



# Safety of a New Intranasal Vaccine Against Equine Influenza: A Study of the Stability of the *Ts Ca* Phenotype of the Reassortant Strain in a Natural Host

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**Abstract** | We tested our intranasal live-attenuated vaccine from a cold-adapted strain of equine influenza (*ts ca* phenotype) in a natural host in order to establish 1) its safety and stability during serial horse to horse passage, and 2) the possibility of spontaneous transmission from horse to horse. To study stability, the vaccine was administered to 5 yearlings. Vaccine viruses isolated from the last group retained their cold-adaptation and temperature sensitivity phenotype, without reversion to the wild-type virus phenotype or occurrence of clinical influenza disease. The vaccine virus did not spontaneously spread in any of the unvaccinated horses when these were mixed with vaccinates in the same field. These results demonstrate that the developed influenza vaccine is safe and phenotypically stable, not transmitted spontaneously. Viruses isolated from the latter group retained the phenotype of the complete attenuation without reversing to the phenotype of the wild-type virus or causing a clinical flu disease.

**Keywords** | Equine influenza virus, Vaccine, Cold adapted, Stability, *Ts ca* phenotype

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## INTRODUCTION

Vaccination is a key strategy to prevent or control equine influenza (EI), an important respiratory disease caused by equine H3N8 influenza virus (EIV) and continues to cause significant outbreaks worldwide, despite several decades of surveillance and prevention (Rodriguez et al., 2018; Tabynov et al., 2014a).

In 2012, we developed a highly effective means of specific prevention of EIV - an innovative live modified cold-adapted viral vaccine based on cold-adapted (*ca*) strain A/HK/Otar/6:2/2010. The fundamental difference between our vaccine and the closest and only commercial analogue of Flu Avert® lies in the method for producing

the vaccine strain. In our case, we used the classical genetic reassortment method using the highly reproductive strain A/Hong Kong/1/68/162/35CA (H3N2) as an attenuation donor. The vaccine formed a pronounced virus-specific T-cell immune response, with the production of secretory IgA antibodies, and also provided good clinical (in terms of intensity and duration of disease signs evaluated by the scoring system) and virological (in terms of virus titer in nasal swabs) protection against a homologous wild strain A/equine/Otar/764/2007 (H3N8) compared with the control group (Townsend et al., 2001).

Moreover, the vaccine even in vaccinated horses provided a continuous protective immune response against the homologous wild-type virus A/equine/Otar/764/2007 (H3N8) with a duration of 12 months (Tabynov et al.,

2014b). When horses used a two-dose immunization regimen with an interval of 42 days, the level of animal protection was even more pronounced, a distinctive feature of which was the formation of a three-month sterile immune response against a homologous virus.

Moreover, vaccinated horses provided good cross protection against the heterologous wild-type virus A/equine/Sydney/2888-8/2007 (H3N8, American lineage Florida sublineage clade 1), 12 months after double immunization. The developed vaccine made it possible to differentiate infected animals from vaccinated (meets the DIVA strategy), which was not previously reported in relation to this type of vaccine.

In addition, the reproducibility of the developed technology for preparing the vaccine under the conditions of scaling according to the approved regulatory and technical documentation for the biological product was established (Assanzhanova et al., 2019).

But at the same time, the safety and stability of the vaccine with repeated passaging on horses, which can be of critical importance in the case of its wide practical use, have not yet been studied.

The aim of the current work was to assess the safety and *ca* *ts* phenotypic stability of the vaccine by testing its ability to cause clinical manifestations of the disease during passage on horses and on the transmission of the vaccine virus from a vaccinated horse to an unvaccinated one under conditions of their joint maintenance.

## MATERIALS AND METHODS

### VACCINE STRAIN AND VACCINE PREPARATION

The modified-live EIV vaccine based on the reassortant *ca* strain A/HK/Otar/6:2/2010 was created and prepared as described previously (Tabynov et al., 2014a).

### ETHICS STATEMENT

This study was carried out in compliance with national and international laws and guidelines on animal handling. Horse experiments were approved by the Committee on the Ethics of Animal Experiments of the Research Institute for Biological Safety Problems (Protocol no. 2018-0153).

### PASSAGE ON YEARLINGS

Male and female yearlings of mixed breed were used. During experiments the animals were housed in the experimental department of The Research Institute for Biological Safety Problems *in vivo* and had not been previously vaccinated for EIV. All horses were seronegative for EIV H3N8, as measured by hemagglutination inhibition assay (HI) prior

to the start of the study.

To evaluate the *in vivo* stability of *ts ca* phenotype of A/HK/Otar/6:2/2010 H3N8 live attenuated influenza vaccine (LAIV), initially, horses (n=5) were vaccinated intranasally with the vaccine containing live-modified reassortant *ca* strain A/HK/Otar/6:2/2010 at a tenfold dose of  $4 \times 10^{9.7}$  EID<sub>50</sub>/animal using a nasal applicator (Boehringer Ingelheim, USA). As sentinels for spontaneous spread of the vaccine, other yearlings (N = 3) were left unvaccinated as a control. Vaccine safety was tested daily by the observation of clinical signs, measurement of rectal temperatures for 14 days post-vaccination (PV) using the point system, in which the parameters are scored as described previously (Tabynov et al., 2014a). Clinical signs of yearlings were observed for 10–20 min daily. The presence of virus in nasopharyngeal swabs were determined prior to vaccination (day 0) and for 9 days thereafter. The specificity of the virus was determined using the commercial Directigen Flu A rapid assay (Becton Dickinson, Franklin Lakes, NJ, USA). Samples of nasal swabs (taken 2-5 days after vaccination) with the highest titer of the virus were used to administer to 3 other EIV seronegative horses the same way. Also, a control group (N=2) of yearlings was again organized as sentinels. Samples of nasal swabs from 2nd passage horses with the highest titer of the virus were pooled and concentrated by a L-80XP high-speed centrifuge (Beckman Coulter) at 27,000 rpm for 1 hour. The pellet was resuspended in 10 ml PBS. The concentrated virus was used to administer to 5 other EIV seronegative horses by nebulizer Flexineb (Nortev, Ireland) in a volume of 2 ml/animal.

### ISOLATION OF THE VIRUS IN HEN EGGS

The presence of virus in nasal swabs was determined by injection into hen eggs (EID<sub>50</sub> titration). Viruses isolated from the nasal swabs of the first and final passage were characterised by their ability to replicate in hen eggs at 28°C and 32°C, but not at 38°C in comparison with the parent virus A/equine/Otar/764/2007 and the difference in the values of the infectious activity in log<sub>10</sub> EID<sub>50</sub>/ml was calculated.

### SEROLOGY AFTER VACCINATION

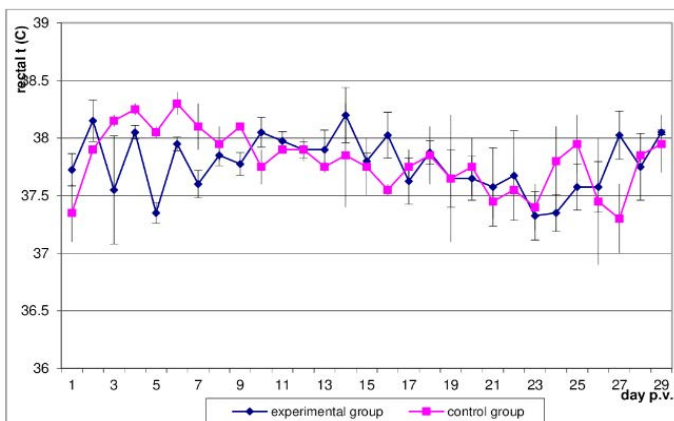
Blood samples were collected from the animals in each group 21-days PV for the detection of antibodies against EIV using the HI assay. Before sampling, the animals were sedated with 20–40 µg/kg detomidine (Cayman Chemical). Blood samples were collected via jugular venipuncture into serum clot activator tubes (VACUETTE®, Greiner Bio-One) for isolation of serum. The HI assay was performed according to Ref. (Tabynov et al., 2014b) using chicken red blood cell suspensions (1%).

### VACCINATION AND PASSAGE

We evaluated the safety through stability of the *ts ca* phenotype induced by our LAIV in horses, its natural host. To this end, 5 horses were vaccinated i.n. with  $4 \times 10^{9.7}$  EID<sub>50</sub> of EIV and monitored for clinical signs such as cough, nasal discharge, respiration and depression, rectal temperature as well as viral shedding during the 14 days after vaccination. None of the horses showed significant adverse effects. Two of the five horses showed a slight, unilateral serous nasal discharge at days 1 to 3 p.v. and a single incidence of coughing was observed, however rectal temperatures remained normal (within  $37,3^{\circ}\text{C} \pm 0.2$  and  $3,82^{\circ}\text{C} \pm 0.2$  from day of vaccination to 14 p.v. day). To measure the presence of EIV LAIV in nasopharyngeal swabs collected at days 0–9 p.v., isolation in eggs was performed on each swab (two swab of each horse per day). Virus shedding was detected in nasopharyngeal swabs collected on days 1–7 p.v. showing a peak at days 2–6 p.v., indicative of viral replication, but not determined on other days. Following vaccination, no HI antibodies were detected in the sera from the all horses, including vaccinated and unvaccinated group (<10), as expected. These results demonstrate the safety profile of our EIV in horses and their ability to replicate in the upper respiratory track, necessary for the induction of immunity.

### CLINICAL SIGNS

The main criterion for determining the reversion of the vaccine virus to virulence was the presence of clinical signs corresponding to equine influenza after administration to animals. No reversion to virulence occurred in any of the 13 yearlings. There were occasional cases of non-zero clinical scores both before and after inoculation, primarily nasal discharge. At the same time, no increase in rectal temperature was observed in the studied (Figure 1).



**Figure 1:** Rectal temperature of control and vaccinated horses of Passage 1. Vaccinated yearlings received a tenfold dose of vaccine ( $10^{9.7}$  EID<sub>50</sub>/animal)

### VIRUS SHEDDING

Overwhelming majority, except yearlings last passage, shed detectable virus at least once during the study, based on analysis of embryonated eggs. The shed virus concentrations ranged from  $1.1 \times 10^1$  to  $3.7 \times 10^4$  EID<sub>50</sub> units/ml in fluid collected from individual swabs (Table 1). The amount of virus transmitted between passages varied from  $7,4 \times 10^4$ /yearlings (in the Passage group 2) to  $3.2 \times 10^2$ /yearlings (in the Passage group 3), which is indicative of the tendency to reduce the spread of the virus during sequential passage.

### PHENOTYPIC STABILITY

At the same time, the study of the phenotypic stability of the vaccine virus through nasal passages by titration under various temperature conditions showed a statistically significant difference in comparison with the wild strain. The virus was classified as *ts ca* if its titer at the studied temperatures was statistically significantly different from the parent wild strain A/equine/Otar/764/2007 ( $P < 0.05$ ). The results of the studies (Table 2) confirmed that the vaccine virus retained the phenotype of the attenuated donor (A/Hong Kong/1/68/162/35), i.e. it was cold-adapted (*ca*) and temperature sensitive (*ts*).

### SEROLOGY

All yearlings were seronegative for equine influenza-2, initially and by the 21 day after inoculation, which confirms that our vaccine does not produce a detectable humoral immune response (Tabynov et al., 2014a).

### DISCUSSION

Equine influenza, currently caused by H3N8 EIV, is the most common and important respiratory infectious disease of horses (Daly et al., 2011; Timoney, 2000). H3N8 EIV is highly contagious and can spread rapidly through groups of naive horses in aerosol droplets that disperse when coughing (Daly et al., 2011; Timoney, 2000). H3N8 EIV infections of horses have been responsible for disrupting major equestrian events and significant economic losses (Daly et al., 2011; Timoney, 2000). The equine population is highly mobile, and horses travel long distances by road and/or air for competitions and breeding. When an infected horse is introduced into a susceptible population, the spread of H3N8 EIV can be explosive. Large outbreaks of H3N8 EIV are often associated with the concentrations of horses at equestrian events. Their dispersal after these events can lead to further widespread dissemination of the virus. It is currently estimated that H3N8 EIV outbreaks are causing economic losses of hundreds of millions of dollars. In endemic countries, the significant economic losses caused by H3N8 EIV infections can be minimized by vaccination of highly mobile horses. Indeed, many racing and equestrian authorities have mandatory vaccination policies that serve

**Table 1:** Virus detection from nasal samples with repeated passage. Titres are EID<sub>50</sub>/ml in nasal swab, detected using embryonic eggs. Bold samples used for administration the next group.

| Passage | ID No. | Day post-vaccination |                       |                       |                       |                       |                       |                       |                       |     |     |
|---------|--------|----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----------------------|-----|-----|
|         |        | 0                    | 1                     | 2                     | 3                     | 4                     | 5                     | 6                     | 7                     | 8   | 9   |
| 1       | 2      | Neg                  | Neg                   | Neg                   | Neg                   | 1.6 x 10 <sup>2</sup> | Neg                   | Neg                   | 1.1 x 10 <sup>1</sup> | Neg | Neg |
|         | 6      | Neg                  | 8.9 x 10 <sup>1</sup> | 5.0 x 10 <sup>2</sup> | 2.8 x 10 <sup>4</sup> | 1.1 x 10 <sup>4</sup> | 8.9 x 10 <sup>1</sup> | 2.8 x 10 <sup>2</sup> | 1.1 x 10 <sup>1</sup> | Neg | Neg |
|         | 5      | Neg                  | 1.6 x 10 <sup>1</sup> | 8.9 x 10 <sup>2</sup> | 2.3 x 10 <sup>2</sup> | 3.7 x 10 <sup>2</sup> | Neg                   | Neg                   | Neg                   | Neg | Neg |
|         | 8      | Neg                  | Neg                   | Neg                   | 2.8 x 10 <sup>4</sup> | 1.1 x 10 <sup>4</sup> | 1.6 x 10 <sup>2</sup> | 2.8 x 10 <sup>2</sup> | Neg                   | Neg | Neg |
|         | 9      | Neg                  | 5.0 x 10 <sup>1</sup> | 3.7 x 10 <sup>4</sup> | 1.3 x 10 <sup>3</sup> | 5.0 x 10 <sup>3</sup> | Neg                   | Neg                   | Neg                   | Neg | Neg |
|         | 3*     | Neg                  | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |
|         | 1*     | Neg                  | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |
| 2       | 4*     | Neg                  | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |
|         | 10     | Neg                  | Neg                   | 5.0 x 10 <sup>1</sup> | 1.6 x 10 <sup>1</sup> | Neg                   | 1.1 x 10 <sup>1</sup> | Neg                   | Neg                   | Neg | Neg |
|         | 11     | Neg                  | 1.6 x 10 <sup>1</sup> | 7.4 x 10 <sup>2</sup> | 1.9 x 10 <sup>2</sup> | 2.8 x 10 <sup>2</sup> | 5.0 x 10 <sup>1</sup> | 2.3 x 10 <sup>2</sup> | Neg                   | Neg | Neg |
|         | 12     | Neg                  | 2.3 x 10 <sup>2</sup> | 1.6 x 10 <sup>2</sup> | 1.1 x 10 <sup>1</sup> | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |
|         | 16*    | Neg                  | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |
|         | 7*     | Neg                  | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |
| 3       | 13     | Neg                  | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |
|         | 14     | Neg                  | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |
|         | 15     | Neg                  | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |
|         | 17     | Neg                  | Neg                   | Neg                   | 1.1 x 10 <sup>1</sup> | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |
|         | 18     | Neg                  | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg                   | Neg | Neg |

\* - negative control

**Table 2:** Comparative phenotypic characterization of reassortant modified equine influenza vaccine virus through nasal passages

| Virus                             | Incubation temperature | Hemagglutinating activity | Infectious activity, log <sub>10</sub> EID <sub>50</sub> /mL |
|-----------------------------------|------------------------|---------------------------|--|
| Vaccine strain A/Otar/HK/6:2/2010 | 28 °C                  | 1:512                     | 7,62 ± 0,08*   |
|                                   | 32 °C                  | 1:1024                    | 8,70 ± 0,14*   |
|                                   | 38 °C                  | 1:4                       | 1,12 ± 0,08*   |
| Wild virus A/equine/Otar/764/2007 | 28 °C                  | 1:2                       | 1,12 ± 0,14  |
|                                   | 32 °C                  | 1:256                     | 6,62 ± 0,22  |
|                                   | 38 °C                  | 1:256                     | 7,03 ± 0,08  |

\*Infectious activity between studied temperature were statistically significant (P<0.05)

as insurance for business. On the other hand, non-endemic countries rely on vaccination of imported horses and quarantine to prevent an entry of H3N8 EIV. Most of these non-endemic countries also vaccinate their horses to reduce the potential impact of an H3N8 EIV invasion.

Traditional vaccination strategies support that vaccine strains must represent viruses in circulation, and it is only through surveillance that vaccine companies decide on which antigens should be used. Thus, EIV surveillance and strain characterization are fundamental for H3N8 EIV control programs based on vaccination. Importantly, vaccine manufacturers need to have a dynamic vaccination approach that allows the rapid generation of novel vaccines to benefit the equine population (Cullinane et al.,

2010; Paillot et al., 2014; Paillot et al., 2016). Results from cross-protection studies indicate that the majority of the inactivated vaccines or the current commercially available LAIV Flu Avert I.N. vaccine would provide poor levels of protection if used in the face of an imminent outbreak because of the antigenic differences between the virus in the vaccine and currently circulating H3N8 EIV strains (Paillot et al., 2016). Notably, some recent H3N8 EIV outbreaks occurred in previously vaccinated animals, where the vaccine strain did not match the circulating virus (Daly et al., 2011; Timoney, 2000; Garner et al., 2011). The frequency of H3N8 EIV outbreaks, the continuous antigenic variation (antigenic drift) of H3N8 EIV and examples of vaccine breakdown due to poorly antigenic match demonstrate the periodic need to update EIV vaccines to prevent



equine influenza in the equine population. Moreover, EIV vaccines should include both clade 1 and clade 2 representative strains of the Florida sublineage, as recommended by the OIE (Paillot et al., 2016).

Regarding fears about the safety of the developed live vaccine associated with the risk of a possible reversal of the vaccine virus or its reassortment with the circulating wild-type virus, followed by the emergence of new pathogenic viruses, we carried out a number of studies on the stability of the *ca* and *ts* phenotype of our vaccine strain. In order to confirm the preservation of the *ca* and *ts* phenotype of the vaccine strain, 3 consecutive passages were carried out on horses, nasal swabs were performed daily, from where the virus was isolated, and then the level of virus replication in the upper respiratory tract of horses was assessed on chicken embryos at optimal/permissive (32 °C), elevated (38 °C) and lower (28 °C) temperatures and the difference in the values of the infectious activity in log<sub>10</sub> EID<sub>50</sub> was calculated.

The virus was classified as *ts* and *ca*, if titer at the studied temperatures was statistically significantly different from the parent wild strain A/equine/Otar/764/2007 (P < 0.05). This is confirmed by data from previous studies (Tabynov et al., 2014b), where also was shown genetic stability for 20 consecutive passages in chicken embryos. These results suggest that the new reassortant *ca* strain A/HK/Otar/6:2/2010 obtained is a good candidate for a live modified *ca* vaccine against equine influenza. Our data are consistent with the positive practical experience of Paillot et al. (2006) and Chambers et al. (2001) where it was confirmed that EIV *ca* strains showed the stability of the temperature sensitivity phenotype (*ts*) *in vivo* for five consecutive passages from horse to horse, as well as inhibit virus replication wild type in the upper respiratory tract of horses. In our works, we experimentally confirm this hypothesis with serial passaging on horses. At the same time, our vaccine virus had lower titers of excretion from the upper respiratory tract, and was almost not detected by the 3rd passage.

We found that the vaccine virus spread only with difficulty from the animal to which it was originally administered. In the present study, a trend of reduced shedding was evident and detectable replication wasn't evident already by the third group of passage.

We tested the ability of the vaccine virus to spread from horse to horse by natural exposure during an outdoor field trial, by inclusion of non-vaccinated sentinel animals. Wild-type equine influenza spreads easily to contact horses via aerosol transmission. However we found that spread of the vaccine virus to sentinels does not occur at all passage levels. The probable reason for the vaccine virus's

inability to spread is that there is no highly efficient viral replication in vaccinated animals, they are seronegative and do not cough.

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## CONFLICT OF INTERESTS

The authors have declared no conflict of interest.

## AUTHORS CONTRIBUTION

ANN designed the experiment, analyzed the data and wrote the manuscript. RSZ, KEM, BDA, MAM, KZK performed fieldwork and wrote the draft. ANN read, edited and approved the final manuscript.

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