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VACCINES AGAINST EQUINE INFLUENZA
(review)

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Abstract

Equine influenza is a highly infectious disease that can rapidly spread and induce high morbidity in susceptible horse populations (K.P. Yurov, 2009; S.P. Waghmare et al., 2010). Equine influenza is caused by RNA viruses are belonged to the genus *Influenzavirus A* of the family *Orthomyxoviridae* (A.D. Zaberezhnyi et al., 2017). Two different equine influenza virus (EIV) subtypes have been recognized based on antigenic properties of the envelope glycoproteins (HA and NA), the H7N7 subtype (equi-1) and the H3N8. The H7N7 subtype was first isolated in Czechoslovakia in 1956 (prototype strain: A/eq/Prague/1/56). The last confirmed outbreak occurred in 1979 in Italy. The H3N8 subtype of EIV is still circulating in the most countries of the world and has caused outbreaks of disease US and Europe (R. Paillot, 2014; B. Cowled et al., 2009; C.O. Perglione et al., 2016; A.I. Kydyrmanov et al., 200;). Vaccination is one of the most effective tools, alongside isolation, movement restriction and basic biosecurity measures, to prevent EIV infection or to limit its consequences (S.S. Wong et al., 2013). The main goal of vaccination against equine influenza is a significant reduction in clinical signs of disease, virus replication and shedding. Potent EIV vaccines reduce virus transmission and increase resistance to infection (D.J. Baker, 1986). Because of effectiveness EIV vaccines depends on antigenic homology between vaccines and circulates strains of EIV all equine influenza vaccines should contain epidemiologically relevant strains recommended by the OIE (OIE Expert Surveillance Panel on Equine Influenza Vaccine Composition, 2017; R. Paillot, 2014). In accordance with last OIE recommendations EIV vaccines should contain both clade 1 and clade 2 viruses of the Florida sublineage. Clade 1 continues to be represented by A/eq/South Africa/04/2003-like or A/eq/Ohio/2003-like viruses. Clade 2 continues to be represented by A/eq/Richmond/1/2007-like viruses. It is not necessary to include an H7N7 virus or an H3N8 virus of the Eurasian lineage in vaccines (R. Paillot, 2014; OIE Headquarters, 2017). This review gives actual data about the types of licensed vaccines against equine influenza. Whole inactivated/sub-unit, live-attenuated and viral-vector based vaccines are considered. Numerous experimental EIV vaccines developed with modern molecular biology technique have been reported. Reverse genetics techniques which provide a good tool for the generation of recombinant influenza viruses and develop both inactivated and live-attenuated influenza vaccines are also discussed (E.-J. Jung et al., 2010; E. Hoffmann et al., 2010; Y. Uchida et al., 2014). Reverse genetics allows generation of artificial recombinant influenza viruses and provides the possibility to rapidly and easily modify the antigenic characteristics of the vaccine strain by genetic manipulation.

Keywords: equine influenza, vaccines, vaccination, whole inactivated vaccines, sub-unit vaccines, live-attenuated vaccines, viral-vector based vaccines, recombinant vaccines, reverse genetics

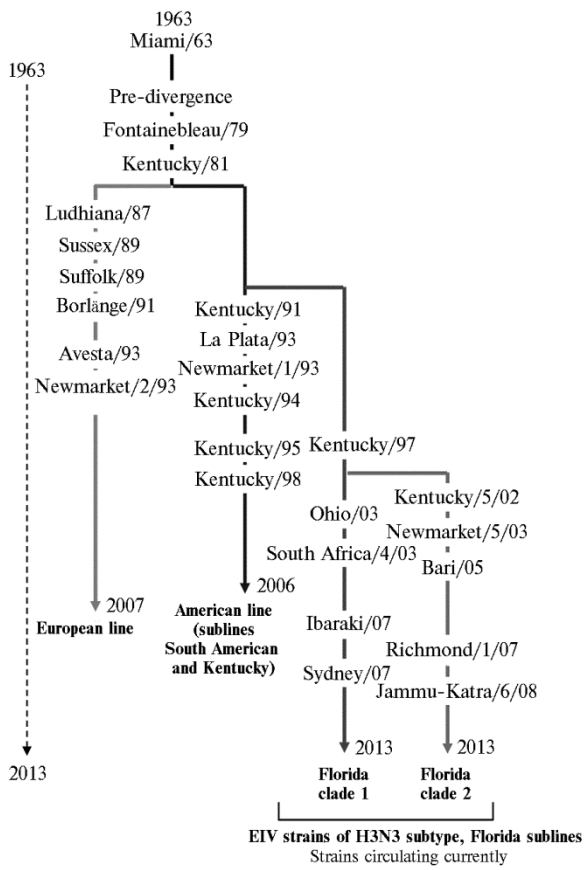
Equine influenza is an infectious disease that affects horses, donkeys, and mules, and causes a body temperature rise to 41 °C, catarrh of the upper respiratory tracts, painful dry cough, rhinotracheitis, or even pneumonia in severe cases. Other notable clinical signs include myalgia, lack of appetite, and enlarged submaxillary lymph nodes [1, 2]. Lethal outcomes mainly occur among younger animals (colts, foals) and donkeys. Death of an adult specimen is usually the result

of a general morbid condition and/or secondary bacterial infection, where pleuritis and pneumonia ensue [3].

Preventive action is the best way to avoid influenza infection; vaccination against equine influenza remains an important tool to control the disease. This makes it imperative to continue research to develop more effective vaccines. The only Russian-made vaccine veterinaries use against equine influenza is a first-generation inactivated vaccine.

This review presents up-to-date information on horse vaccination against influenza; it also covers the types of vaccines, both existing and under development. Today, there exist multiple ways to produce candidate influenza virus strains; one relies upon reverse genetics. The team behind this research used this method to produce Russia's first reassortant recPR8-H3N8eq virus strain, which can be considered a candidate for synthesizing an equine influenza vaccine.

The pathogen is an RNA virus that belongs to the family *Orthomyxoviridae*, genus *Influenzavirus* [4]. The severity depends on the equine influenza virus (EIV) strain as well as on the animal's immunity status. EIV is classified into two subtypes based on the antigenic differences of two envelope glycoproteins: hemagglutinin (HA) and neuraminidase (NA). The first subtype, H7N7, equi-1, is less virulent; its prototype strain is A/equine/1/Prague/56, first isolated in Czechoslovakia in 1956. The last confirmed outbreak occurred in Italy in 1979; however, this subtype was also isolated in India in 1987 [5], as well as in Egypt in 1989 [6]. The other subtype (H3N8, equi-2) is represented by the prototype strain A/equine/2/Miami/63, first isolated in North America in 1963 [7]. It continues circulating in most countries except Australia, New Zealand, and Iceland. Since the late 1980s, H3N8 EIV is classified into the American line and the European line [8]. The American line has three sublines of different antigenic characteristics: South American, Kentucky, and Florida sublines [9]. Further genetic evolution of the Florida subline caused two new viral groups to emerge (Florida clade 1 sublineage, Florida clade 2 sublineage), which contain all the viral isolates that have recently been isolated in the Americas [10, 11], Europe [12-14] and Asia [15, 16] (Fig.).



Equine influenza virus (EIV) **evolution (**main sublines and strains) **[3].**

Note that EIV vaccines have a history of 50 years. Nevertheless, H3N8 viruses are still able to cause enzootics in North America and Europe. Over the last 10 years, multiple major outbreaks occurred in: Sweden (2007), Australia (2007) [17], Japan (2007)

[18, 19], India (2008-2009) [20], South America (2012) [11, 21], Kazakhstan (2007) [22], and Mongolia (2007-2008) [23]. In 2017, an outbreak of influenza caused by A/donkey/Shandong/1/2017(H3N8) EIV was observed in donkeys in the Chinese province of Shandong [24]. In the Russian Federation, EIV epizootics have been registered in the Republics of Khakassia (2007), Buryatia, and Tywa (2008-2009) [25].

Influenza A viruses feature a considerable variability of the virion envelope glycoproteins (HA and NA). Therefore, the more identical are the *HA* genes in the vaccine strain and in the field strain, the more efficiently vaccination will reduce the replication of the influenza virus in the respiratory tract or its release into the environment in case of infection. An efficient vaccine against equine influenza must contain the actual circulating EIV strains [26].

The Vaccine Panel of the World Organisation for Animal Health, OIE, France, publishes annual reports on the laboratory and epidemiological data on EIV strain circulation; the reports present recommendations on vaccine composition. Since 2010, OIE has been recommending that representative H3N8, Florida clade 1 (South Africa/03 or Ohio/03) and Florida clade 2 (Richmond/1/07) strains be included in vaccines. Adding H7N7 and H3N8 (European line) strains is not necessary [3, 27].

Like quarantine and restrictive actions, vaccination against equine influenza is one of the key tools to control the disease [28]. Vaccination mainly seeks to reduce the clinical manifestations of the disease, which improves the animal well-being, shortens the re-convalescence period, and lowers the risk of secondary infections. Besides, vaccination helps reduce the release of the virus into the environment, which curbs the spread of infection [29, 30]. In the Russian Federation, vaccinating horses against influenza is regulated by the Guidelines for Prevention and Tackling of Equine Influenza as approved by the Chief Veterinary Department of the USSR Ministry of Agriculture on September 1, 1980; and by the Veterinary Rules of Transport of Sporting Horses in the Russian Federation as approved by the Ministry of Agriculture of the Russian Federation on May 30, 2003. These documents state the vaccinating horses against influenza is recommendatory rather than mandatory. Subject to preventive vaccination with an inactivated polyvalent vaccine are horses owned by horse farms, sports organization, and circuses; sporting and pedigree horses leaving their farms; horses from any farm or household at risk of influenza. Sporting horses, i.e. horses issued a passport by the Russian Equestrian Federation (REF) or the Russian National Research Institute of Horse Breeding (RNRIHB), as well as circus and theater horses, must be vaccinated at least once every 6 months.

EIV vaccines currently in use can be classified by the production technology in three groups: inactivated whole-virion vaccines, subunit live attenuated vaccines, and vector vaccines (Table) [3].

The first generation of EIV vaccines veterinaries used for decades comprised whole-virion inactivated vaccines that contained aluminum hydroxide as an adjuvant [31]. The primary advantage of this type is that the virus does not replicate, and the vaccine cannot render a horse sick [32]. The immunity this type of vaccines induces is mainly based on stimulating the humoral response in horses. No cytotoxic cell response is induced [33]. The immune system produces antibodies specific not only to variable envelope antigens (HA and NA) but also to more conservative EIV proteins such as NP or M, which are supposedly responsible for cross-protective immunity [3]. Humoral immunity induced by inactivated EIV vaccines does not last long. It has been shown that the conventional inactivated vaccine stimulates the production of short-lived (<100 days) IgG(T) antibodies that cannot fix the complement, whereas a natural EIV infection

mainly causes the production of virus-specific IgA, IgG2a, and IgG2b antibodies. As a result, a horse will need double or multiple vaccinations to have an immune response lasting 12 months [34].

Veterinary-used EIV vaccines classified by the production technology [3]

Name	Produced by	Adjuvant	Antigen	EIV strains
Inactivated virions/cubunits				
DuvaxynTm IE Plus	Elanco (USA)	Carbopol	Whole EIV virions	Newmarket/1/93 (H3N8) Suffolk/89 (H3N8) Prague/56 (H7N7)
Calvenza®-03 EIV	Boehringer Ingelheim Animal Health (Germany)	Carbopol	Whole EIV virions	Newmarket/2/93 (H3N8) Kentucky/2/95 (H3N8) Oiho/03 (H3N8)
Equilis Prequenza (updated 2013)	MSD Animal Health (USA)	ISCOM-Matrix	Whole EIV virions	Newmarket/2/93 (H3N8) South Africa/4/03 (H3N8)
Equilis Prequenza	MSD Animal Health (USA)	ISCOM-Matrix	HA subunits	Prague/56 (H7N7) Newmarket/1/93 (H3N8) Newmarket/2/93 (H3N8)
EquipTM F	Pfizer Ltd. (USA)	ISCOM	HA and NA subunits	Newmarket/77 (H7N7) Borlänge/91 (H3N8) Kentucky/98 (H3N8)
Inactivated Polyvalent Horse Flu Vaccine	Kurskaya biofabrika (Russia)	Гидроокись алюминия	Whole EIV virions	Cambridge-63 (H7N7) France-98 (H3N8)
Live attenuated cold-adapted strains				
Flu Avert® I.N.	Intervet/Schering-Plough Animal Health (The Netherlands)	Нет	Whole EIV virions	Attenuated cold-adapted Kentucky/91 (H3N8)
Vector vaccines				
PROTEQ FLU™	Merial Animal Health Ltd. (France)	Carbomer	HA	Ohio/03 (H3N8) Newmarket/2/93 (H3N8)
PROTEQ FLU™ (updated 2014)	Merial Animal Health Ltd. (France)	Carbomer	HA	Ohio/03 (H3N8) Richmond/1/07 (H3N8)

Note. EIV stands for equine influenza virus; HA stands for hemagglutinin; NA stands for neuraminidase.

Subunit EIV vaccines contain purified viral proteins (HA and/or NA). The adjuvant is an ISCOM or ISCOM-Matrix immunity-stimulating complex. ISCOM consists of 35-nm spherical structures formed by the hydrophobic interaction of amphiphilic antigen (HA and/or NA) molecules with cholesterol, phospholipids, and *Quillaja* saponins. These complexes are more immunogenic than the original proteins [35]. Since ISCOM consists of microparticles, they are easily absorbed by macrophages, where antigens are subsequently processed and presented. ISCOM-Matrix based vaccines are similar, but their viral proteins are not part of the lipid-saponin complex [36]. It has been shown that using inactivated EIV vaccines with a carbomer adjuvant stimulates greater production of protective antibodies [37].

Over the last 15 to 20 years, there has emerged a new generation of EIV vaccines (see Table 1) that trigger both the humoral and the cellular immune response. One example is the live attenuated vaccines based on temperature-sensitive (ts) and cold-adapted (ca) strains. Vaccine EIV strains effectively reproduce in vivo at suboptimal (25 °C) upper respiratory tract temperatures, whereby they induce local and systemic immune response; at the same time, they do not replicate in the lower respiratory tracts at 38-39 °C, where the wild-type virus replication is usually associated with bronchitis, bronchiolitis, interstitial pneumonia, and edema [33].

The first intranasal live attenuated vaccine against EIV (Flu Avert® I.N., Heska Corporation) was developed and licensed in 1999 in the US. The ca strain it was based on had been produced by serial passages of A/Equine/Kentucky/1/91 in chicken embryos (CE) at lower temperatures (34 °C, 30 °C, 28 °C, and 26 °C) [38]. Single vaccination was shown to protect ponies against clinical manifestations of the disease for 3 to 6 months or more in the case of control infection with a homologous wild-type virus [39] or with a heterologous European-line

H3N8 IEV whereas the antiviral antibodies were low [40].

Although such vaccines are clearly advantageous, they pose a risk of vaccine virus reversion or reassortment to the circulating wild-type virus in the horse body, which will cause new pathogenic viruses to emerge. Nevertheless, an experiment, in which five direct consecutive horse-to-horse passages were performed, did not identify any reversions of the Flu Avert® ca strain to the wild type, which indicated stable attenuation and temperature-sensitive phenotype [40].

After a major outbreak of equine influenza caused by H3N8 EIV in Kazakhstan (2007), the St. Petersburg Influenza Research Institute cooperated with Kazakhstani researchers to develop the first Kazakhstan-produced live modified ca vaccine against equine influenza. Classical genetic reassortment methods were used to obtain a ca strain, A/HK/Otar/6:2/2010, which carries genes encoding the envelope proteins (HA, NA) of the A/equine/Otar/764/2007 wild strains (H3N8, American Florida line, clade 2) as well as genes encoding the internal proteins (PB2, PB1, PA, NP, M, NS) of the attenuation donor, the A/Hong Kong/1/68/162/35 (H3N2) ca strain. The safety and efficacy of this vaccine have been studied in horses. As of today, Kazakhstan's horse farms are testing the vaccine in the field [41, 42].

The emergence and advancement of reverse genetics in the late 20th century revolutionized the development of prototype viral strains for inactivated and live attenuated vaccines. When applied to the influenza virus, reverse genetics can quickly produce vaccine candidate strains of any HA or NA subtype. Recombinant vaccines are produced by cloning individual kDNA molecules encoding the eight segments of the virus A RNA into a special plasmid. Then the plasmids are transfected into eukaryotic cells [43, 44]. Reverse genetics can not only produce reassortant viruses of required antigenic properties and lower virulence but also modify them to match the changing antigenic properties of the circulating field strains. Veterinary research has used this method to produce vaccine candidate strains for swine [45] and avian influenza [33, 46].

Mutating the genes of the internal viral proteins and removing the virulence factors of highly pathogenic influenza viruses can produce attenuated strains [45, 47]. Quinlivan et al. [48] thus obtained three recombinant EIV with *NS1* deletions, which attenuated them in relation to the original wild-type A/eq/Kentucky/5/02 (H3N8) and rendered them unable to replicate in interferon-competent cells in vitro. Testing recombinant EIV as candidate vaccines confirmed that vaccinating horses with a mutant NS1-126 virus provided effective clinical protection of animals infected with a wild-type virus. Vaccinated horses had far weaker and fewer clinical manifestations, as well as a shorter viral release period, as compared to the controls [49].

In early 2018, Rodrigueza et al. [50] reported developing a temperature-sensitive (ts) live attenuated vaccine against equine influenza. The researchers applied reverse genetics to make mutations in the polymerase genes *PB1* and *PB2,* A/equine/Ohio/1/2003 H3N8 (Florida sub-lineage clade 1); the mutations they made were responsible for the ts/ca/att phenotype of the attenuation donor A/Ann Arbor/6/60 H2N2 from the human live attenuated vaccine FluMist (MedImmune, US). The resultant recombinant virus was able to effectively replicate at lower temperatures (33 °C); both in vivo and in vitro, it had a phenotype similar to that of the live attenuated vaccine Flu Avert®. Single intranasal administration of the recombinant virus effectively protected the horses against a control exposure to a homologous wild-type virus [50, 51].

Vector vaccines are produced by inserting the required gene of a particular pathogen along with a set of regulatory elements in the viral vectors. Viral antigens are expressed and synthesized de novo in the infected cells [52, 53].

ProteqFlu® (Merial Ltd., France) is the only live recombinant vector vaccine against equine influenza that has been in use by horse breeders since 2003 [33]. The vaccine uses recombinant canarypox virus, ALVAC, as a vector to express HA genes of the A/equine/Ohio/03 (H3N8) and A/equine/Richmond/1/07 (H3N8) equine influenza viruses [54, 55]. The vaccine is safe to use, as the recombinant canarypox virus causes an abortive infection in mammalian cells [56]. Vaccinated horses develop a humoral and cellular immune response. One-vaccinated ponies had far fewer and weaker clinical manifestations of the test infection as compared to the controls. The viral release was significantly lesser but not shorter. After double vaccination with a 35-day interval, the virus-neutralizing IgGa and IgGb antibodies preserved their protective level for 4 months. Therefore, triple vaccination is necessary to induce a 12-month protective immune response [57, 58].

Van de Walle et al. [59] synthesized a recombinant vaccine viral vector based on the abortive NYO3 EHV-1 strain that expresses the *HA* EIV A/eq/Ohio/03 gene. Double immunization with this recombinant virus with a 5-week interval produced specific IEV antibodies in horses, which were detectable for 18 weeks after the second vaccination. However, the authors did not research the protectiveness of the potential vaccine against experimental IEV infection; the safety of the viral vector was not tested either [59].

Reverse genetics is a state-of-the-art and efficient approach to synthesizing vaccine strains of required properties [60]. The Russian Ministry of Health's Gamaleya National Research Center for Epidemiology and Microbiology is also researching the use of reverse genetics to produce recombinant influenza viruses. Thus, they produced a reassortant recPR8-H5N1strain that contains the *HA* gene from the highly pathogenic A/Kurgan/05/2005 (H5N1) avian influenza virus isolated in Russia. The Institute has researched the reproductive, antigenic, and virulent properties of the reassortant. The lab-synthesized recPR8-H5N1-based inactivated emulsified vaccine was shown to protected chicks, aged 6 weeks, against control administration of A/Kurgan/05/2005 (H5N1), which is a highly pathogenic virus [46]. As of today, the Institute's Molecular Diagnostics Laboratory is trying to obtain a reassortant IEV strain with *HA* and *NA* genes from A/equine2/Bitza/07 (H3N8), other genes from the highly productive A/Puerto Rico/8/34 (H1N1) strain.

Thus, equine influenza is a highly infectious disease that tends to spread fast and have high incidence in sensitive specimens. Its outbreaks may significantly impact horse breeding, especially that of sporting horses. Vaccination against equine influenza is an effective prevention tool. First-generation vaccines were inactivated whole-virion and subunit vaccines that induced the production of protective antibodies. Then they invented the second generation (live attenuated and vector vaccines), which could stimulate a humoral and cellular immune response to imitate the protective immune response a natural virus would cause in horses. Reverse genetics is one of the promising methods for the production of recombinant live attenuated vaccines; it can obtain reassortant viruses of the required antigenic properties. It can also modify such viruses to tailor them to the changing antigenic properties of the circulating field strains.

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