was ANT against diphtheria, we will primarily focus on that. Diphtheria is a potentially fatal disease caused by *Corynebacterium diphtheriae* (Gram-positive aerobic bacterium), and subsequent effect of a potent exotoxin on the body[13]. Diphtheriae toxin causes a local tissue death, and hematogenous spread of the toxin leads to neuropathy and cardiotoxicity. Despite the successful conduct of vaccination, diphtheria disease poses a serious danger. Diphtheria ANT is particularly needed during diphtheria epidemics, which are associated with a

global threat due to the relocation of susceptible to diphtheria people. Diphtheria epidemics with high mortality rates (more than 10%) were reported in Haiti, Nigeria and Laos. Diphtheria morbidity and mortality are significantly reduced by rapid administration of ANT to neutralize the diphtheriae toxin and further prevent tissue damage. Human monoclonal anti-diphtheria ABs are in development. Even today, there is a worldwide shortage of diphtheria ANT[14, 15]. Supplies of diphtheria ANT for therapeutic use are limited. Affinity-purified ABs from the plasma of adults who received a booster dose of a vaccine containing diphtheria toxoid may also be a potential source of ANT[16].

2.2 Antitoxin against Tetanus

The intoxication with *Clostridium tetani* (Gram-positive anaerobic bacterium which is a causative agent of tetanus) leads to a severe pathological process, resulting in neurological disease with a mortality rate of up to 40%. ANTs are the only effective means of preventing and treating tetanus in the first two weeks after infection. A critical issue in tetanus ANT production is the purification of AG for immunization of horses. Due to the high toxicity of tetanus toxin, it is difficult to purify the native toxin and obtain its high purity form even after detoxification with formaldehyde[3]. Moreover, the presence of impurities in *C. tetani* AG leads to the formation of low-effective antibodies against tetanus toxin during immunization and subsequent hyperimmunization processes, which is due to the fact that the use of such AG leads to gradual liver degeneration and cells necrosis despite the inactivation of residual toxicity and the use various purification methods. The horse's liver gradually enlarges and breaks down which results in internal bleeding and death of the animal. Therefore, horses could be highly effective producers for no more than 2-3 years, depending on the immunization scheme and the type of AG. Various methods for the purification of tetanus AG, including chromatography, have been used[17]. The search of immunogenic and non-toxic AGs for tetanus ANT production is underway. The use the non-toxic carboxy-terminal fragment of the tetanus toxin heavy chain (fragment C) was proposed to obtain specific ABs against *C. tetani*. Fragment C can be transported to the central nervous system, that's why it was used as a valuable biological carrier of neurotrophic factors to improve the treatment of neurodegenerative diseases. The neuroprotective properties of fragment C have recently been described *in vitro* and *in vivo*, involving the activation of protein kinase B and extracellular signal-regulated kinase signaling cascades through neurotrophin tyrosine kinase receptors[18]. The tetanus toxin is a 150.7 kDa protein consisting of a 52.4 kDa light chain (fragment A) and a 98.3 kDa heavy chain linked by a disulfide bond (Fig.1)[19-21]. The heavy chain includes fragments B and C, and fragment C with a mass of 51.6 kDa is located at the C-end and is responsible for binding to neuronal cells.

The research of the possibility of using tetanus ANT is ongoing. Thus, the study of tetanus ANT safety was conducted in 20,000 patients with associated injuries in Ethiopia in 2015-2019. The tests were successful and showed non-significant adverse events after ANT administration[22].

The fragment C of tetanus ANT is attractive for researchers because of its non-toxic nature, immunological properties, and the ability to bind neurons[19]. As a derivative of tetanus ANT, fragment C retains its immunogenic properties without toxicity. It is also a perspective carrier protein due to its effectiveness and relative simplicity of isolation[20]. Thus, the native fragment C of tetanus ANT is a very attractive candidate for tetanus vaccine development. The immunogenicity of fragment C in the production of antitetanus ABs was studied using only fragment C and in various combinations of it with tetanus toxoid for immunization[19]. Horses with a body weight of 250-400 kg were injected into the back or neck with the C-fragment of *C. tetani* toxin with Freund's incomplete adjuvant in a water-oil emulsion. Titers reached 1000 IU/ml after two stages of immunization. The fragment C of *C. tetani* toxin demonstrated extremely low toxicity and allergenicity. Today, many countries produce tetanus ANT, for example, Behring's tetanus ANT contains 1000 IU/ml of an-

ti-tetanus antibody (170 mg/ml protein).

2.3 Antitoxin against Gangrene

Gas gangrene is a life-threatening disease that has severe manifestations such as myonecrosis, gas formation and sepsis. The causative agents of gas gangrene is gram-positive anaerobic bacteria of the genus *Clostridiim*, which includes several strains (*Cl. perfringens*, *Cl. oedematiens*, *Cl. histolytycum* etc.). The disease is primarily caused by *Cl. perfringens*, which is present in soil and in human gastrointestinal flora. The infection usually occurs due to traumatic wounds, contamination with soil, and intestinal surgery. The infection destroys erythrocytes, thrombocytes, and polymorphonuclear leukocytes, causing extensive damage to cell membranes and capillaries, which defines the typical pathophysiology. Initially, the wound swells, the affected skin becomes blistered with hemorrhagic bullae, and then becomes bronze, brown or black in color. A foul-smelling, brownish-red or bloody discharge emanates from the affected tissue or wound. Over time, signs of sepsis, toxemia of septic shock and multiple organ failure become apparent[23, 24]. Gas gangrene, caused by *Cl. perfringens,* leads to death within 48 hours after infection. The mortality may be due to the presence of both *Cl. perfringens* toxins, namely α-toxin (CPA) and perfringolysin O, which are important virulence factors of gas gangrene. *Cl. perfringens* is detected in approximately 80% of patients with traumatic gas gangrene, less common are *Cl. septicum*, *Cl. novyi*, *Cl. gistoliticum*, etc.[25].

Between 1940 and 2000, a number of research were devoted to the obtaining of AG for immunization of horses and the development of the polyvalent ANT against gangrenous infections[7, 10, 11, 26-29].

Although the effectiveness of gangrenous ANT is now questioned, a number of countries, such as India, Russia, etc., continue to produce it for the prevention and treatment of gas gangrene[30]. The ANT is released in the form of polyvalent serums containing 25,000 or 30,000 units of ANT per vial. However, in another countries, such as the USA, this product is no longer available due to low effectiveness and severe allergic reactions[31]. At the same time, the use of ANT in severe cases of gas gangrene have recently reported. In these cases, specific ANTs against α-toxin of *Cl. perfringens* are recommended to be administered along with antibiotics as early as possible to neutralize the gangrenous toxin. The authors have concluded that the optimal treatment for gas gangrene includes antibiotic therapy, specific ANTs administration, and surgery[31]. In context of limited therapeutic options, natural products for the treatment of gangrene and neutralization toxins are constantly being studied, for example amentoflavone and glycoside verbascoside effectively neutralize α-toxin and perfringolysin O[32, 33].

2.4 Antitoxin against Botulism

A special place among toxins is occupied by the botulism neurotoxin (BNT). BNTs produced by *Clostridium botulinum* are the most toxic substances known, but the number of currently approved medicines to fight these neurotoxins is quite small[34]. Several types of BNTs (from A to G) are known. Diseases in humans are often caused by serotypes A and E, rarely by serotypes B, E, F, and G. Serotypes C and D can cause diseases only in mammals and waterfowl. However, *Cl. botulinum* IBCAIO-7060 strain that produces BNT serotype H was recently discovered. Among this 8 exotoxins, it is the most potent, followed by serotypes B and F. BNTs consist of a 50 kDa light chain (LC) and a 100 kDa heavy chain (HC) linked by disulfide bond (Fig.2). The LC has zinc metalloprotease catalytic activity, and the HC contains two functional domains, N-terminal translocation domain (HCN) and C-terminal receptor-binding domain (HCR).

BNTs can penetrate to peripheral nerves, where serotypes B, D, F and G proteolyze VAMP (synaptobrevin, vesicle associated membrane protein), serotypes type A, C and E cleave SNAP-25 (synaptosomal-associated protein, 25-kDa), that leads to paralysis. These membrane proteins are components of the SNARE protein complex, which mediates the fusion of synaptic vesicle with presynaptic membrane of the neuron with subsequent release of acetylcho-

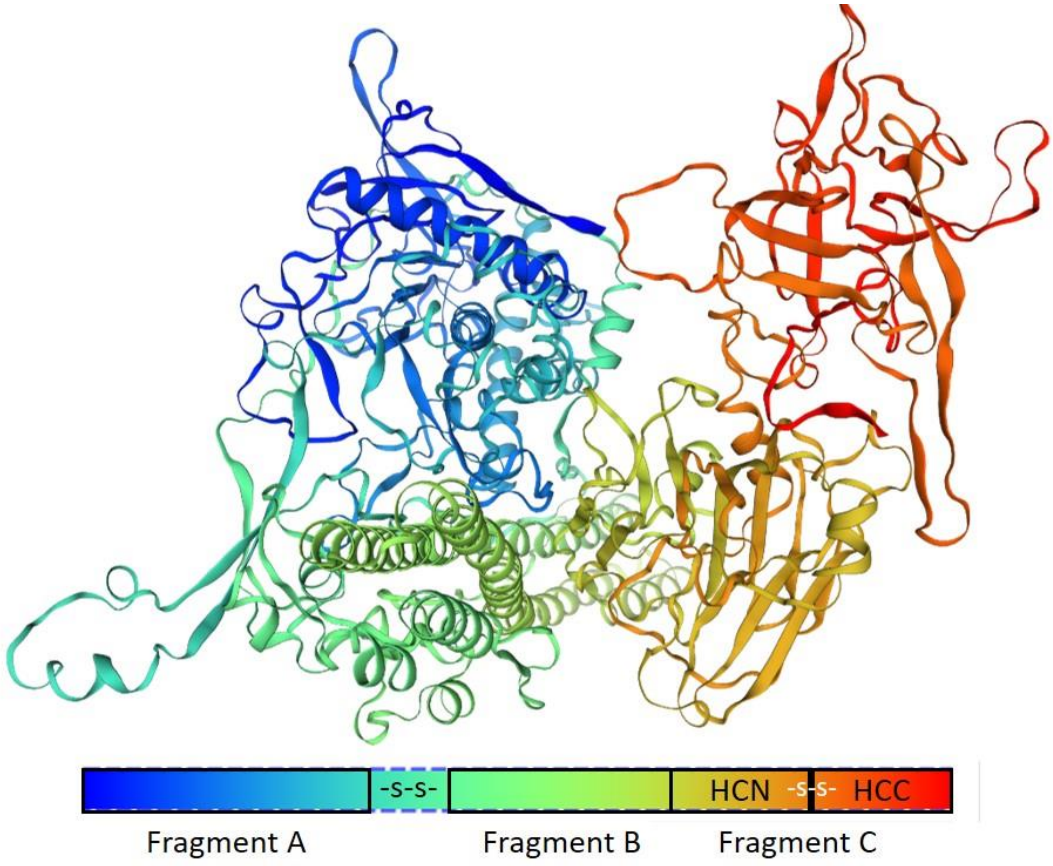

Figure 1: Tetanus toxin structure and schematic representation of protein domains. The figure was created using SWISS-MODEL database[21].

line. The action of BNTs leads to inhibition of acetylcholine exocytosis, thereby causing neuromuscular paralysis[36, 37].

Considering the high toxicity of BNTs, it is necessary to focus on the main drug for the prevention and treatment of botulism – ANTs against *Cl. botulinum* neurotoxins. This ANTs are produced by immunizing horses with botulinum toxoids. ABs to serotypes A, B, E and F have been obtained[38]. ANTs against BNTs serotypes A and B were obtained by immunizing horses with formalin-detoxified botulinum toxoids. Antibody titers were determined *in vitro* (using ELISA) and *in vivo* using neutralization reaction. The titers of antibodies in horses blood were between 1 and 5 IU/ml after primary immunization and reached peaks of approximately 2000 IU/ml for serotype A and 150-625 IU/ml serotypes B after additional booster injections of toxoid[39].

South Korean researchers proposed an original method for botulism ANT production which included immunization of a local horse breed, protein precipitation with ammonium sulfate, and F(ab')2-fragment obtaining using immobilized proteases[40]. Halla horses were immunized with the C-terminal receptor-binding domain of BNT serotype A1 (BNT/A1-HCR) expressed in *E. coli* instead of the inactivated botulinum toxoid. Aluminum hydroxide was used as an adjuvant. 3 ml of AG solution with concentration of 1 mg/ml was administered into the right side of cervix of horse three times at 2-week intervals and

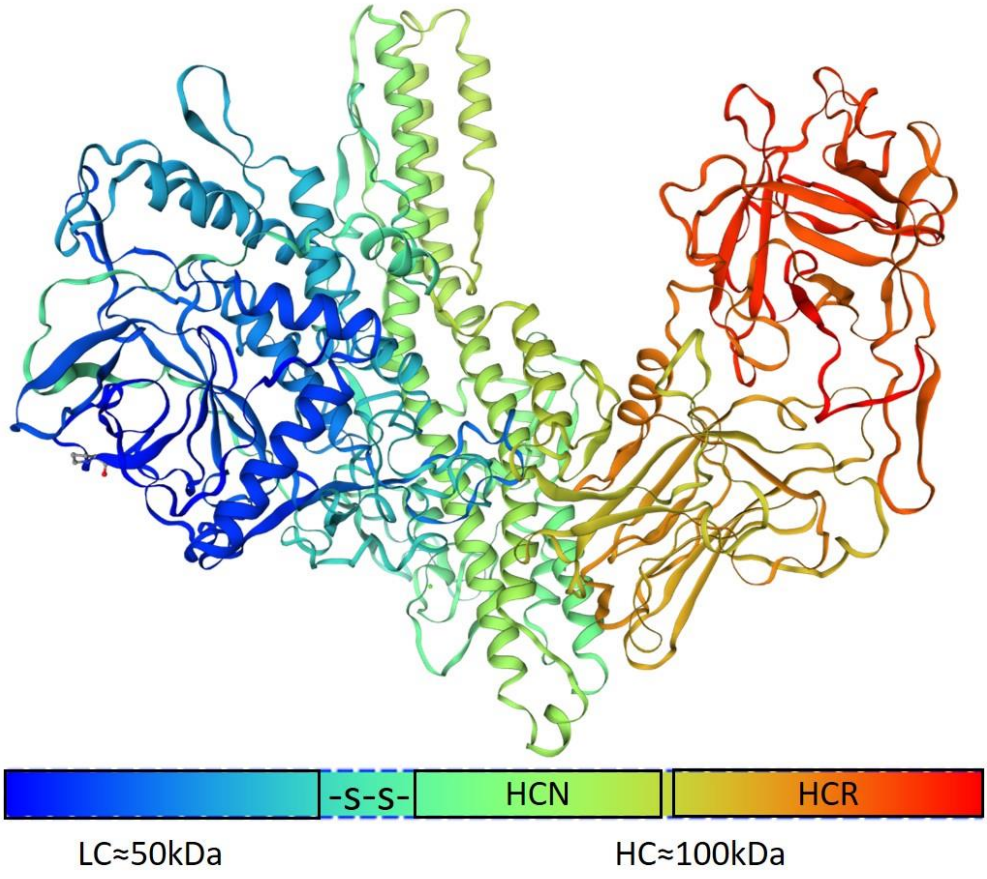

Figure 2. Botulism toxin A structure and schematic representation of protein domains. The figure was created using SWISS-MODEL database[21].

then 4 times. To obtain F(ab')2-fragment of botulism ANT, a saturated solution of ammonium sulfate (4.1 M, 25 °C) was slowly added to horse serum to achieve a concentration of 70% at 4 °C. The precipitated protein was separated by centrifugation at 4 °C, 5000 g for 30 minutes, and the pellet was suspended in MabSelect SuRe resin buffer A (20 mM sodium phosphate, 150 mM NaCl, pH 7.2), bound to MabSelect SuRe resin, washed in 5 column volumes of buffer A, and eluted in 10 column volumes of 100% buffer B (0.1 M sodium citrate, pH 3.0) in a linear gradient. The eluate was collected in a tube containing 10% of the eluate volume of 1.0 M Tris HC1 (pH 8.0). The elution fractions buffer was replaced with pepsin digestion buffer (20 mM sodium acetate, pH 4.0) and treated with pepsin. F(ab')2 was prepared using a Pierce F(ab')2 Preparation Kit, which is designed to cleave of IgG with the formation of F(ab')2-fragments that retain antigen-binding activity. Pepsin immobilized agarose resin beads ensured the hydrolysis of IgG. Using SDS-PAGE, the degree of hydrolysis and the purity of F(ab')2-fragments were determined. The product was concentrated by ultrafiltration on the Viva-spin system. The AB titer against BNT/A1-HCR increased rapidly by the 4th week after immunization and then maintained for several weeks after booster immunization. At week 24, horse sera showed neutralizing activity *in vitro* more than 8 IU of reference horse ANT. Furthermore, 100 μg of purified horse F(ab')2-fragment demonstrated

Table 1: Commercial antitoxins from animal blood

Name of the drug	Composition of the drug	Auxiliary substances	Manufacturer
Diphtheria Antitoxin (Equine)	Enzyme refined, equine diphtheria antitoxic immunoglobulin fragments – not less than 10000 IU/ml (in 10 mL vial)	Cresol – 0.25 %, glycine – 22.5 mg/ml, NaCl – 9 mg/ml, water for injection	VINS Bioproducts Ltd., India
Tetanus antitoxin (Equine)	Enzyme refined, equine tetanus antitoxic immunoglobulin fragments – not less than 1,000 or 1,500 IU/ml (10 mL vial)	Cresol – 0.25 %, glycine – 22.5 mg/ml, NaCl – 9 mg/ml, water for injection	VINS Bioproducts Ltd., India
H-BAT™ (Botulism Antitoxin Heptavalent (A, B, C, D, E, F, G) (Equine))	F(ab')2 and F(ab')2-related equine antibody fragments: serotype A – 4,500 IU/vial, serotype B – 3,300 IU/ vial, serotype C – 3,000 IU/ vial, serotype D – 600 IU: serotype E – 5,100 IU/ vial, serotype F – 3,000 IU/ vial,serotype G – 600 IU/vial (50 mL vial)	NaCl – 9 mg/ml, water for injection	Emergent BioSolutions Inc., USA
Anti-Gas Gangrene Serum (Polyvalent Equine Purified Concentrated Liquid)	30,000 IU/ampoule: Cl. perfringens – 10,000 IU, Cl. oedematiens – 10,000 IU, Cl. septicum – 10,000 IU.	NaCl – 9 mg/ml, water for injection	Microgen, Russia

100% neutralization of 10,000 LD50 *in vivo*[40].

The receptor-binding domain (RBD) of BNT heavy chain instead of inactivated botulism toxoids was used for production of botulism ANT in horses[41]. AGs of BNTs serotypes A, B, E, and F were expressed in recombinant *E. coli*. For comparison, AG of BNT serotype B from the *S. cerevisiae* yeast was used. Ten horses were subcutaneously immunized with 2 mg of recombinant purified AGs, and anti-RBD ABs were detected. IgG was isolated from the serum and used to obtain F(ab')2-fragment. For this purpose, the serum was diluted 1:2 (v/v) with water for injection, pH was adjusted to 3.0 using 1.0N HCl, pepsin was added to the serum from 6-8 horses in concentration of 6 U/ml and incubated for 30 minutes at a temperature of 37 °C. Then 15% (w/v) ammonium sulfate was added to the mixture, the pH was adjusted to 5.4 and incubated in for 30 minutes at 58 °C to precipitate ballast proteins. The resulting mixture was cooled to 45 °C, 0.8% perlite (volcanic rock) was added and mixed, the sorbent was filtered. The pH of the resulting filtrate was adjusted to 7.2, ammonium

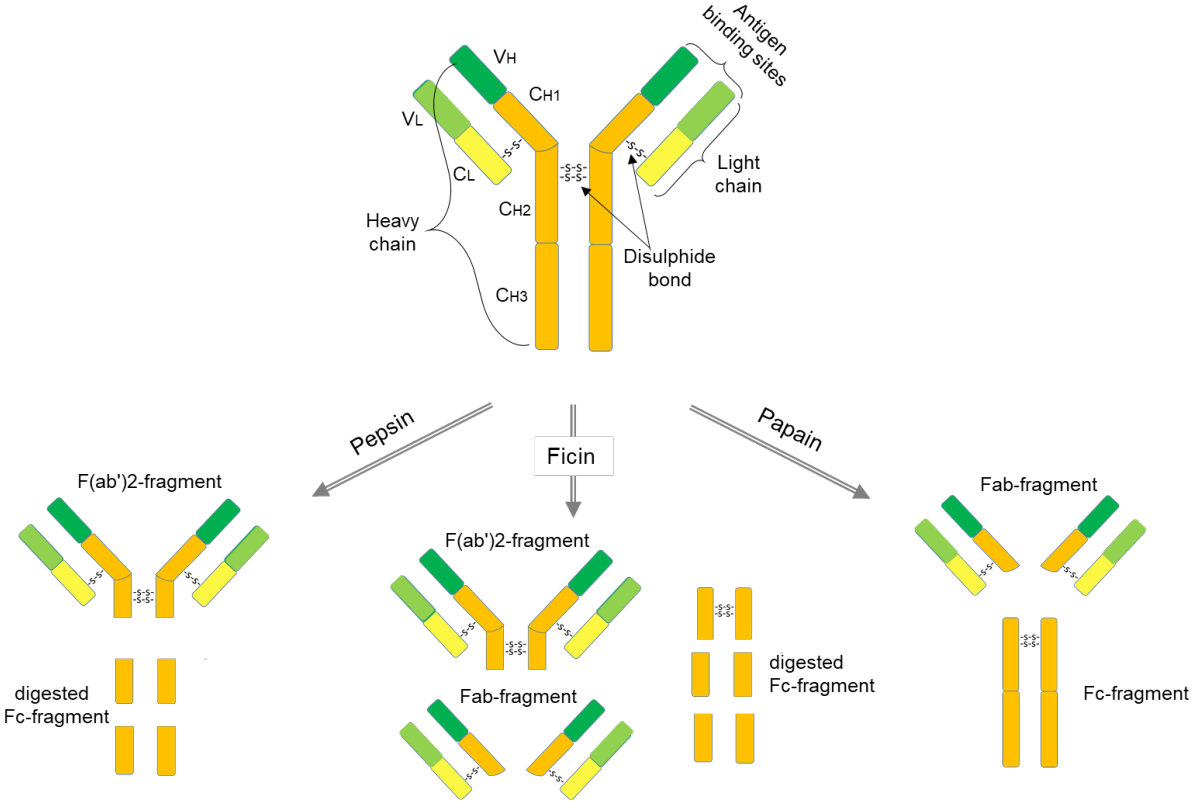

Figure 3: Structure of IgG molecule and fragments obtained by proteolytic digestion with papain, pepsin or ficin.

sulfate was added to a final concentration of 20% and incubated for 45 minutes to precipitate Fc-fragments. The mixture was stirred for 60 minutes, followed by press filtration. An ultrafiltration device with a cutoff value of 50 kDa was used for further F(ab')2-fragment separation and concentration. The resulting F(ab')2-fragment solution was standardized (pH 7.0) and filtered through 0.22 μm. The final product was stored at 4°C.

In recent years, the creation of ANTs against various types of BNT has continued[40-42]. Thus, the botulism ANT against neurotoxin E was developed by F(ab')2-fragment purifying using pepsin digestion of serum IgG from immunized horses[42]. The recombinant heavy chain domain of the neurotoxin E (rEHc) expressed in *E. coli* was used as AG. The AG was purified by chromatography. Aluminum hydroxide was used as an adjuvant. ANT containing F(ab')2-fragments showed the protective effect against BNT serotype E both *in vitro* and *in vivo*[42]. ANTs production using GMP rules is also underway[43, 44].

In the USA, an ANT against seven types of BNT (septivalent) has been proposed. In this case, each individual horse was immunized against one type of BNT, and the plasma from immunized horses was collected separately. IgGs of each serotype of BNT in equine plasma were digested with pepsin to obtain mainly the F(ab')2-fragment. Only after AB titer determination all seven ANTs were mixed into a heptavalent product, which were placed in single-use vials. In March 2013, the FDA has approved a drug based on equine serum containing Abs, that is capable of neutralizing the specified types of BNT for the symptomatic treatment of botulism in adults and children[45]. The drug includes F(ab')2 and F(ab')2-related fragments of IGs and is available only in the

Table 2: IG preparations from the blood of immunized animals

Name of the drug, producer, manufacturer	Composition of the drug	Purpose of the drug
Anti-rabies IG solution (equine), "Biolik", Ukraine	IG – 100 mg/ml, 150 IU/ml; glycine – 25 mg/ml, NaCl -10 mg/ml, water for injection. *	The treatment and prevention of rabies when bitten by rabid or suspected to be rabid animals
"Equirab" – anti-rabies IG solution (equine), "Bharat Serums And Vaccines Limited", India	IG – 100 mg/ml, 300 IU/ml, glycine – 25 mg/ml, NaCl - 10 mg/ml, cresol – 0.25 %; water for injection. *	
"ATGAM®" – anti-human thymocyte IG sterile solution. (equine), "Pfizer", Germany.	IG – 50 mg/ml; glycine, NaCl, water for injection. *	As immunosuppressor for the prevention graft rejection after renal transplantation, the treatment of aplastic anemia in the absence of indications for bone marrow transplantation; to delay the first episode of graft rejection after renal transplantation. The half-life is 5.7±3 days. Ig reduces the number of circulating thymus-dependent lymphocytes (recommended by FDA)
"ATG-Fresenius S" – concentrated anti-human T-lymphocyte immunoglobulin preparation derived from (rabbits immunized with a human T-lympoblast cell line), "Fresenius", Germany.	IG – 5 mg/ml, glycine – 10 mg/ml, NaCl – 2.5 mg/ml, D-mannitol – 10 mg/ml. *	
"Thymoglobulin" – anti-thymocyte IG (rabbit) , powder for solution, "Sanofi", Canada	IG – 5 mg/ml, glycine – 10 mg/ml, NaCl – 2.5 mg/ml, D-mannitol – 10 mg/ml, pH 6.5-7.2, monomers and dimers – not less then 95 %, g-globulin – not less then 95%. *	
Antibotulinum IG against types A and B (equine), concentrate, "Sanofi Pasteur Limited", Canada	7,500 IU of type A and 5,500 IU of type B (one vial), NaCl – 0.9 %, phenol – 0.4 %.	For prevention or treatment of botulism, types A and B (recommended by FDA)
Heptavalent antibotulinum IG against types A, B, C, D, E, F, G (equine), concentrate, "Cangene Corporation", Canada	4,500 IU of type A, 3,300 IU of type B, 3,000 IU of type C, 600 IU of type D, 5,000 IU of type E, 3,000 IU of type F, and 600 IU of type G (one vial), NaCl – 0.9 %. Represented by F(ab')2-fragments.	For prevention or treatment of botulism, types A, B, C, D, E, F, G (recommended by FDA)
"Anascorp®" (Centruroides immune F(ab')2) – IG against scorpion venom (equine), lyophilisate, "Rare Disease Therapeutics", USA	One vial contains 120 mg of total protein and nor less than 150 LD_{50} (mouse) neutralizing units, no less than 85% of F(ab')2-fragments, no more than 7% of F(ab)-fragments, no more than 5% of intact IG; NaCl – 80 mg, glycine – 95 mg, sucrose – 38 mg, cresol – 0.41 mg.	For the treatment of clinical signs of scorpion envenomation (recommended by FDA)

Table 2. (continued)

"Anavip®" (Crotalinae immune F(ab')2) – IG against North American pit viper venom (equine), lyophilisate, "Rare Disease Therapeutics", USA	One vial contains 120 mg of total protein, no less than 85% of F(ab')2-fragments, no more than 7% of F(ab)-fragments, no more than 5% of intact IG; NaCl – 57 mg, glycine – 52 mg, sucrose – 86 mg.	For the treatment of clinical signs of Crotaline snakes (recommended by FDA)
"DIGIFab" – Digoxin immune Fab (ovine), lyophilisate, BTG International, USA.	One vial contains 40 mg of digoxin immune Fab (monovalent) IG fragments, 75 mg of mannitol.	For the treatment of patients with life-threatening or potentially life-threatening digoxin toxicity or overdose (recommended by FDA)

Notes. * – an intradermal test is performed with a diluted IG solution before administration.

USA. The ANT is quite expensive and is available in limited quantities for export outside the USA, that leads to the need to produce botulism ANTs using local domestic horses[40, 46]. Each vial contains 4,500 units of ABs to BNT serotype A, 3,300 units to B, 3,000 units to C, 600 units to D, 5,100 units to E, 3,000 units to F, and 600 units to G[47].

Currently, antitoxins are produced in several countries around the world, such as India (Vins Bioproducts Ltd.), USA (Emergent BioSolutions Inc.), Japan (KM Biologics Co., Ltd.), Russia (Microgen), Brazil, etc. Table 1 shows the compositions of commercial antibacterial antitoxins for prophylaxis and treatment in humans.

3. Antitoxins against Viral Infections

The use of monoclonal ABs yields positive results in the treatment of viral diseases, in particular Ebola, that revived interest in therapy based on specific ABs, in particular equine ones. Equine ANT against the Ebola virus was prepared under GMP conditions. Ebola virus-like particles were used as AG emulsified in Freund's complete adjuvant. Equine serum was precipitated with ammonium sulfate for protein purification, treated with pepsin (5-10 U/ml) for 45-90 minutes, and F(ab')2 fragments were purified up to 95% (SDS PAGE detection method). Each horse produced between 120 and 250 g of purified ANT[48]. The effectiveness of the F(ab')2-fragments and native ABs was compared in a mouse model. Mice were infected with the Ebola virus and then injected with 2 mg of ANTs. Protection of mice from the virus was shown in both cases. Similar results were demonstrated in a guinea pigs model of Ebola. Moreover, the use of equine IGs specific to the Ebola virus glycoprotein showed the effective post-exposure prophylaxis of primates infected with the Ebola virus[49].

ANTs for the treatment of SARS-CoV-2 infection is underway. Equine ABs showed broad-spectrum and high neutralizing activity against SARS-CoV-2 infection in rodents[50, 51]. The ammonium sulfate precipitation was used to purify IgG from horse serum. Pepsin was used to hydrolyze the Fc-region and obtain purified F(ab')2-fragments. Column chromatography with protein A was used to separate intact residual IgG. The use of the ANT based on purified equine of F(ab')2-fragments was completely effective *in vivo,* all SARS-CoV-2 infected mice survived. ANT against the SARS-CoV-2 virus was effective in a decrease in lung pathology in hamster model. Recombinant spike glycoprotein was used as AG for production of specific ABs, which were treated with pepsin to obtain F(ab')2-fragments and separate Fc-fragments[52]. The authors have proposed this drug for COVID-19 treatment.

A number of viruses accumulated mutations in the receptor-binding domain (RBD) of the spike protein, which interacts with the angiotensin-converting enzyme2 (ACE-2) receptor, that enhances cell penetra-

tion. The RBD is the primary target for neutralizing ABs. Equine ANT against SARS-CoV-2 bound RBD and N-proteins of mutated virus variants and inhibited the binding of RBD to ACE-2 receptor[53].

VINCOV-19 is an ANT obtained by immunization of horses with the spike glycoprotein of the inactivated SARS-CoV-2 virus. It demonstrated high neutralizing activity of the virus[54]. Human trials of this ANT was announced by VINS Bioproducts Limited, which produces equine IGs for the treatment of snake, dog, scorpion bites, diphtheria, tetanus, gas gangrene and other infections.

Thus, the possibility of production equine specific ABs to fight viral infections is real today, as evidenced by heterogeneous ANTs against rabies and influenza.

4. Immunoglobulin Preparations from Animal Blood

IG preparations obtained from the blood of immunized animals have been successfully used in prevention and treatment of various infectious diseases for many years[3]. Horses, oxen, goats, sheep, rabbits, and camels can be used as a producers if specified IGs. IGs against rabies virus, tick-borne encephalitis virus, Ebola virus, O-antigen of *B. antracis* (pathologic agent of anthrax), various serotypes of *Leptospira*, smallpox virus, etc. are currently known[55-57].

Smallpox is a severe infectious disease caused by the smallpox virus which quickly spreads among people, causing a mortality rate of up to 30% within 15-20 days of infection. In 1980, the WHO declared the eradication of smallpox and vaccination was stopped. Currently, most people are not vaccinated, and vaccine immunity in people vaccinated before 1980 is waning. The issue of creating ANT against the smallpox virus is relevant, considering the situation in the world and the growth in the number of terrorist organizations and conflicts. ANT against smallpox containing F(ab')2-fragments of equine IGs against vaccinia virus TianTan (VVTT) was developed using modern biotechnology methods of production, purification, and immunization of horses[57]. VVTT was cultured in chicken embryo fibroblast cells, purified and concentrated by ultrafiltration (300 kDa membrane), centrifuged in a sucrose gradient at 30,000 g for 6 hours. Horses were immunized with at least 10-point injection of purified inactivated viral AG in an oil adjuvant through the submaxillary and inguinal lymph nodes. The purified IGs were more than 90% pure (analyzed by HPLC). The titer of the neutralizing ABs against the smallpox virus exceeded 1:3200 in a culture of virus-sensitive cells. ANT demonstrated a high protective effect in prevention and treatment of animals that are susceptive to the smallpox virus. Safety control of ANT met the requirements of the Chinese Pharmacopoeia, the level of side effects was reduced to 1%.

IG preparations are used to create passive immunity. The production of this drugs begins with bacterial or viral AG preparation, development of an animal immunization scheme, and production of immune plasma or serum. Isolation and purification of IGs are carried out using various methods and their modifications, such as Cohn method[58], precipitation using rivanol[3] or caprylic acid[59, 60]. Fragments of IGs are obtained using pepsin, including immobilized one[61] and a number of other technologies. Rabies IG is well known for target neutralization of rabies virus in human bitten by a rabid animal or an animal suspected to be rabid. Rabies IG is produced by both the rivanol-alcohol and the Cohn method. IGs against tick-borne encephalitis virus are also obtained using the rivanol-alcohol method. Rivanol (ethacridine lactate) is used in the first stages of precipitation, which reduces of the number of stages and the duration of the technological process. Thus, the production of IGs against tick-borne encephalitis using the rivanol-alcohol method include 8 technological stages, in contrast to 12 stages when obtained by the Cohn method. The rivanol-alcohol method is also less labor-intensive and cheaper[3]. It consists of following stages: viral AG preparation; preparation, immunization, and exploitation of horses; blood taking from animals, immune plasma or serum obtaining; precipitation of ballast proteins by treating with a rivanol solution, at pH 5.2-5.8 (albumin, α- and β-globulins precipitate); filtration of the precipitate; NaCl is added to the filtrate, and the mixture

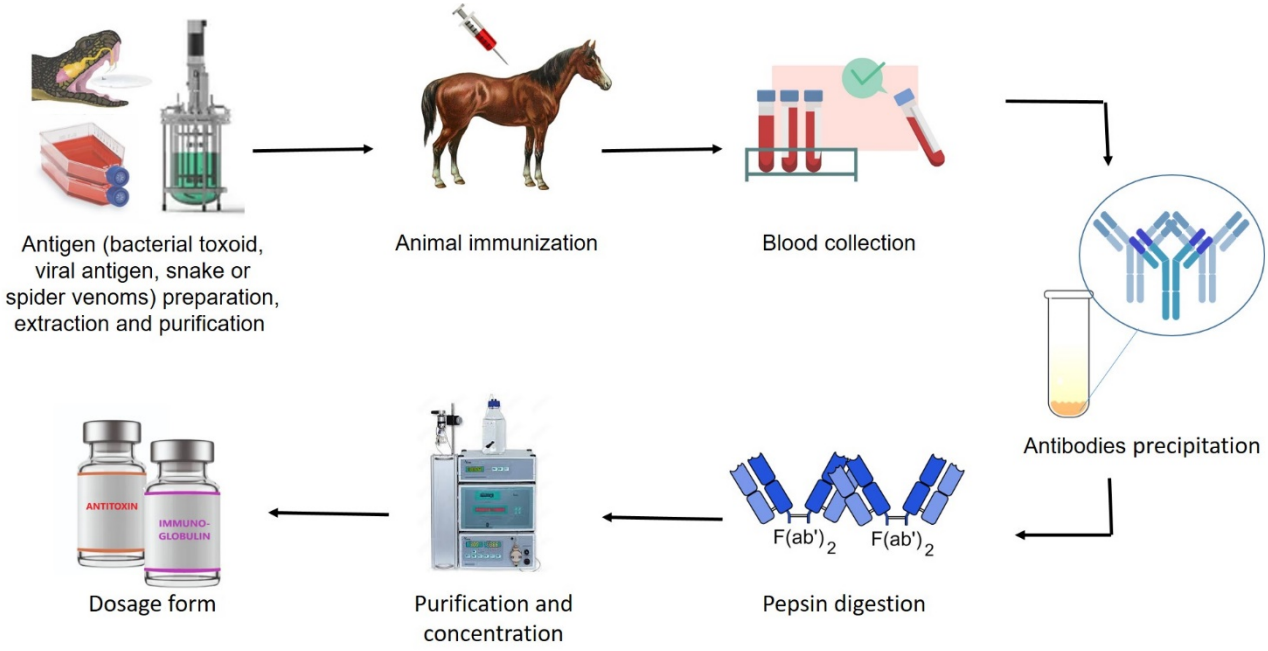

Figure 4: The general technology for producing both ANTs and IG preparations against various bacterial and viral agents, as well as against snake and insect venoms.

is incubated for 12-15 hours at 10-15 °C to form a precipitate of water-insoluble rivanol chloride; the resulting precipitate is separated, the sterilizing filtration is carried out. A strict adherence to temperature conditions and minimal foaming during mixing and centrifugation is an important condition for production stable animal IGs, as is the case with IGs isolation from human blood.

Using the HPLC gel filtration method, the fractional composition of anti-rabies IGs obtained by the rivanol-alcohol method and Cohn method was compared. IGs obtained using rivanol contained 92-94% of monomers and dimers, 3.2-4.25% of aggregates, up to 2.2% of F(ab')2-fragments, while IGs obtained using the Cohn method contained 85-88% of monomers and dimers, 4.0-7.6% of aggregates, up to 4.1% of F(ab')2-fragments. During storage, the content of monomers and dimers decreases, the content of aggregates and fragments increased, and the drug obtained by the Cohn method was more stable[3, 62]. Moreover, IGs by the Cohn method also demonstrated higher purity of precipitation, electrophoretic homogeneity, percent of total protein and virus neutralizing activity, but no differences in authenticity, pH, endotoxins, harmlessness, pH, protein, glycol were observed[3].

According to WHO, at present, heterologous anti-rabies IGs are currently high-purified and safe, but side effects may occur in 1-2% of patients[63]. The use of various enzymes, such as pepsin, papain, plasmin, chymopapain and enzymes of microorganisms (bacteria *S. aureus V8*, fungi of the genus *Aspergillus*), for IG proteolysis have been reported. When pepsin acts on IG molecule, one F(ab')2-fragment and one Fc-fragment are formed (Fig.3). The F(ab')2-fragment is able to bind AG, while the Fc-fragment can fix a complement and bind with tissue receptors. Thus, the removal of the Fc-fragment leads to the elimination of undesirable interactions with Fc-receptors in tissue, which reduces the frequency of side effects. Hydrolysis is carried out by mixing IGs with immobilized pepsin in 0.1 M acetate buffer (pH 4.5) for 24 hours at 37 °C. The optimal IG concentration in the reaction mixture is about 20-30 mg/ml.

F(ab')2-fragments were separated by chromatography with SP-Sepharose-XL, concentrated, and sterilized by sterilizing filtration. In comparative study on white mice, the neutralization activities of IGs and an ANT based on F(ab')2-fragments were 359 IU/ml (titer of specific AB – 1:3119) and 283 IU/ml (titer of specific AB – 1:2460), respectively, with a norm of at least 150 IU/ml[64]. The anti-rabies IG solution containing F(ab')2-fragments also demonstrated the absence of toxicity in white mice[64]. In anti-rabies IG production, when receiving, viral AG for horses immunization were obtained in a kidney cell culture of Syrian hamster. The residual content of AG in kidney cells was less than 0.5 μg/ml. Aluminum phosphate was used as an adjuvant. Plasma was monitored for the absence of anaphylactic and pyrogenic reactions in rabbits. Specific plasma activity was not less than 75 IU/ml. The IG preparation was obtained by fractionation using the caprylate-alcohol method followed by proteolysis with pepsin. The product contained at least 90% of F(ab')2-fragments and had an electrophoretic purity of at least 90%. The specific IG activity was no less than 400 IU/ml.

A lymphocytic IG (antithymocyte IG) obtained by immunizing horses with human thymic lymphocytes is of interest (see Table 2), as it is used in kidney transplantation and aplastic anemia. Table 1 shows a few commercial IG preparations against bacterial, viral AGs and snake venoms obtained from the blood of immunized animals (horses, rabbits, sheep).

A purification of antitetanus IGs was conducted using caprylic acid[59, 66]. The equine immune plasma was mixed with a buffer solution (sodium acetate / acetic acid) to an ionic strength of 0.2 mol/L and caprylic acid was added to a concentration of 3% and pH 4.5, that allowed to precipitate non-IG proteins, which were separated by centrifugation. IG concentration and purification from caprylic acid was carried out by ultrafiltration. The yield of non-precipitated IGs was 91–95%, the ratio albumin to Ig was 0.019–0.021. The following purification gave the antitetanus IG preparation with a purity of at least 96%[59, 66].

A method for anti-smallpox IG preparation consisted of following stages[67]: smallpox AG was obtained on Vero cell line; the cell culture was separated by centrifugation, and the supernatant was used as AG for immunization of horses; horse plasma was defibrinated with 30% calcium chloride; IGs were precipitated using ammonium sulfate while albumin and low molecular weight impurities remained in the supernatant. The sediment contains at least 80% of IGs and no more than 20% of impurities (analyzed by HPLC). Ammonium sulfate was removed by gel filtration using Sephadex G-25. IGs were further purified on a O Sepharose FF and concentrated on 50 kDa molecular weight cutoff Sartocon Slice 200 cassettes. The resulting IGs were fractionized using gel filtration on a Superdex 200 Prer grade sorbent, and fractions containing IG monomers were pooled. The IG preparation had non-toxicity and high antiviral activity[67].

A significant amount of research has been devoted to the development of various IG purification methods and the investigation of their efficacy[68]. Traditionally, equine IGs are purified by fractionation with ammonium sulfate or caprylic acid, which are based on the selective precipitation of all non-IG serum proteins. F(ab')2-fragments are more preferred as ANTs that native ABs due to its favorable pharmacokinetic profile, i.e. wide volume of distribution and ability to reach tissue compartments faster than IGs due to lower molecular weight and immunogenicity. Ammonium sulfate fractionation requires a lot of time and is rather expensive. In addition, the preparation obtained by ammonium sulfate contains not only F(ab')2-fragments, but also impurities from unsplit IGs. A caprylic acid precipitation method was developed later, which turned out to be more effective in production time, product yield, and content of albumin impurities. A one-step caprylic acid precipitation provides about 70% purity of IGs. IG purification is also carried out using ion exchange chromatography, hydrophobic chromatography, protein A or protein G affinity chromatography. Today, chromatography based on protein A or G makes it possible to obtain highly purified IG preparations. A combination of caprylic acid precipitation and chromatography is recommended for IG purification. For example, the combination of caprylic acid precipita-

tion and affinity chromatography have provided IgG with a purity more than 90% (in one step), while the lowest content of aggregates was present in samples treated with anion exchange chromatography[69]. Albumin was the main contaminant in IG preparations prepared by ammonium sulfate precipitation and cation exchange chromatography. The use of affinity chromatography resulted in the loss of IgG(T)-subclass. Capryloic acid and ammonium sulfate did not affect the distribution of subclasses. Changes in the composition of the IgG subclass affected the specific protective effectiveness of the drug. The use of caprylic acid did not change the effectiveness of the drug. The affinity chromatography using Fc-binding IgG ligands has been proposed[69].

5. Antitoxins against Snake and Insect Venoms

A snakebite is a medical emergency and may result in death or irreversible diseases. The only effective treatment against snakebite is intravenous administration of a polyvalent antivenom. The history of antidote to snake venom begins with the work of the French physician, bacteriologist and immunologist Albert Calmette (one of the authors of the BCG vaccine), who in 1894 proposed obtaining ANTs against snake venom on horses and pigs. In this chapter, we will focus on drugs for the treatment and prevention of diseases caused by snake and spider bites.

1.8 million people are bitten by venomous snakes annually, with approximately 94,000 deaths, mostly in poor tropical countries[70]. For example, in Africa, a number of patients of health centers bitten by poisonous snakes reaches more than 300,000 every year, and in tens of thousands of cases it leads to deaths or amputation of limbs with subsequent disability of people. In the Republic of Guinea, in West Africa, the intoxication from the bites of poisonous snakes is 150-200 cases per 100 thousand population with a mortality rate of 18%, which poses a serious problem for the country's health care.

Thus, the need for ANTs against snake venoms is great. However, the availability of ANTs in most African, Asian and South American countries is clearly insufficient, especially in remote provincial health centers where their availability is vital. So, in Africa, two families of snakes, *Elapidae* (asp family) and *Viperidae* (viper family), pose the biggest threat to human health. The venom of snakes of the *Elapidae* family (cobras and mambas) has neurotoxic properties. The poison causes a blockade of neuromuscular synapses, including the respiratory muscles and diaphragm, which leads to respiratory arrest. The venom of snakes of the *Viperidae* family is rich in enzymes and has a hemolytic effect. Two main components, metalloproteases and serine proteases of viper venom, cause poisoning. At the first stage, the venom causes thrombosis occurs, and at the second stage, a blockade of the blood coagulation system occurs, provoking external or internal bleeding called hemorrhagic syndrome. This syndrome is characterized by varying degrees of swelling and the appearance of watery blisters. Bites of vipers, which are found on all continents, are painful, and with the significant intoxication the tissue necrosis develops, which can lead to amputation of limbs. Given the above, antivenoms against venom toxins are fundamental in the treatment of snake bites.

The only rational and effective therapy in these cases is specific antidotes, the development and production of effective, available and sufficient antidotes are underway. The immunization process, which is rarely described in detail, is one step that needs to be carefully studied and improved, especially for production of multispecific ANTs. The polyspecific nature of a therapeutic antivenoms can eliminate the need to identify the species of snake that bit the patient.

Potent polyspecific ANT against three medically important vipers of Thailand and neighboring countries, namely *Cryptelytrops albolabris* (white-lipped viper), *Calloselasma rhodostoma* (Malayan viper) and *Daboia siamensis* (Russell's viper) was developed[70]. Four horses were immunized with a mixture of three viper venoms using a "low dose, low volume, multi-site" immunization protocol. Using ELISA, the rapid increase of AB titers against was showed the 3 poisons, and a plateau was reached approximately 8 weeks after immunization. The neutralizing activities of the ANT against *Cryptelytrops albolabris*,

Calloselasma rhodostoma and *Daboia siamensis* venoms *in vivo* were 10.40, 2.42 and 0.76 mg/mL, respectively, which were much higher than the minimum activity limits. The corresponding values of neutralizing activities for monospecific ANTs against *Cryptelytrops albolabris*, *Calloselasma rhodostoma*, and *Daboia siamensis* venoms were 7.28, 3.12, and 1.50 mg/mL, respectively. Polyspecific ANTs also effectively neutralized the procoagulant, hemorrhagic, necrotic, and nephrotoxic activities of viper venoms. The authors concluded that proposed immunization method should be useful in the production of potent multispecific ANTs against snake venoms. Injections of bentonite, Freund's complete and incomplete adjuvants mixed with low doses of snake venoms were used as adjuvant in the process of intramuscular or dermal immunization of horses against snake venom. Sublethal doses of *Naja oxiana* and *Naja karachians* venoms were mixed with mineral oil M5904 as adjuvant and used for oral immunization of horses, that provided high titer of neutralizing ABs.

Elapid venoms contain toxins, for example short-chain α-neurotoxins, which are quite numerous, highly toxic and, therefore, play a big role in intoxication processes. The main problem is that α-neurotoxins have weak immunogenicity, and many modern antivenoms show low reactivity against them. A recombinant short-chain consensus α-neurotoxin (ScNtx) was developed based on the sequences of the most lethal snake venoms from the Americas, Africa, Asia and Oceania. The antivenom obtained by immunizing horses with ScNtx can successfully neutralize the lethality of both pure recombinant and native short-chain α-neurotoxins, as well as whole neurotoxic venoms of elapids from various genera, such as *Micrurus*, *Dendroaspis*, *Naja*, *Walterinnesia*, and *Ophiophagus*[71, 72]. These results provide proof of the efficacy of recombinant proteins with rationally designed consensus sequences as universal AGs for the development of next-generation antivenoms with higher potency and wider neutralizing capacity.

In Europe, the equine ANT against European viper venom, containing antitoxic neutralizing IGs against venom of one or more viper species, is produced. The drug must neutralize the poison of *Vipera ammodytes*, *Vipera aspis*, *Vipera berus*, *Vipera ursinil* or mixture of these poisons. One milliliter of the drug must contain a sufficient amount of antitoxic IG to neutralize at least 100 (mouse) LD_{50} of the of venom of *Vipera ammodytes*, *Vipera aspis* or other viper species. Purified concentrated liquid ANTs, containing specific AB fragments against the venom of gyurza, efa, and Central Asian cobra, are also produced. ANTs are available as monovalent or polyvalent, and contain 150 antitoxin units (AU) to cobra venom, 500 AU to viper venom, 250 AU to epha venom. In the USA, a polyvalent ANT against all pit vipers and ANT against the venom of the American king adder are produced[73].

Sea snake venom is highly toxic and can cause severe respiratory failure, leading to high mortality. Antivenoms against venoms of land snakes have no effect on intoxication after a sea snake bite. The venom of *Hydrophis curtus* was used as an AG for immunizing horses to develop an antivenom for the dominant species of sea snakes in the Chinese seas[74]. The drug obtained from immune horse plasma had a high AB titer, showed the high neutralizing activity, including cross activity to the venom of another sea snake. Today, this product is at the study stage.

ANTs can be produced on liquid or freeze-dried form for intramuscular or intravenous administration. ANTs against snake and insect venoms are commonly available in large zoos around the world. In addition, there are specialized organizations that deal with information issues, for example, the Oklahoma Center for Poison and Drug Information provides a 24-hour consultation service for doctors. In various countries, ANTs against venoms of local snake species have been proposed, for example, "Inoserp Pan-African", manufactured by Inosan Biopharma Africa taking into account WHO recommendations and requirements, covers at least 18 snake species, inhabitating the sub-Saharan Africa.

ANT for the treatment of black widow spider bite was obtained from horse blood, and ANT for the treatment of scorpion bite was obtained from goat blood [74]. In Japan, a large-scale experimental production of equine ANT against the redbacked spider (*Latrodectus hasseltii*), inhabitating the Japan, was conducted[75]. The toxoid was prepared from a crude extract of the venom from 10,000 redback spiders, then mixed with a mineral oil adjuvant, and used to immunize healthy

horses over several weeks. The horse plasma was separated, the γ-globulin fraction was purified, and purified AB concentrate was stored in lyophilized form as an antidote. The quality of the product corresponds to the valid Japanese specifications. The product showed a neutralizing effect against spider venom [75].

The technology for obtaining ANTs against venoms of snakes and spiders using horses include following steps:

1. Preparation of AGs from the venoms by collecting venom from snakes or decapitating snakes followed by removing the poisonous glands, grinding and extracting the poison with water. If necessary, the suspension is centrifuged and the clear supernatant is lyophilized. The venom is inactivated, for example, with a 0.6% formaldehyde solution.

2. An appropriate adjuvant (Freund's adjuvant, potassium alum, calcium phosphate, etc.) for immunization is selected.

3. Immunization of horses is conducted according to established immunization schemes, for example:

a) to obtain the ANT against viper venom, initially 2-fold grund immunization with increasing doses of AG up to 1.0 mg with break of 2 weeks is conducted. Then immunization is carried out 9 times (from 0.5 mg to 70 mg) every 7 days, and after this, hyperimmunization of 90 and 100 mg is started with an interval of 7 days. Blood collecting begins after the activity reaches at least 150 AU/ml;

b) to obtain the ANT against Sea Snake Hydrophis venom, 1 ml (5 mg/ml) of inactivated venom is administered to each horse by multipoint subcutaneous injection. Then immunization is carried out 5 more times, and blood collecting begins.

Considering the species diversity of venomous snakes and spiders in the world's fauna and the possibility of their contacts with people, there is an urgent need for available polyvalent ANTs consisting of highly purified, lyophilized and effective IG fragments against venoms of the main snakes and spiders of certain regions.

6. Technology for Producing Antitoxins and Immunoglobulin Preparations

The general technology for producing both ANTs and IG preparations against various bacterial and viral agents, as well as against snake and insect venoms can be represented by the following scheme (Fig.4):

1. Preparation of AG for animal immunization includes media preparation, cultivation of bacteria or viruses, production of toxins or toxoids, production of viral antigens, etc.

2. Preparation, immunization and exploitation of animal producers (horses, cattle, mules, oxen, rabbits, etc.[76]).

3. Grund immunization of animals is a selection of animals capable of producing ABs against the administered AG in high titers.

4. Hyperimmunization of animal producers is a parenteral administration of increasing doses of AG to animals in order to obtain a maximum immune response in the animal. AG is administered subcutaneously or intramuscularly in several places near the lymph nodes. Adjuvants are used for immunization, and the adjuvant selection is an independent issue. Dozens of adjuvants for the production of hyperimmune serums have been proposed in the literature, including aluminum hydroxide, aluminum phosphate, potassium alum, aerosil, oil emulsion adjuvants, oil emulsions containing inactivated *Mycobacterium tuberculosis*, saponins of *Quillaja saponaria*, nanoparticles of cholesterol, phospholipids and ISCOMs, liposomes, etc.[77-81]. The mechanisms of action of adjuvants are varied and are constantly being studied. Some of them cause an inflammatory reaction, others promote depositing, others are carriers of AG, etc.

5. Blood collection from animals is usually conducted 7-10 days after the last immunization, when the titer of specific IGs is maximum. Blood is taken from the jugular vein in an amount of 10-13% of the total blood mass, taking into account the condition of the animal. Blood sampling is carried out by venipuncture or plasmaphoresis.

6. Purification and concentration of ANTs are carried out using precipitation with ammonium sulfate at various concentrations, treatment with pepsin to obtain F(ab')2-fragments, concentration by ultrafiltration, removal of pepsin and ammonium sulfate, pressing of the precipitate, centrifugation or separation, sterilizing filtration[30, 40, 82–84]. These operations can be carried out in different orders at different tem-

peratures, pH and ratio of components.

7. Purification and concentration of IG preparations are carried out using precipitation with ethanol, rivanol, caprylic acid, treatment with pepsin to obtain F(ab')2-fragments (if necessary), centrifugation or separation, sterilizing filtration[3, 66, 84, 85.] Various chromatography methods have been proposed for the purification of IGs, such as anion exchange, affinity, exclusion, hydrophobic, etc. [66, 68, 85].

8. The filling of primary packaging with sterile solution of ANT or IG preparation, labeling and secondary packaging.

9. Control of the resulting drug.

This scheme can be used with various modifications.

7. Conclusions

ANTs and IG preparations are currently produced in many countries around the world. It is difficult to imagine the current arsenal of medicines without sufficient quantities of various ANTs and IG preparations (antitetanus, antidiphtheria, antibotulinum, antivenoms against snake and spider, antirabies, anticovid, etc.), obtained by immunization of animal-producers, in particular horses, with appropriate AGs. In recent years, cases of botulism, including food-borne botulism, diphtheria and tetanus have been reported. The creation of ANTs against COVID-19 is also quite promising[51-53, 86]. Equine polyclonal ABs have been shown to be an effective, low-cost, scalable treatment for COVID-19 with broad coverage. ABs recognize a variety of epitopes on viral proteins and have high binding affinities, that provides broader recognition and neutralization than in plasma of vaccinated or convalescent people[86].

The events of the recent years in Ukraine and in the whole world are cause for concern about using BNT or the smallpox virus as a bioterrorist weapon. In this context, as well as in case of normal exposure to pathogenic microorganisms on human, numerous therapeutic products have been developed or are currently being developed to treat or prevent a number of infections, such as botulism[67]. Works aimed at creating bioweapons based on botulism are known[43, 87]. Importantly, *C. botulinum* toxins were among the first candidates for bioterrorism because they can be produced, stored, and distributed. Need to realize that BNT is a biological warfare agent which affects only living organisms without damaging everything around them. The state must have a sufficient guaranteed supply of BNT and other therapeutic drugs to protect the population from infectious agents. The work on standardization of well-known ANTs is still being carried out today[88].

Currently, a commercial process for obtaining horse antiserum and its derivatives has been developed in many countries. Removal of Fc-fragment from IgG allows equine F(ab')2 to be an acceptable immunotherapeutic agent with reduced side effects in complex and hard-to-treat diseases, especially snakebites and highly pathogenic infectious diseases[84-86]. □

References

1. Popoff M.R., Faure G., Legout S., Ladant D. Animal Toxins: A Historical Outlook at the Institut Pasteur of Paris. *Toxins.* 15, 462, 2023. https://doi.org/10.3390/toxins15070462
2. Cholakov I. (1987). Nobel Prizes. Scientists and discoveries. *Moscow, World*, pp. 369.
3. Krasnopolsky Yu.M., Borshchevskaya M.I. (2008). Biotechnology of immunobiological preparations. *Kharkiv, Farmitek*, pp. 267-288.
4. Ek N. Serum levels of the immunoglobulins IgG and IgG(T) in horses. *Acta veterinaria Scandinavica*. 15, 609-619, 1974. https://doi.org/10.1186/BF03547230
5. Wagner B. Immunoglobulins and immunoglobulin genes of the horse. *Dev. Comp. Immunol*. 30, 155-164, 2006. https://doi.org/10.1016/j.dci.2005.06.008
6. Burgasova P.N.(ed). (1978) Guide to vaccine-serum production. *Moscow, Medicine*, pp. 439.
7. Karpov S.P., Prager S.M., Sinelnikov G.E., Fedorov Yu.V. (1976). Hyperimmune serums. *Tomsk, Publishing house TSU*, pp. 376.
8. Korshun S.V., Nedrigailov V.I., Ostryanin G.Ya. (1902). On obtaining anti-diphtheria serum rich in antitoxins. In: *Materials of reports at the Pirogov Congress*. Moscow.
9. Golbets I.I. (1998). To the 100th anniversary of the Biolek enterprise. Scientific research. *In: Collection of reports of the scientific and practical conference "Standardization, control and production of immunobiological and medicinal products", dedicated to the 100th anniversary of the Kharkov enterprise "Biolek"»*. Kharkiv, pp. 12-35.
10. Petrenko M.D., Cherkas G.P., Ponomarenko M.G. Obtaining polyvalent serums against gas gangrene and tetanus. *Collection of works of the Kharkov Mechnikov Research Institute of Vaccines and Serums.* 27, 141-144. 1963.
11. Golbets I.I., Kazarova T.A., Orlova G.L.. Dynamics of biochemical and immunochemical properties of purified toxoid Cl. oedematiens in the process of its manufacture. *Vaccines and Serums*. 7, 66-78, 1973.
12. Golbets I.I. On the issue of fractionation of botulinum antigens types A and B. *Theoretical and practical issues of vaccine-serum business : Republican Interdepartmental Collection*. 3, 89-100, 1969.
13. Dalin M.V., Fish N.G. (1980). Protein toxins of microbes. *Moscow, Medicine*, pp. 224.
14. Sonobe M.H., A Trezena.G., Guilhen F.B., Takano V.L., Fratelli F., Sakauchi D., Morais J.F., Prado S.M., Higashi H.G. Determination of low tetanus or diphtheria antitoxin titers in sera by a toxin neutralization assay and a modified toxin-binding inhibition test. *Braz. J. Med. Biol. Res.* 40, 69-76, 2007. https://doi.org/10.1590/s0100-879x2007000100009
15. Smith H.L., Saia G., Lobikin M., T. Tiwari, S.C. Cheng, D.C. Molrine. Characterization of serum anti-diphtheria antibody activity following administration of equine anti-toxin for suspected diphtheria. *Hum. Vaccin. Immunother*. 13, 2738-2741, 2017. https://doi.org/10.1080/21645515.2017.1362516
16. Bermejo-Martin J.F., Avila-Alonso A., González-Rivera M., Tamayo E., Eiros J.M., Almansa R. Post-booster Antibodies from Humans as Source of Diphtheria Antitoxin. *Emerg. Infect. Dis*. 22, 1265-1267, 2016. https://doi.org/10.3201/eid2207.151670
17. Mezin I.A., Menzeleev R.F., Krasnopolsky Yu.M.. Purification of tetanus toxin by affinity chromatography on reversed-phase sorbents with adsorbed gangliosides. *Biotechnology*. 4, 22-25, 1992.
18. Calvo A.C., Oliván S., Manzano R., Zaragoza P., Aguilera J., Osta R. Fragment C of tetanus toxin: new insights into its neuronal signaling pathway. *Int. J. Mol. Sci*. 13, 6883-6901, 2012. https://doi.org/10.3390/ijms13066883
19. Yu R., Ji C., Xu J., Wang D., Fang T., Jing Y., Kwang-Fu Shen C., Chen W. The Immunogenicity of the C Fragment of Tetanus Neurotoxin in Production of Tetanus Antitoxin. *BioMed Res. Intl.* 2018, 6057348, 2018. https://doi.org/10.1155/2018/6057348
20. Helting T.B., Zwisler O.. Structure of tetanus toxin. I. Breakdown of the toxin molecule and discrimination between polypeptide fragments. *J. Biol. Chem.* 252, 187-193, 1977.
21. Bienert S., Waterhouse A., de Beer T.A., Tauriello G., Studer G., Bordoli L., Schwede T. The SWISS-MODEL Repository-new features and functionality. Nucleic Acids Res. 45(D1), D313–D319, 2017.

https://doi.org/10.1093/nar/gkw1132
22. Joseph M., Woldeamanuel Y., Medhin G., Manyazewal T., Fekadu A., Makonnen E.. Safety of equine tetanus antitoxin for prophylactic use in Ethiopia: a retrospective multi-center study. *Trop. Med. Health.* 51, 23, 2023. https://doi.org/10.1186/s41182-023-00518-8
23. Giri B., Kole L. (2015). Combating the Insidious Enemy: Epidemiology, Pathophysiology, and Treatment of Clostridial Gas Gangrene. *In: Gopalakrishnakone P., Balali-Mood M., Llewellyn L., Singh B.R. Ed(s). Biological Toxins and Bioterrorism. Toxinology.* Dordrecht, Springer, pp. 425–448. https://doi.org/10.1007/978-94-007-5869-8_36
24. Fu Y., Alenezi T., Sun X. Clostridium perfringens-Induced Necrotic Diseases: An Overview. *Immuno.* 2, 387-407, 2022. https://doi.org/10.3390/immuno2020024
25. Aggelidakis J., Lasithiotakis K., Topalidou A., Koutroumpas J., Kouvidis G., Katonis P..Limb salvage after gas gangrene: a case report and review of the literature. *World J. Emerg. Surg. : WJES.* 6, 28, 2011. https://doi.org/10.1186/1749-7922-6-28
26. Becker M. On the production of gas gangrene serum in general and of polyvalent gas gangrene aphylacto-serum of the horse especially using the simultaneous immunization method with the 4 known gas gangrene types in uno. *Arch. Exp. Veterinarmed..* 21, 519-546, 1967.
27. Yanamoto A., Ito A., Murata R., Uematsu N., Nagai K. Hyperimmunization of horses with alpha toxoid of Clostridium perfringens. *Jpn. J. Med.l Sci. Biol.* 23, 111-115, 1970. https://doi.org/10.7883/yoken1952.23.111
28. Orlova G.L., Krasnopolsky Yu.M., Golbets I.I. (1981). Standardization of methods for monitoring and specific activity of gangrenous toxins and anti-gangrenous serums. *In: Current Issues of Immunoprophylaxis of viral and bacterial infections*, Tomsk. pp. 179-180.
29. Chai T., Liu X., Wang H., inventors; Shandong Agricultural University, assignee. C and D type C. perfringens antitoxin serum and preparation method thereof. China patent 104829712A. 2015.
30. Komarovskaya E.I., Perelygina O.V. Current incidence of certain clostridial infections: gas gangrene and tetanus. *Biopreparations. Biogical Products Prevention, Diagnosis, Treatment.* 21, 31-38, 2021. http://dx.doi.org/10.30895/2221-996X-2021-21-1-31-38
31. Hifumi T., Koido Y., Takahashi M., Yamamoto A. Antitoxin treatment for liver abscess caused by Clostridium perfringens. *Clin. Mol. Hepatol..* 19, 97-98, 2013. https://doi.org/10.3350/cmh.2013.19.1.97
32. Liu S., Yang X., Zhang H., Zhang J., Zhou Y., Wang T., Hu N., Deng X., Bai X., Wang J. Amentoflavone Attenuates Clostridium perfringens Gas Gangrene by Targeting Alpha-Toxin and Perfringolysin O. *Front. Pharmacol*. 11, 179, 2020. https://doi.org/10.3389/fphar.2020.00179
33. Zhang J., Liu S., Xia L., Wen Z., Hu N., Wang T., Deng X., He J., Wang J. Verbascoside Protects Mice From Clostridial Gas Gangrene by Inhibiting the Activity of Alpha Toxin and Perfringolysin O. *Front. Microbiol.* 11, 1504, 2020. https://doi.org/10.3389/fmicb.2020.01504
34. Wendt S., Eder I., Wölfel R., Braun P., Lippmann N., Rodloff A. Botulism: Diagnosis and Therapy. *Deutsche Medizinische Wochenschrift.* 142, 1304-1312, 2017. https://doi.org/10.1055/s-0043-112232
35. Pellett S., Yaksh T.L., Ramachandran R. Current status and future directions of botulinum neurotoxins for targeting pain processing. *Toxins*. 7, 4519–4563, 2015. https://doi.org/10.3390/toxins7114519
36. Silvaggi N.R., Boldt G.E., Hixon M.S., Kennedy J.P., Tzipori S., Janda K.D., Allen K.N. Structures of Clostridium botulinum Neurotoxin Serotype A Light Chain complexed with small-molecule inhibitors highlight active-site flexibility. *Chem. Biol..* 14, 533-542, 2007. https://doi.org/10.1016/j.chembiol.2007.03.014
37. Matak I., Lacković Z. Botulinum neurotoxin type A: Actions beyond SNAP-25. *Toxicology.* 335, 79-84, 2015. https://doi.org/10.1016/j.tox.2015.07.003
38. Siegel L.S. Evaluation of neutralizing antibodies to type A, B, E, and F botulinum toxins in sera from human recipients of botulinum pentavalent (ABCDE) toxoid. *J.Clin. Microbiol.* 27, 1906-1908, 1989. https://doi.org/10.1128/jcm.27.8.1906-

1908.1989

39. Li D., Mattoo P., Keller J.E.. New equine antitoxins to botulinum neurotoxins serotypes A and B. *Biologicals : Journal of the International Association of Biological Standardization.* 40, 240-246, 2012. https://doi.org/10.1016/j.biologicals.2012.03.004
40. Kim N.Y., Park K.E., Lee Y.J., Kim Y.M., Hong S.H., Son W.R., Hong S., Lee S., Ahn H.B., Yang J., Seo J.P., Lim Y.K., Yu C.H., Hur G.H., Jeong S.T., Lee H.S., Song K., Kang T.J., Shin Y.K., Choi J.S., Choi J.Y. Development of an Equine Antitoxin by Immunizing the Halla Horse with the Receptor-Binding Domain of Botulinum Neurotoxin Type A1. *J. Microbiol. Biotechnol*. 29, 1165-1176, 2019. https://doi.org/10.4014/jmb.1904.04027
41. Shi D.Y., Lu J.S., Mao Y.Y., Liu F.J., Wang R., Du P., Yu S., Yu Y.Z., Yang Z.X. Characterization of a novel tetravalent botulism antitoxin based on receptor-binding domain of BoNTs. *Appl. Microbiol. Biotechnol.* 107, 3205–3216, 2023. https://doi.org/10.1007/s00253-023-12515-2
42. Shi D.Y., Liu F.J., Mao Y.Y., Cui R.T., Lu J.S., Yu Y.Z., Dong X.J., Yang Z.X., Sun Z.W., Pang X.B. Development and evaluation of candidate subunit vaccine and novel antitoxin against botulinum neurotoxin serotype E. *Hum. Vaccin. Immunother.* 16, 100-108, 2020. https://doi.org/10.1080/21645515.2019.1633878
43. Rasetti-Escargueil C., Popoff M.R. Antibodies and Vaccines against Botulinum Toxins: Available Measures and Novel Approaches. *Toxins.* 11, 528, 2019. https://doi.org/10.3390/toxins11090528
44. Wheeler M.W. Production of Monovalent Botulinus Antitoxic Serum Types A and B. *J. Immunol.* 8, 501–505, 1923. https://doi.org/10.4049/jimmunol.8.6.501
45. Rao A.K., Sobel J., Chatham-Stephens K., Luquez C. Clinical Guidelines for Diagnosis and Treatment of Botulism, 2021. *Recommendations and Reports*. 70, 1-30, 2021. https://doi.org/10.15585/mmwr.rr7002a1
46. Barker D., Gillum K.T., Niemuth N.A., Kodihalli S.. Therapeutic efficacy of equine botulism heptavalent antitoxin against all seven botulinum neurotoxins in symptomatic guinea pigs. *PloS one.* 14, e0222670, 2019. https://doi.org/10.1371/journal.pone.0222670
47. Beliveau M., Anderson D., Barker D., Kodihalli S., Simard E., Hall C., Richardson J.S.. Exposure-Response Modeling and Simulation to Support Human Dosing of Botulism Antitoxin Heptavalent Product. *Clin. Pharmacol. Ther.* 112, 171-180, 2022. https://doi.org/10.1002/cpt.2620
48. Zheng X., Wong G., Zhao Y., Wang H., He S., Bi Y., Chen W., Jin H., Gai W., Chu D., Cao Z., Wang C., Fan Q., Chi H., Gao Y., Wang T., Feng N., Yan F., Huang G., Zheng Y., N Li., Li Y., Qian J., Zou Y., Kobinger G., Gao G.F., Qiu X., Yang S., Xia X. Treatment with hyperimmune equine immunoglobulin or immunoglobulin fragments completely protects rodents from Ebola virus infection. *Scientific reports.* 6, 24179, 2016. https://doi.org/10.1038/srep24179
49. Wang H., Wong G., Zhu W., He S., Zhao Y., Yan F., Rahim M.N., Bi Y., Zhang Z., Cheng K., Jin H., Cao Z., Zheng X., Gai W., Bai J., Chen W., Zou Y., Gao Y., G Gao.F., Yang S., Xia X., Qiu X. Equine-Origin Immunoglobulin Fragments Protect Nonhuman Primates from Ebola Virus Disease. *J. Virol..* 93, e01548-18, 2019. https://doi.org/10.1128/JVI.01548-18
50. Hansda A., Biswas D., Bhatta A., Chakravorty N., Mukherjee G. Plasma therapy: a passive resistance against the deadliest. *Hum. Vaccin. Iimmunother.* 18, 2006026, 2022. https://doi.org/10.1080/21645515.2021.2006026
51. Li E., Han Q., Bi J., Wei S., Wang S., Zhang Y., Liu J., Feng N., Wang T., J Wu., Yang S., Zhao Y., Liu B., Yan F., Xia X. Therapeutic equine hyperimmune antibodies with high and broad-spectrum neutralizing activity protect rodents against SARS-CoV-2 infection. *Front. Immunol..* 14, 1066730, 2023. https://doi.org/10.3389/fimmu.2023.1066730
52. Cunha L.E.R., Stolet A.A., Strauch M.A., Pereira V.A.R., Dumard C.H., Gomes A.M.O., Monteiro F.L., Higa L.M., Souza P.N.C., Fonseca J.G., Pontes F.E., Meirelles L.G.R., Albuquerque J.W.M., C Sacramento.Q., N Fintelman-Rodrigues., Lima T.M., Alvim R.G.F., Marsili F.F., Caldeira M.M., Zingali R.B., G. de Oliveira A.P., Souza T.M.L., Silva A.S., Muller R Rodrigues., D.D.R.F., Jesus da Costa L., Alves A.D.R., Pinto M.A., Oliveira A.C., Guedes H.L.M., Tanuri A.,

Castilho L.R., Silva J.L. Polyclonal F(ab')2 fragments of equine antibodies raised against the spike protein neutralize SARS-CoV-2 variants with high potency. *iScience.* 24, 103315, 2021. https://doi.org/10.1016/j.isci.2021.103315

53. Andrade S.A., Batalha-Carvalho J.V., Curi R., Wen F.H., Covas D.T., Chudzinski-Tavassi A.M., Moro A.M. Equine Anti-SARS-CoV-2 Serum (ECIG) Binds to Mutated RBDs and N Proteins of Variants of Concern and Inhibits the Binding of RBDs to ACE-2 Receptor. *Front. Immunol*. 13, 871874, 2022. https://doi.org/10.3389/fimmu.2022.871874
54. Liu X., Liu Y., Jin X., He Z., Huang Z., Sun S., Gao Y., Li J., Ning Q., Xie Z., Jin N., Liu M. Rapidly developable therapeutic-grade equine immunoglobulin against the SARS-CoV-2 infection in rhesus macaques. *Signal Transduct. Target. Ther.* 7, 219, 2022. https://doi.org/10.1038/s41392-022-01095-8
55. Lub M.Yu., Shevtsov A.N., Kozhukhov V.V., Logvinov S.V., Sedelnikov I.N., Kozlova T.N., Fokina V.V., inventors; Federal State Institution "48 Central Research Institute of the Ministry of Defense of the Russian Federation", assignee. Serum immunobiological preparation for emergency anthrax prevention and treatment. Russian Federation patent 2381037C1. 2010.
56. Mikhailov V.V., Borisevich N.A., Timankova A.D., Krasnyansky V.P., Potryvaeva N.V., Lebedinskaya E.V., Chernikova N.K., inventors; Virology Center of the Research Institute of Microbiology of the Ministry of Defense of the Russian Federation, assignee. Preparation containing immunoglobulin against abol fever from horse blood serum and liquid abol immunoglobulin. Russian Federation patent 2130318C1, 1999.
57. Shi B., Han H., Tan L., Liu Y., Yan F., Li B., Zheng N., Li M., Zhao L., Li H., Fan T., Zhang Z., Li X., Tian C., Zheng Y., He W., Zhao Z. Preparation of Equine Immunoglobulin F(ab') 2 against Smallpox and Evaluation of its Immunoprotective Effect. *Zoonoses.* 3, e992, 2023. http://dx.doi.org/10.15212/ZOONOSES-2022-0048
58. Cohn E.J., Oncley J.L., Strong L.E., Hughes W.L., Armstrong S.H. Chemical, clinical, and immunological studies on the products of human plasma fractionation. I. The characterization of the protein fractions of human plasma. *J. Clin. Invest*. 23, 417-432, 1944. https://doi.org/10.1172/JCI101508
59. Cruz A.M., Oscar O.A., Dailin D.C., Oliver P.M. (2015). Caprylic Acid in the purification of horse tetanus IgG immunoglobulin. *Conference Abstract: IMMUNOCOLOMBIA2015 - 11th Congress of the Latin American Association of Immunology. Frontiers in Immunology.* https://doi.org/10.3389/conf.fimmu.2015.05.00241
60. Al-Abdulla I., Casewell N.R., Landon J. Single-reagent one-step procedures for the purification of ovine IgG, F(ab')2 and Fab antivenoms by caprylic acid. *J. Immunol. Methods*. 402, 15-22, 2014. https://doi.org/10.1016/j.jim.2013.11.001
61. Puvanakrishnan R., Bose S.M. Immobilization of pepsin on sand: preparation, characterization and application. *Indian J. Biochem. Biophys.* 21, 323-326, 1984.
62. Sitnik N.P. (2007). Development of a highly purified anti-rabies immunoglobulin preparation from horse plasma. Ufa, Ph.D. thesis in biology. pp. 20.
63. WHO Expert Consultation on Rabies: first report. Geneva, 2004, pp. 121.
64. Generalov S.V., Abramova E.G., Nikiforov A.K., Hramkova E.M., Shepelev I.A., Savitskaya L.V., Minaeva L.N., Galkina M.V., Miheeva T.A., Kochkalova N.N., Kireev M.N.. F(ab')2-Fragments of Antirabic Immunoglobulin Production Using Immobilized Pepsin. *Problems of especially Dangerous Infections.* 97, 53-56, 2008.
65. Mótyán J.A., Tóth F., Tőzsér J. Research applications of proteolytic enzymes in molecular biology. *Biomolecules*. 3, 923-942, 2013. https://doi.org/10.3390/biom3040923
66. Miranda-Cruz A.R., Sánchez-Artigas R., Otero-Alfaro O., Góngora-Amores W., Cobos-Valdes D., Goya-Batista Y., Balboa-González J., Pérez-Martín O. Using the caprylic acid in obtaining the horse immunoglobulin anti tetanus toxin. *VacciMonitor.* 23, 63-72, 2014.
67. Borisevich S.V., Kutaev D.A., Rozhdestvensky E.V., Gordeev E.V., Khmelev A.L., Nazarov S.V., Melnikov S.A., Nimirskaya S.A., Chernikova N.K., Podkuiko V.N., inventors; Federal State Institution "48

Central Research Institute of the Ministry of Defense of the Russian Federation", assignee. Method of producing smallpox immunoglobulin from horse blood serum. Russian Federation patent 2770425C2. 2021.

68. Fernandes A., Kaundinya J.O., Daftary G., Saxena L., Banerjee S., Pattnaik P. Chromatographic purification of equine immunoglobulin G F(ab)2 from plasma. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 876, 109-115, 2008. https://doi.org/10.1016/j.jchromb.2008.10.030
69. Mateljak Lukačević S., Kurtović T., Lang Balija M., Brgles M., Steinberger S., Marchetti-Deschmann M., Halassy B. Quality-Related Properties of Equine Immunoglobulins Purified by Different Approaches. *Toxins.* 12, 798, 2020. https://doi.org/10.3390/toxins12120798
70. Sapsutthipas S., Leong P.K., Akesowan S., Pratanaphon R., Tan N.H., Ratanabanangkoon K. Effective equine immunization protocol for production of potent poly-specific antisera against Calloselasma rhodostoma, Cryptelytrops albolabris and Daboia siamensis. *PLoS neglected tropical diseases.* 9, e0003609, 2015. https://doi.org/10.1371/journal.pntd.0003609
71. de la Rosa G., Olvera F., Archundia I.G., Lomonte B., Alagón A., G. Corzo. Horse immunization with short-chain consensus α-neurotoxin generates antibodies against broad spectrum of elapid venomous species. *Nat. Commun.* 10, 3642, 2019. https://doi.org/10.1038/s41467-019-11639-2
72. Wang B., Liu G., Luo M., Zhang X., Wang Q., Zou S., Zhang F., Jin X., Zhang L. Preparation and Evaluation of a Horse Antiserum against the Venom of Sea Snake Hydrophis curtus from Hainan, China. *Toxins.* 14, 253, 2022. https://doi.org/10.3390/toxins14040253
73. Pucca M.B., Cerni F.A., Janke R., Bermúdez-Méndez E., Ledsgaard L., Barbosa J.E., Laustsen A.H. History of Envenoming Therapy and Current Perspectives. *Front. Immunol.* 10, 1598, 2019. https://doi.org/10.3389/fimmu.2019.01598
74. Dart R.C., Bush S.P., Heard K., Arnold T.C., Sutter M., Campagne D., Holstege C.P., Seifert S.A., Lo J.C.Y., Quan D., S Borron., Meurer D.A., Burnham R.I., McNally J., Garcia-Ubbelohde W., Anderson V.E. The Efficacy of Antivenin Latrodectus (Black Widow) Equine Immune F(ab')2 Versus Placebo in the Treatment of Latrodectism: A Randomized, Double-Blind, Placebo-Controlled, Clinical Trial. *Ann. Emerg. Med.* 74, 439-449, 2019. https://doi.org/10.1016/j.annemergmed.2019.02.007
75. Mori S., Horita A., Ginnaga A., Miyatsu Y., Sawabe K., Matsumura T., Ato M., Yamamoto A., Shibayama K., Arai S., Yamagishi T., Takahashi M., Taki H., Hifumi T. Venom and Antivenom of the Redback Spider (Latrodectus hasseltii) in Japan. Part II. Experimental Production of Equine Antivenom against the Redback Spider. *Jpn. J. Infect. Dis.* 70, 635-641, 2017. https://doi.org/10.7883/yoken.JJID.2017.125
76. Manteca Vilanova X., Beaver B., Uldahl M., Turner P.V. Recommendations for Ensuring Good Welfare of Horses Used for Industrial Blood, Serum, or Urine Production. *Animals.* 11, 1466, 2021. https://doi.org/10.3390/ani11051466
77. Freund J., Thomson K.J. Antibody formation and sensitization with the aid of adjuvants. *J. Immunol.* 60, 383-398, 1948.
78. Cox J.C., Liefman C.E., Premier R.R., Chandler H.M., Herrington R.W., Middleton H.D., Hurrell J.G. Immune response and reactions to various dose regimens for raising hyperimmune antisera in sheep. *Vet. Immunol. Immunopathol*. 7, 65-72, 1984. https://doi.org/10.1016/0165-2427(84)90028-x
79. Krasnopolsky Yu.M., Pylypenko D.M. (2022). Pharmaceutical biotechnology: present and future. *Kharkiv, Printing House "Madrid".* pp. 151.
80. Krasnopolsky Y., Pylypenko D. Licensed liposomal vaccines and adjuvants in the antigen delivery system. *Biotechnologia.* 103, 409-423, 2022. https://doi.org/10.5114/bta.2022.120709
81. Grygorieva G., Pylypenko D., Krasnopolsky Y. Liposomes: from August Wassermann to Vaccines Against COVID-19: Review. *ADMET and DMPK*. 11, 487–497, 2023. https://doi.org/10.5599/admet.1926
82. Buer A.W. A method for production of high-titre tetanus serum. *Acta Pathologica Microbiologica Scandinavica*. 23, 293-298, 1946. https://doi.org/10.1111/j.1699-0463.1946.tb00543.x

83. Pan X., Wu Y., Wang W., Zhang L., Xiao G. Development of horse neutralizing immunoglobulin and immunoglobulin fragments against Junín virus. *Antivir. Res.* 174, 104666, 2020. https://doi.org/10.1016/j.antiviral.2019.104666
84. Mashin V.V., Sergeev A.N., Martynova N.N., Oganov M.D., Sergeev A.A., Kataeva V.V., Zagidullin N.V. Ensuring Viral Safety of Equine Immunoglobulins during Production. *Pharm. Chem. J.* 56, 283-288, 2022. https://doi.org/10.1007/s11094-022-02632-z
85. Mottate K., Yokote H., Mori S., Horita A., Miyatsu Y., Torii Y., Kozaki S., Iwaki M., Takahashi M., Ginnaga A.. Retrospective survey to evaluate the safety and efficacy of Japanese botulinum antitoxin therapy in Japan. *Toxicon : official journal of the International Society on Toxinology.* 110, 12-18, 2016. https://doi.org/10.1016/j.toxicon.2015.11.010
86. Moreira-Soto A., Arguedas M., Brenes H., Buján W., Corrales-Aguilar E., Díaz C., Echeverri A., Flores-Díaz M., Gómez A., Hernández A., Herrera M., León G., Macaya R., Kühne A., Molina-Mora J.A., Mora J., Sanabria A., Sánchez A., Sánchez L., Segura Á., Segura E., Solano D., Soto C., Stynoski J.L., Vargas M., Villalta M., Reusken C.B.E.M., Drosten C., Gutiérrez J.M., Alape-Girón A., Drexler J.F. High Efficacy of Therapeutic Equine Hyperimmune Antibodies Against SARS-CoV-2 Variants of Concern. *Front. Med.* 8, 735853, 2021. https://doi.org/10.3389/fmed.2021.735853
87. Bozheyeva G., Kunakbayev Y., Yeleukenov D. (1999). Former Soviet biological weapons facilities in Kazakhstan : past, present, and future. *USA, Center for Nonproliferation Studies, Monterey Institute of International Studies, Monterey, CA.* pp. 29.
88. Expanded Access Investigational New Drug (IND) Application Protocol: Use of Diphtheria Antitoxin (DAT) for Possible Diphtheria Cases BB-IND 11184. Version Number 12.0. Centers for Disease Control and Prevention, Atlanta, 2023, pp. 19.