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Reviews, challenges

ANTHRAX: LIFE CYCLE, MECHANISMS OF PATHOGENESIS AND PROSPECTS IN THE DEVELOPMENT OF VETERINARY VACCINES

(review)

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Abstract

Anthrax is an acute *especially* dangerous disease of agricultural and wild animals, as well as humans. Anthrax is induced by the gram-positive spore-forming bacterium *Bacillus anthracis*. This infection is global, but the incidence rate of livestock and people varies depending on the environmental situation and the implementation of control strategies (C.J. Carlson et al., 2019). Historical and modern experience suggests that uncontrolled outbreaks of anthrax can have disastrous consequences. This review describes the life cycle of the pathogen, environmental features of the anthrax spread and mechanisms of pathogenesis. Given these factors we discuss the optimal strategies that have been developed over the years taking into account the cost and outcome for combating the dangerous infection. The timely disposal of dead animals and the vaccination of healthy livestock, used together, can effectively stop the spread of the disease. Thus, the development of highly effective, safe and low-cost vaccines is extremely relevant and, moreover, in fact the only promising method for improving the epizootic situation with this hazardous disease. Vaccination of farm animals for several decades has significantly reduced the risk of anthrax, but it is not mandatory in many countries and is often used only after onset of the disease, and not to prevent it. Despite a significant decrease in the incidence rate, the current situation with anthrax in the Russian Federation is characterized as unstable (A.G. Ryazanova et al., 2018; E.G. Simonova et al., 2018). Animal epizootics and human cases are still being recorded in the country due to the presence of natural soil reservoirs of the pathogen and incomplete coverage of vaccination for farm animals. Currently, only live attenuated vaccines are used to vaccinate animals. The review summarizes their effectiveness and safety, as well as the limitations associated with the use of attenuated vaccines. Although existing vaccines have been shown to be effective, they have several serious flaws. Certainly, the relevance of the development of more effective veterinary vaccines against anthrax, based on modern approaches, is fully justified. In particular, there is a need to design a veterinary vaccine that does not contain the pathogen in any form and is compatible with the use of antibiotics, which are necessary, both during the outbreak of anthrax and for regular use in the treatment of various animal diseases. The application of new approaches, the devising modern recombinant vaccines and the rejection of the use of pathogens in an attenuated form is an important and promising task. This review provides an analysis of studies on the development of new candidate vaccines against anthrax. The main attention is paid to the development of subunit vaccines using *B. anthracis* recombinant antigens obtained in various expression systems, including vaccines for oral administration and compatible with antibiotics.

Keywords: anthrax, *Bacillus anthracis*, veterinary vaccines, recombinant antigens

Anthrax is an extremely dangerous zoonose disease caused by a gram-positive spore-forming bacterium *Bacillus anthracis* (pathogenicity group 2).

Nearly all mammals, including humans, are susceptible to anthrax [1]. Soil containing *B. anthracis* spores is a source of infection for herbivores. High resistance of anthrax spores to the environmental factors, their ability to persist in the soil for a long time and to turn into a vegetative form under certain conditions makes the fight against this infection an extremely difficult task of medicine and veterinary. Humans get infected by direct contact with contaminated objects of animal origin. From 2000 to 20000 human anthrax cases are reported annually [2]. It is considered that 1.83 billion people live in the areas with the risk of anthrax outbreak [3]. In some regions disease outbreaks are a consistent and predictable ecosystem peculiarity and take their place at the certain stage of the seasonal cycle, in other areas epizootics are rare and are capable to cause massive livestock and wild animals deaths [4-7]. Climatic conditions are considered to be a generally accepted factor of the activation of natural foci and a risk factor of anthrax epizootics among herbivores, especially in endemic regions [8-12]. Despite the fact that anthrax has been being studied for about 130 years, there are many unresolved issues related to the pathogenesis and epidemiology of the disease [13].

Nowadays modern veterinary vaccine invented in 1937 [14] is used in most countries. This vaccine is a non-encapsulated toxigenic Sterne 34F2 strain or its analogues (the Russian Federation, China, Romania). Although existing vaccines have been shown to be effective, they have several disadvantages. In particular, the parenteral route of vaccine administration is impractical for mass immunization of animals, especially for animals on free grazing. However, peroral administration of Sterne 34F2 vaccine is turned out to be ineffective [15]. The design of new concepts and the rejection of attenuated pathogens as a vaccine basis are important and prospective research tasks.

This review discusses the relevance of new-generation recombinant anthrax vaccines and modern methods and approaches used for their development. The effectiveness and safety of live attenuated vaccines use is also discussed in current paper.

Lifecycle of the causative agent, ecological and epizootic features of anthrax. Anthrax is a natural-focal sapronous disease [16, 17]. The disease mainly affects herbivores. Cattle, horses, deer, sheep and goats are the most susceptible to *B. anthracis* animals. In certain cases anthrax epizootics taking place in the wild can lead to infection of farm animals and humans. *B. anthracis* occurs in two forms – as vegetative cells and as spores. This fact determines crucial anthrax epizootic features. It is generally accepted, that alimentary infection rout is the main one. In alimentary rout spores enter the animal's organism with food or water. However, the infection through the skin and respiratory tract is possible as well. At the same time, anomalies that often occur in anthrax epizooties are difficult to explain by the direct transition of spores into organism during cattle grazing. Apparently, insects are able to be spore carriers in unpredictable disease outbreaks [18-20]. Human's activities and ecological factors can also play an important role in the occurrence of anthax epizootics [12, 21-23]. After infection and death of animals *B. anthracis* vegetative cells enter the environment (with blood and other biological fluids) and turn into spores when exposed to oxygen. Seeding the soil with spores after the death of sick animals is obligatory for the continuity of anthrax causative agent circulation. *B. anthracis* spores can be translocated from primary soil foci to other areas (including ones that previously were free from the infection) with the participation of scavengers or insects (biotic processes) or due to abiotic factors such as water or wind [19, 20, 22, 24]. Spores are extremely resistant to environmental factors and can persist in the soil for decades [23]. An outbreak of anthrax in deer in the Yamalo-Nenets Autonomous Okrug in 2016 (75 years after the last known case in the region) indicates that the

potential duration of *B. anthracis* persistence in the environment may exceed centuries [11]. However, the quantity of spores in soil and water samples in the areas of burials of animals that died because of anthrax rapidly decreases to a level that is below the detection threshold, which can be explained by an effective dispersion of spores by animals, rainwater and wind alongside with a relatively low viability of *B. anthracis* vegetative cells and spores in the environment [8]. It was hypothesized that *B. anthracis* reproduces in the soil in so-named incubation zones before the contamination of animals. Calcium and organics-rich soils at pH > 6.0 and temperature above 15 °C favor the bacteria reproduction in topographically well-defined areas in which the infection of animals and anthrax outbreaks occur [8, 10]. Under the modeled environmental conditions it has been shown that the spores of the virulent Ames strain and vaccine Sterne strain were able to germinate and to reproduce intracellularly in a free-living soil amoeba (*Acanthamoeba castellanii*). The 50-fold increase in the number of spores was observed after 72 hours of inoculation [25]. In another work it was demonstrated that inoculation with the spores of rhizosphere can also lead to their germination [26].

It turned out that bacteriophages can play an important role in the anthrax lifecycle as well. Lysogeny may stimulate or, conversely, block sporulation (depending on the bacteriophage) inducing phenotypic changes in *B. anthracis* and providing a long-term bacterial colonization both in the artificial soil environment and in the manure worm (*Eisenia fetida*) intestines [27]. It has been shown that vegetative cells can persist in the soil for up to 120 hours, and a mixture of vegetative cells and spores inserted into the upper soil layer remains in it for 56 days [28]. The constant host-soil-host cycle is a basis for recurrent outbreaks. People are infected with anthrax through the contact with infected animals, and several cases of the disease transmission through insect bites were described as well [1]. The possibility of transmission of anthrax from a sick human to a healthy one has not been documented. Any anthrax outbreak can be disastrous if left uncontrolled. Anthrax usually develops very quickly in most animals and the successful treatment is not always possible. The disease is characterized with a global spread, but the incidence of anthrax in the farm animals and humans varies depending on local ecology and the implemented disease control strategies [3]. The decades of vaccination of farm animals in economically developed countries have reduced the risk of anthrax significantly. The refusal to vaccinate reindeer over the previous 9 years was the main reason for anthrax epizootic in the Yamalo-Nenets Autonomous Okrug in 2016, when over 2600 reindeer died and 36 people fell sick (with one lethal outcome) [11]. Most developed countries report sporadic anthrax cases in livestock and humans, but this disease still remains enzootic in some regions of Africa, in the Middle East and in Central Asia. Despite the significant decrease in incidence, current anthrax situation in the Russian Federation is still characterized as unstable [29-31]. Anthrax epizootics and human anthrax cases are still documented in Russia. This is due to the presence of natural soil reservoirs of the pathogen and incomplete vaccination of farm animals coverage. In the Russian Federation there are over 35 000 stationary unfavorable anthrax points (SUP). At the same time, there is a high probability of the presence of a significant number of unaccounted burials [32]. Most of anthrax outbreaks documented in recent years have been occurred in accounted SUPs, and in some of them the activity of the anthrax soil foci has not been manifested for the last 40-60 years. At the same time, some epizootic foci have appeared in the territories considered to be safe [33]. There are also reports of anthrax outbreaks in the wild in various ecosystems all over the world [5, 7, 34].

It is a mistake to treat anthrax a legacy of some sanitary troubles of the past like improperly utilized livestock buries etc. Apparently, there are multiple

natural reservoirs of the pathogen, which has phases of rest and phases of active development in its lifecycle. Spores can be activated due to natural and climatic processes: floods or droughts, dramatic shift in temperature, thawing of the soil, landslides etc. In particular, it can be confirmed by the periodic mass deaths of wild animals from anthrax in the protected wild areas in Africa. For instance, hundreds of hippos died from anthrax in 2017 in Bvabvata National Park in northeastern Namibia during an extremely low water level in rivers [7]. Yamal anthrax outbreak is also associated with an unusual heat, which contributed to an increase in seasonal thawing of permafrost to 1 m and the movement of spores from deep layers to the soil surface. Such soil conditions are favorable for the sporulation and vegetation of the pathogen. Climatic factors additionally contributed to an increase in the number of the blood-sucking insects, which are considered as carries and a reason for the rapid spread of epizootics [11, 16, 36). The activity of emergent focus is only manifested in the disease and death of animals. At the same time, natural foci themselves often cannot be detected and localized. Common practice for controlling infection in cattle includes the utilization of animal carcasses and vaccination of grazing animals.

The search for optimal strategy to combat the spread of the disease both in terms of cost and effectiveness has recently been performed using modern methods of mathematical modeling [36]. The results of American researchers' calculations indicate that only the combination of timely disposal of animal carcasses with vaccination of healthy livestock is able to stop the spread of anthrax. This makes the development of modern cheap and effective anthrax vaccines extremely relevant and, moreover, the only prospective way to improve the epizootic situation with such a dangerous disease as anthrax.

Pathogenesis mechanisms. Once *B. anthracis* spores enter the organism of a susceptible host (via gastrointestinal or inhalation rout or through the skin) they can locally germinate into a vegetative form at primary contact sites. Also, spores can be captured by macrophages and be translocated into the lymph nodes, where they germinate, migrate into the bloodstream and release the system effects-causing toxins. The main virulence factors of the vegetative form of *B. anthracis* are a capsule of poly-γ-D-glutamic acid and anthrax toxins. The capsule has low immunogenicity and makes the bacterium resistant to phagocytosis and the complement system. Capsule components are encoded by the pXO2 plasmid. As a result, pathogen becomes invulnerable to the host's immune system [37, 38]. Anthrax toxins are three proteins encoded by pXO1 plasmid – protective antigen (PA), lethal factor (LF) and edema factor (EF), which combine into binary complexed PA/LF and PA/LF to form a lethal toxin (LT) and edema toxin (ET), respectively [39].

PA is an 83 kDa protein (PA83). After PA83 binds to cellular receptors (capillary morphogenesis protein 2 — CMG2 and tumor endothelial marker 8 — TEM8), it undergoes proteolytic hydrolysis by cellular surface furin-like proteases. As a result of the proteolysis, a non-engaged in the receptor binding N-terminal 20 kDa PA fragment is being split off leaving only the 63 kDa C-terminal PA fragment bound to the receptor. CMG2 receptor has a higher affinity to PA83, is widely expressed in various types of cells and is considered to be the main anthrax toxin receptor mediating *in vivo* lethality, while TEM8 plays a minor role in the pathogenesis of anthrax [40]. Two anthrax receptors have a high degree of identity with each other and contain a conservative domain that binds PA [41, 42]. CMG2 is highly conservative for various animal species (for example the degree of identity of human and mouse CMG2 is 82%), it is found only in vertebrates. It can be assumed that the conservatism of cellular receptors and PA is one of the reasons for the lack of resistance to this pathogen in a huge variety of mammal species. It

has been shown that PA83 is a calcium-dependent serine protease, like furin is, and that PA83 is potentially capable to use this activity to bind to TEM8 [43]. PA63 within a complex with the receptor forms oligomers (heptamers or octamers), which bind three or four LF, EF or LF and EF simultaneously. The toxin complex undergoes endocytosis. Under the acidic endosomal pH conditions a number of conformational changes occur in the structure of PA63. This changes lead to the formation of a channel in the endosomal membrane through which LF and EF are transferred to the cytosol, where they manifest their toxicity due to their enzymatic activity [44-46]. Recently, a new function of PA20 has been described and a PA20-mediated mechanism has been proposed, by which insects-carriers of *B. anthracis* gain anthrax resistance due to activation of innate immunity. According to the authors, a similar mechanism may potentially exist in mammals [47].

LF is a zinc-dependent metalloprotease that cleaves mitogen-activated protein kinases MAPKKs, MEKs and MKKs, thus disrupting the activation of signaling pathways of mitogen-activated cascades, including ERK (Extracellular signal-regulated kinase) pathway 1/2, JNK/SAPK (c-Jun N-terminal kinase/Stress activated protein kinase) and p38. Those cascades are crucial for numerous cellular functions such as proliferation and cell cycle regulation, as well as for immunomodulation and survival in toxic strokes [48-50]. EF is a highly effective calmodulin-dependent adenylate cyclase that is about 1000 times more active than adenylate cyclase of mammals and causes a sustained increase in cAMP level. High cAMP concentrations disrupt key cellular functions, leading to negative consequences for the host [51-54].

Nowadays, there is no doubt that anthrax toxins are crucial in anthrax pathogenesis. The release of toxins takes place in early stages after spore germination: PA mRNA is detectable 15 min after the initiation of germination [55]. However, the mechanisms of pathogenesis differ from one animal species to another and depend on the type of disease and other factors. In the initial stages of the infection combined LT and EF activity blocks the host innate immune response. For example, the phenomenon of LT-induced macrophages death is known in some mouse strains. In the later stages, when high LT and ET concentrations are reached, they can cause host death directly through affecting various vital systems, in particular the cardiovascular system and the liver [40, 56]. Experiments on mice have shown that expression of the main toxin receptor CMG2 in vascular smooth muscle cells and in cardiomyocytes is required for the LT-induces lethal outcome. ET-induced mortality is due to disruption of another type of cell, mainly hepatocytes. Targeting endothelial cells with any of the toxins does not contribute to mortality from *B. anthracis* as significantly as it has been considered previously [56]. Thus, both LT and ET are lethal to mice, while each of three toxin components (PA, LF and EF) individually are not toxic [57]. At the same time, experiments on macaques have shown that pathological effects that lead to lethal outcome are mainly due to the activity of anthrax LT [58]. A study of the influence of LT an ET on the subgroups of human alveolar phagocytes and leucocytes with low CMG2 and TEM8 expression has demonstrated that all cell types bound PA in a dose-dependent manner. The cells were invulnerable to LT-induced apoptosis or necrosis at toxin concentrations below 1000 ng/ml. However, exposure to toxins have inhibited spore internalization. Authors suppose that in pulmonary anthrax, ET prevents spore phagocytosis in the initial stages of infection and in the later stages high concentration of LT in the bloodstream suppresses pathogen phagocytosis by leukocytes, thus ensuring rapid *B. anthracis* proliferation in the blood [59]. Perhaps the same mechanism exists in some other animal species. Long-term storage of active enzymes (LF) in endosomal vesicles and their slow release into the

external environment, which is possible even in the absence of bacteria, is an important characteristic of the high virulence of *B. anthracis*. Apparently, this is the reason for lethal outcomes even after a successful elimination of bacteria during antibiotic treatment [60].

Live attenuated vaccines. Anthrax was one of the first bacterial diseases to be controlled by vaccination. The first live attenuated anthrax vaccines were invented by Louis Pasteur in 1881 and have been being used effectively to vaccinate animals in Europe and South America for 50 years. Live attenuated vaccines for veterinary use can be divided into three main categories: Pasteur vaccines, Sterne vaccines and Carbozoo vaccines. The division is based on different mechanism of pathogen attenuation [61]. It used to be assumed that the attenuation according to Pasteur scheme (Pasteur vaccines) leads to the elimination of pXO1 plasmid encoding the main virulence factors (PA, LF and EF), which results in the nontoxigenic encapsulated ($pXO1^-/pXO2^+$) vaccine strain. Currently it is believed that Pasteur vaccines were mixed cultures containing a small percentage of completely virulent bacteria ($pXO1^+/pXO2^+$) [62-64]. Mechanism of attenuation implemented in Carbozoo vaccines is still unknown, however, studies have demonstrated the presence of both plasmids ($pXO1^+/pXO2^+$) in such bacteria strains. These strains are toxigenic and encapsulated [65].

In the 1930s, Pasteur vaccines were driven out by vaccines based on attenuated non-encapsulated ($pXO1+/pXO2^-$) *B. anthracis* Sterne strains. In Sterne vaccines *B. anthracis* lacks pXO2 encoding the formation of capsule components. Worldwide, most anthrax vaccines for animals contain the toxigenic non-encapsulated *B. anthracis* Sterne 34F2 strain obtained from virulent isolate from cattle. In Russia, China and Romania other similar toxigenic non-encapsulated strains are being used [1]. In the USSR, live vaccines based on non-encapsulated STI1 and GNKI strains were used for vaccination in 1940s-1980s, which lead to a significant decrease in the incidence of anthrax in both humans and animals. Since the 1980s the attenuated *B. anthracis* 55-VNIIVViM ($pXO1^+/pXO2^-$) strain has been being used in Russia as a vaccine strain for cattle. In Italy and Argentina, Carbozoo vaccines based on the Italian vaccine strain Carbosap [66] and the Argentine strain A [67] are used to vaccinate animals.

Sterne-like strains (34F2 and its analogues in Russia, China, and Romania) lack the genes for capsule formation while still producing the toxin. These strains are more about to have low virulence than to be avirulent, thus maintaining the capability to cause a certain rate of morbidity [1]. An overdose of vaccines based on these strains is dangerous and can lead to sever consequences up to the death of animals. Some animals, for example goats, are more susceptible to infection and death in such cases, thus requiring a particularly thorough dosage control [1, 14]. It has also been reported that vaccination with the Sterne 34F2 strain can cause the death of miniature horses [68] and llamas [69]. For the Russian strain 55-VNIIVViM, vaccination of horses is recommended only from 9 months of age. It is not recommended to vaccinate pregnant, sick or weak animals. Vaccination is also prohibited less than 6 weeks before slaughtering animals for meat. Other factors limiting the use of currently existing vaccines are the high cost of the drug, the time-limited effect (in epidemically unfavorable regions, the vaccination should be repeated annually), the need for parenteral vaccine administration (a decrease in the protectivity and the appearance of adverse effects are possible even in case of minor deviations from the recommendations on dosage and vaccination regulation). Moreover, the presence of live pathogen spores in the vaccines requires special training of personnel, and all used tanks and glassware should be subsequently sterilized and disinfected [1]. In 2020, the development of a vaccine based on the spores of the microencapsulated, attenuated B. anthracis Sterne 34F2

strain for oral administration was reported. The studies on mice have demonstrated immunogenicity and neutralizing activity against a lethal dose of LT *in vitro* after a single immunization via gavage [70]. This research direction may turn out to be prospective. However, it is desirable to further study the protectivity of microencapsulated spores in farm animals and the safety of such strain for the environment when used to vaccinate wild animals and animals on free-range.

In addition, live attenuated vaccines are not effective enough during anthrax outbreaks, because only 80% of vaccinated animals gain immunity sufficient to resist *B. anthracis* infection 8 days after the first vaccination [71]. Since the immunity develops more than 1 week after vaccination, a long course of antibiotics is recommended before the vaccination. Administration of the vaccine is incompatible with antibiotics: antibiotics should not be prescribed two weeks before and after the vaccination due to their inhibitory effect on the immune response development and to possible anaphylactic reactions [1, 72]. Antibiotics are widely used to treat various disease of farm animals and can be contained in animal feed. This fact also limits the possibilities of effective vaccination against anthrax using live attenuated vaccines [1].

Therefore, the development of an alternative economically efficient vaccine that is safe to use and is compatible with antibiotics is desirable.

Recombinant vaccines. Recombinant vaccines are a promising approach that allows to overcome the limitations of traditional vaccines. Many successfully designed recombinant subunit and vector veterinary vaccines against various pathogens are already used for vaccination [73].

In recent years, recombinant anthrax vaccines for both medical and veterinary purposes have been being actively developed. The potent use of *B. anthracis* spores as a biological weapon has triggered intensive research in the area of creation of new-generation anthrax vaccines for humans. In our review published in «Expert Review of Vaccines» in 2019 it is mentioned that significant efforts were made to develop new approaches to anthrax vaccination and to study recombinant anthrax vaccines being developed [74]. The main direction of modern research in this area is focused on the creation of recombinant anthrax vaccines containing recombinant anthrax protective antigen (PA), which is the main anthrax antigen. PA is a central toxin component and plays a key role in the protection against toxigenic and encapsulated *B. anthracis* strains. Anti-PA antibodies induction is the main immune response after animal vaccination with Sterne vaccine [75-77]. In many studies it has also been shown that vaccines protectivity correlates with PA-induced neutralizing antibodies titer. Most of epitopes, antibodies to which have toxin-neutralizing activities, are mapped to PA [74].

Anthrax vaccines candidates are being developed using various approaches that can be divided into four groups: adenovirus-based vaccines expressing full-size recombinant PA (rPA83) [78, 79]; vaccines based on live bacterial vectors, such as *Lactobacillus* spp., and on attenuated *Salmonella* spp. strains expressing rPA83 [80-84]; DNA vaccines [85-87]; vaccines based on *B. anthracis* recombinant antigens obtained using in vitro expression systems. Most of the vaccines currently being developed are based on the latter approach. Of the 10 vaccines in clinical trials, 8 are created using purified rPA83. Subunit purified rPA83-based vaccines are characterized with high safety and protective properties [74]. In return, subunit vaccine candidates can be divided into three groups: vaccines based in rPA83 obtained in various expression systems [88-92]; vaccines based on chimeric proteins obtained by fusion or conjugation of rPA83m or separate PA domains with additional antigens, such as LF domains [93-97], LF+EF [98), antigenic component of spores [99, 100] or a capsule component — poly-γ-D glutamic acid [101-104], to enhance the immune response; vaccines based on rPA83m mixed with

other components, such as bacterial surface S-layer recombinant protein [105] or *B. anthracis* spores [106, 107]. New approach was implemented during the development of a vaccine based on spores of probiotic *Bacillus subtilis* using a technology that makes it real to effectively express heterologous rPA83 protein in the sporulation phase with further attachment or adsorption of the protein to the spore surface. The effectiveness and safety of such vaccine has been demonstrated for various models of administration, including oral, internasal and sublingual [107]. In addition to these strategies, the possibility of using various adjuvants in order to increase rPA83-based vaccines stability is being investigated [74, 108]. A number of new adjuvants are currently in clinical trials, and data is being accumulated on their effectiveness comparing to aluminum hydroxide, which is an adjuvant that is used in many vaccines [109]. One of the main problems in the development of subunit vaccines based on rPA83 is the instability of this protein. rPA83 contains two sites that are sensitive to proteolytic cleavage. It has also been shown that the instability of rPA83 is associated with spontaneous deamidation of several asparagine residues in the protein and the rate of deamidation increases significantly when aluminum hydroxide is used as an adjuvant. Based on a number of data that has been published recently, it can be argued that rPA83 adsorbed on aluminum hydroxide is unstable and loses its ability to induce neutralizing antibodies during storage [110-112]. New adjuvants together with the use of mutant rPA83 forms that are resistant to deamidation and proteolysis can solve the abovementioned problem. Search for a new adjuvant and rPA83 molecule stabilization are among the main directions of a new effective anthrax vaccine development. The study by Ryabchevskaya et al. [113] has demonstrated the possibility of simultaneous implementation of two approaches to stabilize rPA83 through the adsorption of rPA83 on the surface of spherical particles obtained from a plant virus and through a directed mutagenesis of sites that are a reason for protein destabilization. Thus, over the years, with the development of genetic engineering methods, creation of various vector platforms, DNA vaccines and adjuvant systems used to increase the immunogenicity and the stability of subunit vaccines, there are all required conditions for the creation of new, safer and more effective anthrax vaccines.

Our research on scientific publications devoted to experimental veterinary vaccines with studies on farm animals have revealed only a few articles. However, according to many authors, methods and approaches used for medical vaccines development are also optimal for veterinary vaccines creation. In the study performed by Fasnella et al. [71] rPA83 and mutant forms of LF (mLF-Y728A; E735A) and EF (mEF-K346R) with inactivated enzymatic activity have been cloned and expressed in *Escherichia coli* system successfully. Two vaccine candidates, monovalent containing PA83 and trivalent (TV) containing PA83, mLF and mEF were used to immunize rabbits in the presence of Marcol 52 (ESSO) and Montane 80® (SEPPIC) adjuvants, commonly used as adjuvants for veterinary use. New Zealand rabbits single time immunized subcutaneously with rPA83 and TV have produced high levels of antibodies against PA (rPA83 and TV vaccines), LF and EF (TV vaccine), and both vaccines have demonstrated 100% protection of rabbits against virulent *B. anthracis* strain 0843 (200LD50) 1 week after vaccination. Sterne-vaccinated rabbits have demonstrated lower anti-PA, anti-LF and anti-EF antibodies level comparing to those induced by experimental vaccines. Also, when tested under the same experimental conditions, Sterne vaccine protected only 80% of infected rabbits 1 week after vaccination. Thus, both vaccine candidates have been proven to be more effective than Sterne vaccine. The possibility to use them with antibiotics simultaneously due to the lack of a live pathogen in their composition is another significant advantage of these vaccine candidates. However, it should be mentioned that advantages of the trivalent vaccine over the vaccine containing

a single rPA83 antigen have not been shown. Moreover, there is data showing that the use of catalytically inactivated through a single spot mutation mutant EF in conjunction with PA may be unsafe [114].

In the study by Koehler et al. [115] the immunogenicity of two recombinant multicomponent vaccines has been evaluated in goats using a lipopeptide adjuvant. These vaccines have contained rPA and an antigenic spore somponent BclA (bacillus collagen-like protein of anthracis) expressed in *E. coli* systems alongside with spores inactivated with formalin (FIS). Goats are extremely susceptible to *B. antracis*. Goats, three times subcutaneously immunized with rPA+rBclA or rPA+rBclA+FIS have demonstrated 50% and 80% protection, respectively, against a lethal dose of virulent *B. anthracis* strain spores. Further studies performed by the same group of scientists have demonstrated that immune serum from goats vaccinated with rPA+rBclA or rPA+rBclA+FIS is capable to protect approximately 70% of mice against lethal dose of anthrax spores [116]. Thus, the experiments carried out have demonstrated the efficacy of two recombinant anthrax vaccines and the induction of a protective immune response in vaccinated goats. Preliminary data from serological studies on goats have confirmed the reliability of immunogenicity of these vaccines when administered simultaneously with antibiotics.

Currently, the same scientific group continues their studies on the vaccine candidate in cattle. Jauro et al. [106] have compared the immunogenicity and protectivity of a vaccine candidate based on rPA83 combined with FIS and with aluminum hydroxide-containing adjuvant (Emulsigen-D/Alhydrogel) and a vaccine based on the spores of an attenuated Sterne 34F2 strain. After the vaccination of cows the immunogenicity and protectivity *in vitro* in a toxin-neutralizing test and *in vivo* in a mouse model with passive immunization have been evaluated. It has been demonstrated that the antibody titers were similar in case of both vaccines. However, it has been shown that, in contradistinction to Sterne vaccines, vaccine candidate is effective when used simultaneously with antibiotics [117].

Several scientific groups implement various approaches to rPA83 obtaining in a plant expression system [74]. Gorantala et al [118] have attempted to develop a universal oral vaccine suitable both for veterinary and medical use. To achieve this aim, transgenic mustard plants (*Brassica juncea*) have been obtained for the expression of rPA83, the leaves and stems of these plants can be used fresh for human consumption and for cattle feeding. In addition, flour obtained from *B. juncea* is used as feed for cattle in many countries [118]. The presence of standard transformation protocols for *B. juncea*, large plant biomass, long-term stability of the transgene, and safe storage of the antigen in seeds indicate that transgenic mustard plants can effectively express rPA83 and become the basis for the development of a vaccine against anthrax. In has been shown that transgenic *B. juncea* plants after repeated oral administration to mice for 1 month with a final booster dose of rPA83 obtained in *E. coli* (also administered orally) in the presence of mucosal adjuvant have stimulated both systemic and mucosal immune responses. Also, 60% of the mice have survived the lethal dose of *B. anthracis* (Sterne strain). In control experiments using rPA83 from *E. coli* being administered orally (by gavage) 80% of the mice have survived the lethal dose of *B. anthracis*. The need for rPA83 booster dose may be due to the low protein expression level in transgenic mustard (0.3-0.8% of a total soluble protein fraction) [118]. According to the authors, further research on improving the protein expression is required. In particular, the creation of transplastomic mustard plants with PA83 gene integrated into the chloroplast genome is proposed. In the same study, transplastomic tobacco plants have been obtained with rPA83 expression levels equal to 2.5-4% of the total soluble plant protein fraction. In addition, the authors believe that the

peculiarities of the digestive processes in ruminants can contribute to the effective impact of the antigen on the lymphoid tissues associated with the intestine, and thus enhance the immune response of the intestinal mucosa. As already mentioned, after entering the organism spores germinate turning into a vegetative form locally at the primary pathogen-host contact sites, for example, in the intestinal mucosa. Therefore, the development of an oral recombinant veterinary vaccine in a prospective and important research direction.

In another study, transplastomic tobacco plants have been obtained in which the level of rPA83 expression in mature leaves has reached 14.2% of the total soluble plant protein fraction mass. Calculations have shown that one acre (0,405 ha) of transplantomic tobacco plants expressing rPA83 is capable to produce up to 360 million doses of anthrax vaccine [119]. The expression of anthrax antigens using plant expression system is a promising direction for vaccination of free-range ruminants. This direction, undoubtedly, deserves further development.

So, the particularities of the life cycle and ecology of the anthrax causative agent indicate that it is not yet possible to eliminate this disease completely. Vaccination is the main way to combat anthrax. Live attenuated vaccines that have been being used for decades are effective but are also characterized with several limitations. In particular, they are incompatible with antibiotics, which are necessary both during anthrax outcomes and for the treatment of other animal diseases. Therefore, the use of recombinant vaccines that can be administered simultaneously with antibiotics is extremely relevant and, moreover, in fact is the only promising way to improve epizootic and epidemic situation with this dangerous disease. The central direction of modern anthrax vaccine development is focused on the creation of subunit vaccines containing the sequence of protective antigen (PA) — the main anthrax toxin antigen. The undoubted priority is given to the creation of safe and effective vaccines for humans, but the same approaches and antigens are potentially suitable for vaccination of farm animals. Recombinant vaccines, including those for oral administration, is a prospective direction of the development of veterinary vaccines. On farm animals, data has been obtained on the effectiveness of vaccine candidates based on PA83 mixed with inactivated *B. anthracis* spores, and it has been proven that, in contradistinction to Sterne vaccine, the vaccine candidate is highly effective when administered simultaneously with antibiotics. Subunit PA83-based vaccines are promising, both vector ones with *in vivo* expression and ones based on recombinant antigen obtained *in vitro* and stabilized without loss of immunogenicity. In our opinion, mutant forms of rPA83 that are resistant to proteolysis are promising when combined with new adjuvants and/or carrying platforms. Another important direction of recombinant veterinary vaccines development, which requires attention, is the use of probiotics (*Lactobacillus* spp.) as vectors for the delivering of the antigen or for the antigen exposing on the surface of *B. subtilis* spores with the possibility of oral administration.

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