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**Efficacy of a recombinant turkey herpesvirus (H9) vaccine against**

**H9N2 avian influenza virus in chickens with maternal-derived antibodies**

Xue Pan1,2\*, Qinfang Liu1, Shiqi Niu1, Dongming Huang1, Dawei Yan1,Qiaoyang Teng1, Xuesong Li1, Nancy Beerens3, Maria Forlenza4,

Mart C. M. de Jong 2 and Zejun Li1

\*lead presenter

1pan.xue@outlook.com, Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China.

2 Quantitative Veterinary Epidemiology, Animal Sciences Group, Wageningen University and Research,Wageningen, Netherlands.

3Wageningen Bioveterinary Research, Wageningen University and Research, Lelystad, Netherlands.

4Host-Microbe Interactomics Group, Animal Sciences Group, Wageningen University

and Research, Wageningen, Netherlands.

**Abstract:** Although vaccines have been widely used for many years, they have failed to control H9N2 avian influenza virus (AIV) in the field in China. The high level of maternal-derived antibodies (MDAs) against H9N2 AIV contributes to the H9N2 AIV vaccine failure in poultry. The study aimed to generate a new vaccine to overcome MDAs interference in H9N2 AIV vaccination in chickens. We used turkey herpesvirus (HVT) as a vaccine vector to express H9 hemagglutinin (HA) proteins. The recombinant HVT expressing H9 HA proteins (rHVT-H9) was successfully generated and characterized in primary chicken embryonic fibroblasts (CEFs). Western blot (WB) and indirect immunofluorescence assay (IFA) showed that the rHVT-H9 consistently expressed HA proteins. In addition, the rHVT-H9 had similar growth kinetics to the parent HVT. Preliminary animal experiments showed that compared to the conventional inactivated whole virus (IWV) vaccine, the rHVT-H9 stimulated robust humoral immunity in chickens with passively transferred antibodies (PTAs) that were used to mimic MDAs. Transmission experiments showed that the rHVT-H9 induced both humoral and cellular immunity in chickens with PTAs. Furthermore, we used mathematical models to quantify the vaccine’s efficacy in preventing the transmission of H9N2 AIV. The results showed that the rHVT-H9 reduced the virus shedding period and decreased the reproduction ratio (R) value in chickens with PTAs after homologous challenge, although the vaccination in this trial did not yet bring R < 1. In summary, we generated a new rHVT-H9 vaccine, which stimulated strong humoral and cellular immunity, reducing virus shedding and transmission of H9N2 AIV even in the presence of PTAs in chickens.

**Background/Objective:** H9N2 avian influenza virus (AIV) is the most widespread and prevalent low pathogenicity avian influenza virus (LPAIV) subtype of influenza virus in the world, posing a serious threat to both the global poultry industry and human health. Vaccination is the main strategy for managing H9N2 AIV in poultry, however maternal-derived antibodies (MDAs) interfere with immune responses contributing to H9N2 AIV vaccination failure. Turkey herpesvirus (HVT) is considered a potential vector for polyvalent live vaccines in chickens to overcome MDAs interference. When assessing the efficacy of vaccines in the management of infectious diseases, especially zoonotic diseases, the ability to control the transmission of viruses at the population level is important. Therefore, the aim of the present study is to generate a new vaccine to overcome MDAs interference in H9N2 AIV vaccination in chickens.

**Methods:** The H9N2 AIV HA gene with CMV promoter was cloned into the genome of HVT. The recombinant strain (rHVT-H9) was generated by using crispr cas9. The H9N2 AIV (109.25 EID50/0.1 ml) was inactivated with 1:2000 β-propiolactone (BPL) by constantly shaking for 16 h at 4°C. To generate H9N2 IWV vaccine, the inactivated H9N2 virus was then mixed with water-in-oil Montanide VG71 (0.85 g/cm3) adjuvant (SEPPIC, France) at a volume ratio of 3:7 following manufacturer instructions. PCR, western blot and indirect immunofluorescence assay (IFA) analysis were used to identify rHVT-H9 in vitro. Three groups of chickens were used. Each group consisted of 13 one-day-old SPF chickens with PTAs applied in the same way to mimic MDAs. Chickens were subcutaneously inoculated with 0.1 ml of 5,000 PFU rHVT-H9 (group 1), H9N2 IWV vaccine (group 2) or PBS (group 3). Sera were collected weekly and HI titers against H9N2 were determined by HI assay. Three chickens in each group were sacrificed 28 days after vaccination to identify the replication of the rHVT-H9 in chickens with PTAs and to test the efficacy of cellular and mucosal immunity. Five chickens in each group were challenged with 0.1 ml of 106 EID50 of H9N2 virus intranasally 28 days after vaccination. The other five chickens in each group were added 24 h post-challenge (p.c). Oronasal and cloaca swabs were taken every 2 days until 14 days p.c. The stochastic SIR model was used as a model to estimate the transmission of animal experiments.

**Results:** The sequence analysis showed no mutation in the recombinant rHVT-H9 and the whole recombinant sequences were fully consistent with the expectation. PCR, WB and IFA assay showed that the rHVT-H9 successfully expressed HA proteins in vitro. Growth curve of the rHVT-H9 indicated that the rHVT-H9 had similar growth kinetics to the parent HVT in CEFs. To examine the cellular immunity of vaccinated chickens with PTAs, a ch-IFN-γ ELISpot assay was performed following the manufacturer’s instruction. The results showed that the rHVT-H9 induced significantly higher ch-IFN γ secretion than the H9N2 IWV vaccine in chickens with PTAs, which suggested that the rHVT-H9 could induce potential T cell immunity even in the presence of MDAs. Cytokine mRNA expressions in splenocytes were tested by real-time PCR. All cytokines including ch-IFN-α ch-IFN-β ch-IFN-γ and ch-IL-12p40 from the rHVT-H9 vaccinated chickens were significantly higher than those from the H9N2 IWV vaccine immunized chickens with PTAs. After challenging with H9N2 AIV virus, chickens immunized with the H9N2 IWV vaccine continued shedding virus until 8 days p.c which was similar to PBS inoculated chickens after challenge. However, the rHVT-H9 vaccinated chickens stopped shedding virus earlier, at 4 days p.c. The reproduction ratio (R) of the rHVT-H9 group was 1.75 (0.71–5.51), smaller than the R 12.76 (4.42–37.88) of the H9N2 IWV vaccine-immunized group and that of the PBS group (0.85- ∞), with estimates based on final size and significance on GLM.

**Conclusion:** We are the first to study the efficacy of the recombinant rHVT-H9 vaccine in reducing the transmission of H9N2 AIV in the presence of PTAs in chickens. The rHVT-H9 stimulated strong humoral and cellular immunity, reducing virus shedding and transmission of H9N2 AIV in the presence of PTAs in chickens but not yet sufficiently since the R value was over 1. Future rHVT-H9 studies should assess the efficacy of rHVT-H9 in commercial chickens in poultry and determine if a booster vaccination with the commercial H9N2 IWV vaccine may totally prevent transmission of H9N2 AIV.

**Keywords:** maternal-derived antibodies, recombinant vaccine, turkey herpesvirus (HVT), immune responses, transmission