Safety and immunogenicity of a novel cold-adapted modified-live equine influenza virus vaccine

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Objective To design and evaluate the safety and immunogenicity of a modified-live vaccine to prevent equine influenza virus (EIV) infection based on the novel reassortant cold-adapted strain A/HK/Otar/6/2010.

Methods Surface proteins (HA, NA) from the wild-type strain A/equine/Otar/764/2007 (H3N8) and internal proteins (PB2, PB1, PA, NP, M, NS) from the attenuated cold-adapted donor strain A/Hong Kong/1/68/162/35CA (H3N2) were included in the vaccine. Horses were administered 10^6.2 EIDso/mL of the modified-live vaccine or saline solution using a nasal spray. The clinical condition of the animals was assessed throughout the study and nasopharyngeal swabs were collected for virus titration. Two yearlings in each group were euthanased on day 5 post vaccination (PV) for histological examination and measurement of viral titres in the organs. Serum samples and nasal secretions were collected to evaluate serological response. Lymphoproliferation after restimulation in vitro was determined to evaluate cell-mediated immunity. To evaluate the protective capacity of the vaccine, the yearlings in both groups were challenged with the wild-type virus at 28 days PV and their clinical condition and serological response was evaluated. Nasal swabs were collected to assess viral shedding from the upper respiratory tract.

Results Single intranasal administration of a modified-live EIV vaccine caused no adverse effects and vaccinated yearlings and pregnant mares did not form detectable levels of antibodies by days 7, 14 and 28 PV, as indicated by the HI reaction and ELISA. Secretory antibodies could be detected on day 7 and reached maximal levels on day 14 PV. In vitro studies showed that the yearlings and pregnant mares both formed a cell-mediated immune response by day 14 PV. The vaccine protected yearlings against challenge with wild-type virus. We conclude that single intranasal administration of the modified-live EIV vaccine was safe in the yearlings and pregnant mares that we treated, and was immunogenic and protective in the yearlings.

Keywords cold-adapted vaccine; equine influenza virus; horses; immunogenicity

Abbreviations cpm, counts per minute; EID, embryo infectious dose; EIV, equine influenza virus; HI, haemagglutination-inhibition; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; PC, post challenge; PV, post vaccination; SI, stimulation index

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E quine influenza virus (EIV), which is the leading viral cause of respiratory disease in horses, has two subtypes: subtype 1 (H7N7) and subtype 2 (H3N8). The H7N7 subtype has not been reported in recent years, but the EIV H3N8 subtype is currently a significant health risk to horses and an economic problem in horse breeding.1 The current vaccination strategies generally rely on the administration of inactivated or modified-live vaccines.

At the beginning of the 1960s, whole inactivated vaccines and virus-like particles or their subunits were widely introduced after a series of successful field trials in veterinary practice. The main advantages of these vaccines are their safety and the absence of viral replication.2 The main disadvantages of inactivated vaccines are their poor immunogenicity because of the predominantly short-term humoral immunity and the necessity for repeated administration of the vaccine to maintain an immune response.3–5 Some inactivated vaccines administered with adjuvants also may cause adverse local reactions in vaccinated animals.6

These drawbacks have led to the search for more effective vaccines against EIV. Hannant et al.7 showed that the immune response induced by experimental infection with EIV persisted and could protect horses from subsequent re-infection for more than 1 year. Those authors noted that vaccines mimicking the infectious process are more effective than inactivated vaccines. For this reason, the design of live-attenuated8–11 and vector vaccines12,13 has been the dominant approach in the development of effective vaccines against equine influenza over the past 20 years.

The current live-attenuated vaccines include preparations based on cold-adapted (Ca) strains. Under natural conditions, Ca EIV strains can reproduce effectively in the upper respiratory tract, where they cause local and systemic immune responses. The most significant advantage of Ca EIV strains is their inability to reproduce at the higher temperatures in the lower respiratory tract, where replication of the wild-type virus is usually accompanied by the development of bronchitis, pneumonia and pulmonary edema.10 In addition, vaccines developed from Ca strains can generate cross-reactivity of the immune system in vaccinated animals, which is very important in view of the antigenic variation (antigenic drift) observed for EIV.9

Approaches based on the application of Ca strains have been used to create a live-attenuated vaccine against EIV that has been used successfully in North America. This Ca-modified-live vaccine for intranasal administration (Flu Avert™ I.N.; Heska Corporation, CO, USA) was licensed in 1999 and since then has been used widely in North America. The vaccine contains the Ca strain A/equine/Kentucky/91 (H3N8) of North American origin; however, it also protects against the European H3N8 lineage. Despite not stimulating a
high level of antibody response, single administration of Flu Avert™ I.N. vaccine provides long-term protection against infection, compared with inactivated vaccines. The most significant advantage of this vaccine is that it generates an immune response similar to that observed during natural infection.9,10

At the Research Institute for Biological Safety Problems in Kazakhstan, and in Heska Corporation, we designed a live vaccine to prevent EIV infection on the basis of the reassortant Ca strain A/HK/Otar/6:2/2010. The live vaccine contains the surface proteins (HA, NA) from the wild-type strain A/equine/Otar/764/2007 (H3N8) and the internal proteins (PB2, PB1, PA, NP, M, NS) from the attenuated Ca donor strain A/Hong Kong/1/68/162/35CA (H3N2). The purpose of this study was to further assess the application of this vaccine in veterinary practice, by investigating its safety and immunogenicity in horses.

Materials and methods

Vaccine strain
A modified-live EIV vaccine, based on a reassortant Ca strain, with a 6:2 genome structure was produced by classical genetic methods from the donor Ca attenuated strain A/Hong Kong/1/68/162/35CA (H3N2; kindly provided by the Research Institute for Influenza, St Petersburg, Russia) and the wild-type strain A/equine/Otar/764/2007 (H3N8) (GenBank: ADZ55424, the American Lineage Florida Clade 2) (isolated in Kazakhstan in 2007). The method of obtaining the reassortant Ca strain (exemplified by the recombinant strain A/HK/Astana/6:2/2010) has been described previously.14 The reassortant Ca strain A/HK/Otar/6:2/2010 was produced in Kazakhstan at the Research Institute.

Vaccine preparation
The vaccine virus was cultured in 10-day-old specific-pathogen-free chicken embryos (Lohmann Tierzucht GmbH, Germany) at 34 ± 0.5°C. The virus was infected into the allantoic cavity at a dose of 10,000 EID50 (embryo infectious dose)/0.2 mL. After incubation for 48 h, the embryos were cooled to 2–8°C, the allantoic fluid was collected, clarified by centrifugation at 9000g for 30 min and mixed in a 1:1 ratio with sterile stabilising medium containing 12% peptone from casein (Sigma-Aldrich, Germany) and 6% lactose (Sigma-Aldrich). The vaccine mixture was mixed at 300 rpm for 30 min at room temperature and then aliquoted into vials (2.0 mL each). The vaccine vials were stored at 2–8°C for no more than 2 days before use.

Animals
The study included 16 purebred (Kazakh dual-purpose Mugalzhar breed) 4–5-year-old pregnant mares (in the second half of pregnancy). All the mares were seronegative for EIV and were scanned for pregnancy status. They were divided into two groups and housed in separate rooms of the stable. Drinking water and hay were available ad libitum and pelleted feed was provided twice daily. All the mares were treated to control gastrointestinal parasites.

The study group also included 20 purebred (Kazakh dual-purpose Mugalzhar breed) yearlings of both sexes aged 1–1.5 years. All foals were seronegative for EIV and their management was similar to that of the mares. During the challenge infection experiment, the animals were housed in a special isolation ward to prevent the spread of the wild-type virus to the environment.

This study was carried out in compliance with national and international laws and guidelines on animal handling. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Research Institute for Biological Safety Problems (Permit no. 0812/345).

Vaccine safety
Yearlings (n = 10) and pregnant mares (n = 8) in the experimental groups were administered a nasal spray of 10^{9.2} EID50/mL (1 mL per nostril) of the live-modified reassortant Ca strain A/HK/Otar/6:2/2010 vaccine using a system designed for administration of the Flu Avert™ I.N. vaccine. The yearlings (n = 10) and pregnant mares (n = 8) in the control groups were administered normal saline solution in the same manner.

Clinical monitoring
Monitoring of the general clinical condition of the yearlings and pregnant mares in the experimental and control groups was carried out over 21 days post vaccination (PV). The clinical status of the animals was assessed using a point system in which the following parameters are scored:
- General health: normal general state (score = 0), illness/depression/normal appetite (1), illness/depression/loss of appetite (2), dehydration (3), exhaustion (4), inability to stand (5), on the edge of death (6), death (10).
- Respiratory observations: shortness of breath (1), dyspnoea (2), cough: 2–5 times in 10 min (1), 6–20 times in 10 min (2), >20 times in 10 min (3).
- Ocular observations: lacrimation (1), moderate mucopurulent secretion (2), severe mucopurulent secretion (4), mild conjunctivitis (2), strong conjunctivitis (4).
- Nasal observations: serous secretion of mucus nasal discharge (1), moderate mucopurulent nasal discharge (2), severe mucopurulent nasal discharge (4), sneezing 2–5 times in 10 min (1), sneezing 6–20 times in 10 min (2), sneezing >20 times in 10 min (3).
- Rectal temperature: 38.5–39.0°C (1), 39.1–39.5°C (2), >39.6°C (3).

Viral shedding in the upper airway
Nasopharyngeal swabs were collected from the yearlings and pregnant mares in the experimental and control groups on days 1, 3, 5, 7 and 14 PV. The swabs were placed into tubes containing 1 mL of viral transport medium, comprising phosphate-buffered saline (PBS) containing 40% glycerol and 2% antibiotic solution (1000 U/mL benzylpenicillin, 1000 U/mL streptomycin, 250 mg/mL fungizone). The nasopharyngeal swabs were stored at −70°C before analysis. The viral titres were determined using 10-day-old chicken embryos. The viral titre was calculated using the method of Reed and Muench15 and expressed as log_{10} EID50/mL. The specificity of the virus isolated from the nasopharyngeal swabs was determined using the commercial Directgen Flu A rapid assay (Becton Dickinson, NJ, USA).

Pathological and histological analyses
To investigate the possible pathological changes in the organs of the horses after vaccination, yearlings in the experimental (n = 2) and...
To measure the viral titre in the organs, 1:10 suspensions of the organs were prepared in PBS pH 7.3 containing 10% antibiotic solution.

Collection of samples for determination of vaccine immunogenicity

Vaccine immunogenicity, defined as humoral and cellular immunity, was determined in the yearlings and pregnant mares. Blood samples and nasal secretions were collected from the animals in the experimental and control groups on days 0, 7, 14 and 28 PV. Before sampling, the animals were sedated with 20–40 mg/kg of detomidine IV (Pfizer Animal Health, USA). Blood samples were collected via jugular venepuncture into serum separator tubes (Vacutainer; Becton Dickinson) for the isolation of serum. Nasal secretions were subsequently collected by centrifugation at 2000g, aliquoted and stored at −20°C. For the lymphoproliferation experiments, 60 mL of blood was collected via jugular venepuncture into 15 U/mL of heparin (Elkins-Sinn, NJ, USA).

Determination of vaccine immunogenicity

Humoral immunity. The serum samples were subjected to haemagglutination-inhibition (HI) assay and ELISA to determine the accumulation of influenza virus antibodies. The HI assay was performed according to the OIE manual, using a suspension of chicken red blood cells (1%). A native virus A/HK/Otar/6/2010 (working dose of 4 haemagglutinating units) was used as the antigen. The ELISA assay was performed using a commercial AIV Ab ELISA system for the detection of antibodies against influenza A (Anigen Animal Genetics, Korea), according to the manufacturer’s instructions. The serum samples were tested at a 1:10 dilution in PBS.

The presence of secretory antibodies (IgA) in the nasal secretion samples was assayed using the commercial Nori™ Equine IgA ELISA Kit (Genorise Scientific, PA, USA), according to the manufacturer’s instructions. The nasal secretion samples were tested at a 1:100 dilution in PBS.

Cell-mediated immunity. Whole blood samples were examined for lymphoproliferation after restimulation in vitro. Peripheral blood mononuclear cells (PBMCs) in 1 mL of cRPMI (RPMI-1640 containing 0.01 mol/L HEPES, 0.002 mol/L sodium pyruvate, 100 U/mL penicillin, 100 mg/mL streptomycin and 10% fetal bovine serum) were incubated at 37°C for 45 min with or without 10^6.5 EID_{50} of the native virus A/HK/Otar/6/2010. After incubation, the cells were pelleted by centrifugation at 300g for 10 min to remove the virus and the PBMCs were plated (2 × 10^5 cells in 200 μL/well) in triplicate into 96-well microtiter plates (Costar, CA, USA). The cells were incubated in a humidified 37°C, 5% CO₂ incubator for 72 h and then each well was pulsed with 50 μL of media containing 0.02 mCi/mL 3H-thymidine for 10 h. Thymidine uptake was measured as the counts per minute (cpm) using a microplate scintillation and luminescence counter system (Top Count; Packard, CT, USA). Lymphoproliferation was determined as the stimulation index (SI), calculated as:

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SI = \frac{\text{virus-stimulated PBMCs (cpm)}}{\text{unstimulated PBMCs (cpm)}}.
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Determinations of the protective capacity of the vaccine

To further explore the protective efficacy of the Ca virus A/HK/Otar/6/2010 vaccine, yearlings in the experimental (n = 8) and control (n = 8) groups were subjected to infection with the wild-type virus A/equine/Otar/764/07 (H3N8) on day 28 PV. The animals were exposed using a nebulised aerosol generated from 7.5 mL of egg allantoic fluid. The animals were individually exposed to the challenge virus (10^6 EID_{50} per animal) by connecting a nebuliser (SaHoMa™-II mobile ultrasonic nebulizer; NEBU-TEC International med. Produkte Eike Kern GmbH, Germany) to a hood attached to the horse’s head.

Clinical observations and body temperature were monitored daily for 21 days post challenge (PC). The clinical condition of the infected animals was assessed using the point system described earlier. Serum samples were also collected on days 7, 14 and 28 PC to determine the accumulation of influenza virus antibodies using the HI assay and ELISA.

To assess the degree of viral shedding from the upper respiratory tract, nasal swab samples were taken from the experimental and control groups on days 1, 3, 5, 7 and 14 PC. The samples were collected into tubes containing 1 mL of viral transport medium and assayed in 10-day-old chicken embryos.

Statistical analysis

The clinical scores, the number of days on which clinical signs were observed, the number of days on which virus shedding was detected, rectal temperatures and the antibody titres in the vaccinated and control groups were compared using one-way ANOVA. Significant differences were determined at the level of P < 0.05.

Results

Vaccine safety

Yearlings. Intranasal administration of 10^{6.2} EID_{50}/mL (1 mL in each nostril) of the vaccine from the Ca strain A/HK/Otar/6/2010 had no observable clinical side effects in yearlings. The general clinical state of the yearlings in both the experimental (n = 8) and control groups (n = 8) was normal over the 21 days of observation PV; tearing, mucopurulent secretions or nasal discharge, or signs of conjunctivitis were not observed in any of the vaccinated yearlings. The rectal temperatures (unpubl. data) of the yearlings in the experimental and control groups were similar and remained within the normal range (37.5–38.5°C) for the duration of the observations.

In the yearlings from the experimental group, the extent of shedding of vaccine virus in the upper part of the respiratory tract was low. The virus was shedding in four of eight animals on day 1 PV and in three
animals on day 3 PV, at titres ranging from $10^{0.75}$ to $10^{1.0}$ EID_{50}/mL. Histological analysis of the organs removed from two yearlings each in the experimental and control groups at day 5 PV confirmed the absence of any pathological changes. The virus was not detected in any organ, as indicated by assaying the organ suspensions in chicken embryos.

**Pregnant mares.** A single intranasal administration of the vaccine showed no clinical side effects in the pregnant mares. The clinical status of the pregnant mares in both the experimental (n = 8) and control (n = 8) groups was normal over the 21 days of observation PV; tearing, mucopurulent secretion or discharge from the nose, or signs of conjunctivitis were not observed in any of the vaccinated animals. All pregnancies proceeded normally. The mean rectal temperatures of the pregnant mares in the experimental and control groups did not differ during the observation period and remained within the normal range (unpubl. data).

Pregnant mares in the vaccinated group shed less virus from the upper respiratory tract than the vaccinated yearlings. Virus shedding was observed in three of eight mares on day 1 PV and in one of eight mares on day 3 PV, at doses ranging from $10^{0.75}$ to $10^{1.0}$ EID_{50}/mL.

**Immunogenicity**

**Humoral immunity.** Vaccine immunogenicity was determined in blood samples taken from the yearlings and pregnant mares in the experimental and control groups on days 0, 7, 14, and 28 PV. No specific serum antibodies to EIV subtype A/H3N8 were detected by HI assay or ELISA. Secretory immunity was evaluated using ELISA of the nasal secretions taken from yearlings and pregnant mares on days 0, 7, 14, and 28 PV. Secretory immunity was comparable in the experimental groups of yearlings and pregnant mares (Figure 1). IgA antibodies were first detected on day 7 PV, with titres of 1 : 100 to 1 : 200 in four yearlings and three pregnant mares. IgA antibodies reached maximal levels on day 14 PV, with titres ranging from 1 : 100 to 1 : 400 in six of the yearlings and four of the pregnant mares. On day 28 PV, the number of yearlings and mares with detectable IgA titres decreased and was similar to that of day 7 PV. Specific IgA antibodies were not detected in the control groups of yearlings and pregnant mares over the entire period of observation (unpubl. data).

**Cell-mediated immunity.** Cellular immunity in the vaccinated yearlings and mares was assessed as the degree of lymphoproliferation in peripheral blood in vitro in response to repeated stimulation with a specific inducer. The cellular immune responses in yearlings and pregnant mares were comparable (Figure 2). There was a significant difference (P < 0.05) in the proliferative response of cells isolated from the experimental and control groups on day 14 PV, as indicated by the SI values. In vitro stimulation with the specific inducer led to a specific increase in the mitotic activity of PBMCs isolated from the experimental yearlings and pregnant mares, which indicated formation of a pool of PBMCs recognising the antigen during the post-infection period. There was no difference (P > 0.05) in the SI of the experimental and control yearlings and mares when PBMCs isolated on day 28 PV were assayed. The data indicated that a cellular immune response formed on day 14 PV in the experimental yearlings (1.73 ± 0.26) and experimental pregnant mares (1.56 ± 0.18), which then reduced to similar levels as in the respective control groups by day 28 PV.

Clinical signs after challenge infection. Clinical observation of the infected yearlings in the experimental and control groups was performed over 21 days PC (Table 1, Figure 3). After infection with the wild-type virus, five of the eight yearlings in the experimental vaccinated group and all eight of the yearlings in the control unvaccinated group developed disease. The duration of disease was significantly shorter in the experimental group (4.8 ± 1.4 days; P < 0.001) than in the control group (16.8 ± 0.4 days). The severity of disease, as indicated by clinical signs such as general health, respiratory observations and rectal temperature, was milder in the experimental group than in the control animals.

Temperature response after challenge infection. The changes in body temperature for the yearlings in the experimental group (n = 8) and control group (n = 8) over 21 days PC are shown in Figure 4. The rectal temperature was normal during the entire period of observation in the experimental vaccinated group, with the exception of day 2 PC (up to 38.9°C in two animals) and day 3 PC (up to 38.9°C in one animal). In the control group, two peaks of temperature were observed on day 2 PC (39.1 ± 0.15°C) and day 11 PC (38.6 ± 0.19°C).

Virus shedding after challenge infection. Viral shedding from the upper respiratory tract of the yearlings in the experimental and control groups was determined on days 1, 3, 5, 7 and 14 PC (Figure 5). All the infected yearlings in both the experimental and control groups shed the virus, beginning on the first day after challenge. Increased shedding from the upper airway was observed in the control group during the entire period of observation (P < 0.001 for days 1, 3, 5 and
Viral shedding occurred for a longer period of time in the control unvaccinated group, compared with the experimental group in which virus shedding completely was absent at 5–7 days PC.

Serology after challenge infection.

Determination of the serum antibodies in the experimental (n = 8) and control groups (n = 8) of yearlings on days 7, 14 and 28 PC with wild-type influenza virus is shown in Figure 6. Convalescent animals (the control group) had antibody titres of 15.4 ± 1.8 and 30.8 ± 3.6 in the HI assay on days 14 and 28 PC, respectively, and comparable antibody titres in the ELISA (5.6 ± 1.4 and 25.9 ± 4.4, respectively).

The antibody titres in the experimental group were significantly higher in the HI assay (73.3 ± 21.1 and 146.7 ± 42.2) and ELISA (67.2 ± 22.0 and 174.4 ± 42.7) on days 14 and 28 PC (P < 0.025 and P < 0.005, respectively), compared with the control group.

Discussion

We present the results of a study of the safety, immunogenicity and protective effects in horses of the first Kazakhstan live vaccine against EIV generated from the novel reassortant Ca strain A/HK/Otar/6:2/2010. This vaccine was developed in response to a serious epizootic outbreak of equine influenza A (H3N8) in Kazakhstan in 2007,18 when approximately 200,000 horses became ill, of which 50,000 horses (including 40,000 foals) died. Strain A/equine/Otar/764/2007 (H3N8),19 which was recommended by the OIE for the production of a vaccine against equine influenza,20 Previous research showed that
the vaccine strain A/HK/Otar/6:2/2010 retained the $\text{Ca}$ and temperature sensitivity phenotypes and was genetically stabile during 20 consecutive passages in chicken embryos. Moreover, this virus has been demonstrated to be completely safe in laboratory animals. These factors suggested that the novel reassortant strain A/HK/Otar/6:2/2010 may be a good candidate for a live vaccine against equine influenza A (H3N8).

The vaccine strain A/HK/Otar/6:2/2010 was created following the method used by Heska Corporation, which successfully introduced its $\text{Ca}$ live vaccine Flu Avert™ I.N. into veterinary practice. Thus, there are significant differences between the vaccine strains and preparation processes used to generate these vaccine strains. The Flu Avert™ I.N. vaccine was generated from the wild strain A/equine/Kentucky/91 (H3N8), which was attenuated by multiple passages (49 passages) in chicken embryos under low temperature conditions (26°C). Our vaccine was based on the strain A/HK/Otar/6:2/2010, which was attenuated by classical genetic methods with the novel attenuated donor $\text{Ca}$ strain A/Hong-Kong/1/68/162/35CA (H3N2).

The choice of A/Hong-Kong/1/68/162/35CA (H3N2) as an attenuation donor was not a coincidence, as it offers the advantages of the safety and immunogenicity of these $\text{Ca}$ vaccine strains and also has high reproductive activity. For example, the infectious activity titre of the strain A/HK/Otar/6:2/2010 when cultured in chicken embryos is stable at $\geq 9.0 \text{log}_{10} \text{EID}_{50}/\text{mL}$.

One of the main objectives of this research was to investigate the safety of our vaccine in yearlings and pregnant mares. The results demonstrated that a single intranasal administration of the live vaccine generated from the reassortant $\text{Ca}$ strain A/HK/Otar/6:2/2010 was safe for both the yearlings and pregnant mares that we treated, confirmed by the fact that the clinical condition of the vaccinated animals remained normal during the entire period of monitoring and pregnancy proceeded normally in the vaccinated mares. Additionally, a low extent of shedding of the vaccine virus in the upper respiratory tract was observed in the vaccinated yearlings and pregnant mares.

### Table 1. Clinical scores of vaccinated (experimental) and unvaccinated (control) yearlings following challenge with the wild-type equine influenza (EI) virus A/equine/Otar/764/07 (H3N8) 28 days post vaccination

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<th>Res</th>
<th>Ocu</th>
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</table>

Clinical score represent the summed scores of general health (Gen), respiratory observations (Res), ocular observations (Ocu) and nasal observations (Nas). Total score represents clinical score plus rectal temperature score (Temp).
does not lead to generalised infection and that viral replication occurs exclusively in the upper respiratory system. Our vaccine appeared to be safer for horses than the commercial vaccine Flu Avert™ I.N., which induced adverse effects in the form of expiration of the nostrils and eyes and cough in 6.2% of horses aged 3 months to 30 years under field tests. Our results showed that the wild-type virus A/equine/Otar/764/07 (H3N8) did not have high immunogenic properties. In contrast to its commercial analogue (Flu Avert™ I.N.), our vaccine did not generate a systemic humoral response, as specific antibodies could not be detected in the vaccinated yearlings and pregnant mares using either HI assay or ELISA during the period of observation. However, during the same period of time, short-lived (up to 28 days) secretory antibodies (IgA) were detected in 50–75% of the vaccinated animals. It was previously shown that single application of the vaccine Flu Avert™ I.N. in foals provided a low, but detectable, antibody titre in the HI assay.

Cell-mediated immunity, as indicated by SI values, had formed by day 14 PV in both vaccinated yearlings and pregnant mares, but decreased to levels similar to those of the control groups by day 28 PV, which indicated that the cellular immunity generated in the vaccinated animals subsided on day 28 PV. The data obtained in respect of the period of cell-mediated immune response formation (on day 14 PV) are in agreement with a previous study; however, a different duration of immunity was observed in this study. According to Hannant et al., cell-mediated immunity could be detected at 6 months after natural influenza infection; in the present study, this response was absent at 1 month PV. Interestingly, the cellular and humoral immunity responses observed in yearlings (1–1.5 years) and pregnant adult mares (4–5 years) were similar, which leads us to speculate that the vaccine may not only be effective in young horses, but also in adult horses.

Analysis of the protective data showed that a single intranasal application of the vaccine protected yearlings from the wild-type virus A/equine/Otar/764/07 (H3N8). This protection manifested as a significantly (P < 0.001) lower intensity and duration of the clinical signs of disease, including rectal temperature, and of viral shedding in the experimental groups compared with the control animals. A single application of our vaccine exerted a comparable protective effect to that of the commercial vaccine Flu Avert™ I.N. vaccine. Our recent studies (unpubl. data) have shown that intranasal administration of a single dose of 9.0 log10 EID50 of our vaccine is capable of protecting horses (aged 1–2 years) from clinical disease when challenged with the wild-type virus A/equine/Otar/764/07 (N3N8) at 4 months PV (time of observation). This indicates that the duration of immunity offered by our vaccine is not inferior to that of the commercial vaccine Flu Avert™ I.N., or other commercially available vaccines, including inactivated and live-vector vaccines.

A serological response was first detected at 14 days PC in both vaccinated and control yearlings, with both HI and ELISA antibody titres being significantly higher in vaccinated horses than controls. It should be noted that no antibodies were detectable using the HI and ELISA in the vaccinated group of animals prior to challenge with wild-type virus. If we consider the challenge infection as a booster vaccination, it can hypothesised that two vaccinations could induce a measurable serological response. On this basis, experiments are currently being conducted to investigate the immunogenicity of the vaccine after booster immunisation over an interval of 28–30 days.

**Conclusion**

A single intranasal administration of live vaccine derived from a reassortant Ca strain of A/HK/Otar/6:2/2010, developed at the Research Institute for Biological Safety Problems, Kazakhstan, was safe in the yearlings and pregnant mares that we treated, and induced a protective immune response in yearlings against EIV. The results pre-
sented here can be used for further development of live vaccines against EIV derived from Ca strains and obtained by classical genetic methods.

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Competing interests

The authors declare that they have no competing interests.

Authors contributions

Conceived and designed the experiments: KT, ZhK, AS. Performed the experiments: KT, ShR, NA, YK, DI analysed the data: KT, ZhK. Contributed reagents/materials/analysis tools: ShR, NA, NS. Wrote the paper: KT, ZhK. All authors read and approved the final manuscript.

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