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REVIEW

A new safe and effective cold-adapted modified live equine influenza virus vaccine that enables the differentiation of infected from vaccinated animals

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ABSTRACT

An analysis of the main advantages and shortcomings of the existing inactivated and live vaccines against the equine influenza viruses (EIVs) is given in this paper. For the first time, the most important information, concerning the development of a new live modified cold-adapted (*ca*) equine influenza virus vaccine based on the A/HK/Otar/6:2/2010 strain is summarized. We discuss a number of unique features of the developed vaccine that have not previously been reported, and compare the new vaccine with the existing equine influenza vaccines. The properties of the developed equine vaccine include: long-lasting (12 months or more) protective immunity after a single immunization; sterile immunity after double vaccination; cross-protection against the heterologous virus at 12 months after double vaccination and the differentiation of infected from vaccinated animals.

The equine influenza is an acute contagious virus disease characterized by the development of the catarrhal inflammation of the respiratory tract, general depression, short-term fever, and dry sickly cough and in the severe cases – by the development of pneumonia. Among the known equine influenza virus (EIV) subtypes H7N7 and H3N8, the latter is considered the most prevalent. These viruses pose a significant threat to equine health as well as economic problems for horse breeding [1]. At present, the most efficient way to protect horses from influenza is a complex of specific preventive measures, which are accomplished with the use of inactivated and live vaccines.

Starting from 1960s, inactivated vaccines, containing the whole virus particles or their subunits, were developed and widely used in veterinary practice. The lack of virus replication, which ensures the vaccine safety, is the main advantage of this vaccine type [2]. At the same time, the main disadvantages of inactivated vaccines are weak immunogenicity caused by the formation of an exclusively humoral immune response as well as short-term immunity, which requires multiple immunizations [3, 4, 5]. For example, since the antiviral antibodies IgG(T), which are formed in the course of immunization with the inactivated vaccines, are short-lived (not more than 100 days after vaccination), triple immunization (first two with a 4 to 6 week interval and the third on the 5th or 6th month) is needed for the formation of the 12 month humoral immunity in horses [3, 4, 5]. This immunization scheme is critical because, between the second and third vaccination, horses in field conditions are the most vulnerable to influenza virus infection [7]. In addition, immunization with some of the inactivated vaccines containing adjuvants (phosphate or hydroxide of aluminum) leads to undesirable effects such as an inflammatory reaction and pain at the injection site of the intramuscularly vaccinated animals [8]. This reaction is also related to the use of chicken embryos for the vaccine production: even after the purification, the vaccine still contains significant amounts of egg proteins, which can cause undesirable reactions in the case of multiple intramuscular injections [2].

On the other hand, live attenuated vaccines unlike inactivated vaccines show the most promising results in terms of protection efficacy in horses. Among the live attenuated vaccines, the preparations produced on the basis of the cold adapted (*ca*) virus strains play an exceptional role. The live vaccine virus has the ability to replicate in the upper but not in the lower airways, where the temperature is elevated, unlike the wild virus, which replicates in the lower airways, that usually leads to inflammation like bronchitis and pneumonia [9]. The vaccinated horses have the light form of an influenza infection, which leads to the formation of antiviral humoral and cell immune responses. Moreover, the *ca* vaccine, unlike the inactivated vaccines, is capable of causing cross-reactive T-cell immunity in the vaccinated animals, which is quite important considering the high influenza virus antigenic variability (antigenic drift) [10]. The first live vaccine from the *ca* virus strain

against equine influenza was developed and successfully implemented in the USA. The live *ca* vaccine (Flu Avert® I.N., Heska Corporation), which is administered intranasally, is licensed and has been widely used in the USA since 1999 [11]. It contains the North American *ca* strain A/equine/Kentucky/1991 (H3N8), but at the same time it can protect from the viruses of H3N8 subtype of European lineage. In spite of the fact that the Flu Avert® vaccine does not induce a high level of the humoral antibodies, the single immunization with this vaccine provides longer lasting protection (at least 6 months) than inactivated vaccines. Moreover, animals inoculated with this vaccine develop secretory IgA antibodies that are able to neutralize the virus at early stage of the infectious process in the upper airways [9]. Despite all of the advantages, there are some concerns about the safety of *ca* vaccines. The main concern is related to the risk of the possible reversion of the vaccine virus or the generation of the reassortant with the circulating in the equine body wild type virus, resulting in the formation of new pathogenic viruses [2]. Though these apprehensions are justified, this possibility can be excluded based on more than 20 years of positive experience using live intranasal attenuated influenza vaccine in people in Russia, and recently in North America and Western Europe (FluMist®) [2]. In the case of the *ca* EIV vaccine, it has been proven experimentally that the *ca* strains retain the temperature sensitive (*ts*) phenotype *in vivo* in the course of five consecutive passages from one horse to another, and suppress the replication of the wild type virus in the equine upper respiratory tract [10]. Therefore, the risk of reversion or reassortment is minimal, and the *ca* vaccine against EIV could be used as the therapy for an equine influenza infection [11, 12].

One more prophylactic preparation that has a practical application (licensed in the EU in 2003) is a live vaccine based on canarypox recombinant virus (ProtegFlu, Merial Ltd., UK) [2]. This vaccine includes two canarypox virus vectors coding the hemagglutinins (HA) of EIV strains A/equine/Newmarket/2/93 (H3N8) (European lineage) and A/equine/Kentucky/94 (H3N8) (American lineage). Unlike the inactivated vaccines, this vaccine is able to induce a humoral response, including the secretory IgA antibodies as well as the cellular immune response in the vaccinated animals [13, 14]. However, as in the case of inactivated vaccines, the triple immunization with this vaccine (first two – with 35 days interval, and the third – in the course of the 6th month) is necessary for the formation of 12 months long protective immune response in horses [15]. Furthermore, since this vaccine contains the adjuvant Carbomer 974P, it quite often causes local undesirable reactions in vaccinated horses [16, 17]. The most important feature of this vaccine type is the formation of immunity to the corresponding virus vector in immunized animals already after the first immunization. The immunity developed after the second administration of the same virus vector prevents the reproduction of these viruses and, consequently, the expression of foreign proteins [2, 17, 18]. In order to overcome this problem, Breathnach et al. successfully applied the method of cross-immunization [19]. This method involves the vaccination first by the DNA vaccine that codes the HA and the nucleoprotein (NP) of A/equine/Kentucky/1/81 (H3N8) strain, and subsequent vaccinations (on the 6th and 10th week) – by the modified vaccinia virus Ankara that codes the same proteins. However, this scheme did not find practical application due to its complexity and low productivity.

Thus, if we exclude the vaccines that are still in the development stages (DNA vaccines [20, 21] and live attenuated influenza virus vaccines produced by reverse genetics [22, 23]), only inactivated, live *ca*, and vector vaccines have broad practical application against the equine influenza at present. Therefore, when developing a safe and effective vaccine against H3N8 EIV, which in 2007 caused a large influenza outbreak in Kazakhstan (around 200,000 horses fell ill with a lethal outcome in 50,000 cases including 40,000 youngsters [24]), we had to choose from the above-listed preparations. In the course of detailed analysis of advantages and disadvantages of these vaccines, we considered several factors: safety, efficacy, the duration of immunity after a single immunization, availability of efficient production technology, and the price of the product. As a result, we came to the conclusion that the live *ca* vaccine is the most preferential candidate for development in Kazakhstan.

The cornerstone of success in the development of the first live *ca* vaccine against equine influenza in Kazakhstan was the generation of the vaccine strain. This problem was solved in collaboration with the Research Institute of Influenza (RII, Saint Petersburg, Russia) where the *ca* master strain A/Hong Kong/1/68/162/35 (H3N2) was generated. The new *ca* strain A/HK/Otar/6:2/2010 containing two genes coding the surface proteins (HA, NA) from the wild type strain A/equine/Otar/764/2007 (H3N8, American lineage Florida, clade 2), and six genes coding the internal proteins (PB2, PB1, PA, NP, M, NS) from the master strain was obtained using the classical genetic reassortment method. The wild type strain A/equine/Otar/764/2007 (H3N8) was chosen due to the fact that it was isolated during the last 2007 influenza outbreak in Kazakhstan. According to the data of phylogenetic analysis, the HA gene of this virus is highly homologous (99.99%) to HA of the A/equine/Richmond/1/2007 (H3N8) strain, which was recommended by the World Organization for Animal Health (Office International des Epizooties, OIE) for the production of a vaccine against equine influenza in March 2016 [25]. The *ca* strain A/Hong Kong/1/68/162/35 (H3N2) was used already as a donor of attenuation for construction of vaccine strains, which proved to be not only safe and effective, but also showed high reproductive activity in the cultivating system. Based on the *ca* strain A/Hong Kong/1/68/162/35 (H3N2), new actual vaccine strains could be obtained easily in a short time frame. This was confirmed by the successful generation of the vaccine strains A/Saint Petersburg/HK/2009 (H1N1), A/Astana/HK/2009 (H5N1), and A/Perth/HK/2011 (H3N2) [26]. It is noteworthy that the closest and the only commercially available analog – vaccine Flu Avert® – does not have this advantage. The wild strain A/equine/Kentucky/1991 (H3N8) that was

used for the generation of this commercial vaccine was passaged 49 times in chicken embryos at 26°C to obtain the attenuation phenotype. This means that, in order to generate the actual vaccine strain, it will be necessary to perform similar operations with the new wild type EIV that will take at minimum a year [11]. In contrast, the generation of the actual vaccine strain based on the A/Hong Kong/1/68/162/35 (H3N2) virus takes no more than three months by the classic reassortment method. As was shown previously, the vaccine strain A/HK/Otar/6:2/2010, cultivated in chicken embryos at optimal conditions (the infecting dose, temperature, and incubation time), steadily grows up to 9.0 $\log_{10}$ EID_{50}/ml [27]. This virus keeps the *ca* and *ts* phenotypes and shows genetic stability over the course of 20 consecutive passages in chicken embryos. Furthermore, the complete attenuation of this strain was demonstrated in laboratory animals (mice and guinea pigs) [28]. The obtained results prove that the *ca* reassortant strain A/HK/Otar/6:2/2010 is a good candidate for the live modified *ca* vaccine against the equine influenza. Further trials of this vaccine candidate in yearlings and pregnant mares also showed its safety. After a single intranasal immunization (with 2 x $10^{9.2}$ EID_{50}), the animals did not reveal any symptoms, including fever, during the entire follow-up period (21 days). Only an insignificant replication of the vaccine virus was observed ($10^{0.75-1.0}$ EID_{50}/ml) in 12.5–50% of the immunized yearlings and pregnant mares on the first and third days post vaccination. The secretory IgA antibodies were formed in vaccinated animals on the 7th day, while the T-cell immune response – on the 14th day post vaccination. The vaccinated animals showed pronounced clinical (illness severity and duration) and virological (virus titer in the nasal swabs on the 1st-14th day after the challenge) protection from the homologous wild strain A/equine/Otar/764/2007 (H3N8) compared to the control group [29]. It is interesting that before the challenge the antibodies to EIV of H3 subtype were not detected in the blood serum of the vaccinated yearlings and pregnant meres by hemagglutination inhibition assay (HAI) and ELISA.

The duration of the protective immune response against the equine influenza H3N8 in horses after the first and the recurrent immunizations by the live *ca* vaccine is of particular interest. We were the first to demonstrate the ability of this vaccine to generate clinical and virological protection from the homologous wild type virus A/equine/Otar/764/2007 (H3N8) for a period of 12 months after a single intranasal immunization [30]. As was published earlier, the commercial vaccine (Flu Avert®, Heska Corporation) generates only a 6-month protective immune response in horses after a single vaccination [2, 9].

It is important to mention that the double intranasal vaccination with A/HK/Otar/6:2/2010 (42 days interval) can significantly enhance the clinical and virological protection from the wild type virus compared to a single vaccination. In addition, this vaccine generates sterile immunity (the lack of virus in the nasal swabs of animals after the challenge) lasting for three months after the second immunization [30]. The sterile immunity in horses was demonstrated earlier by an immunization scheme patented by Intervet International B.V. (Boxmeer, NL)[31]. In order to achieve the sterile protective immunity, it was necessary to vaccinate horses with the live *ca* vaccine first and then to repeat the immunization with an inactivated vaccine against the equine influenza virus in 8 weeks. Another example of the generation of the sterile protective immunity in horses was reported after immunization with a live vector vaccine based on the canarypox virus containing the adjuvant Carbopol [32]. We report here for the first time that the sterile protective immunity against influenza in horses can be achieved after double immunization using only a live *ca* vaccine. Besides the sterile immunity, the pronounced clinical and virological protection of horses against the heterologous wild type virus A/equine/Sydney/2888-8/2007 (H3N8) was observed 12 months after the double, but not single immunization [30]. The comparative analysis of our results with the literature data [5, 15, 33, 34] demonstrated that in terms of the duration of the protective immune response, the live attenuated vaccine produced from the reassortant *ca* strain A/HK/Otar/6:2/2010 exceeds all the known (inactivated and recombinant) commercial EIV vaccines. The obtained results make it possible to assume that the developed vaccine has the potential to become an excellent alternative for the inactivated and recombinant vaccines that are used in Kazakhstan and other countries at present.

According to the OIE recommendations, vaccine preparations against equine influenza, in addition to being safe and immunogenic, should also enable the differentiation of infected from vaccinated animals (DIVA strategy) [35]. At present, only live recombinant vector vaccines that express the surface influenza virus proteins meet these requirements fully [36]. In the case of immunization with the traditional inactivated vaccines, it is also possible to differentiate the infected from vaccinated animals through the detection of antibodies to the nonstructural NS1 protein of the influenza virus by serological tests [37]. These antibodies are formed only in the case of the live virus replication. With respect to the live attenuated vaccines against equine influenza, the DIVA strategy is impracticable because both the vaccine and the wild type viruses induce similar infectious processes in animals. However, our serological studies clearly show the possibility to differentiate infected from vaccinated animals with the live modified *ca* vaccine strain A/HK/Otar/6:2/2010 [30]. The key distinctive feature that allows for the differentiation of animals is the absence of the antibodies in HAI in the course of 12 months after the first and, more importantly, after the second immunization of horses with this vaccine. However, significant geometric mean titers (GMT) of antibodies were detected in HAI on the 28th day after the challenge with the homologous (A/equine/Otar/764/2007 H3N8) or heterologous (A/equine/Sydney/2888-8/2007 H3N8) viruses. The observed GMT ranged from 168±27 to

672±144 (95% confidence interval) in all the horses immunized once or twice at different time intervals (1, 2, 4, 5, 6, 9, or 12 months) after the vaccination. These data, presented here for the first time, suggest that the developed vaccine enables the differentiation of infected from vaccinated animals using such prevalent and accessible serological test as HAI.

The final step in vaccine development was the production of the control pilot plant batch of this vaccine with consecutive product quality tests (according to all the parameters listed in vaccine specification) and the verification of the vaccine production technology. For this purpose, a vaccine batch of 13,400 ampoules (134,000 doses) was produced. According to the results of the technological and biological controls, this vaccine batch fully complied with the standard specification for vaccine preparation in terms of appearance, level of foreign impurities, the preasure level within ampules, solubility, pH, specific humidity, sterility, infectious titer, safety, and immunogenicity. Based on this result, the technical documentation package, including the specifications for the new vaccine, was prepared and approved by the regulatory authorities [38].

Thus, as a result of the integrated studies started from the generation of the vaccine strain and finished with the production of the pilot vaccine batch, the new, safe, and effective modified *ca* virus vaccine against the EIV was developed. This vaccine significantly outperforms the other commercial preparations in a number of features.

In 2016-2017, field studies of this vaccine on the horse breeding farms in Kazakhstan are planned. The developed vaccine has a high potential to prevent equine influenza epidemics.

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CONFLICT OF INTERESTS

The author has no relationship with any organizations with financial conflicts and/or financial interests in the subjects and data disclosed in this paper, including operations, consultant services, fees, shares, expert certificates, grants, granted patents or patent applications, or royalties. No help was used for the preparation of this manuscript.

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