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# Mutations in the haemagglutinin protein and their effect in transmission of highly pathogenic avian influenza (HPAI) H5N1 virus in sub-optimally vaccinated chickens

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ABSTRACT

*Background:* Transmission of highly pathogenic avian influenza (HPAI) viruses in poultry flocks is associated with huge economic losses, culling of millions of birds, as well as human infections and deaths. In the cases where vaccination against avian influenza is used as a control measure, it has been found to be ineffective in preventing transmission of field strains. Reports suggest that one of the reasons for this is the use of vaccine doses much lower than the ones recommended by the manufacturer, resulting in very low levels of immunity. In a previous study, we selected for immune escape mutants using homologous polyclonal sera and used them as vaccines in transmission experiments. We concluded that provided a threshold of immunity is reached, antigenic distance between vaccine and challenge strains due to selection need not result in vaccine escape. Here, we evaluate the effect that the mutations in the haemagglutinin protein of our most antigenically-distant mutant may have in the transmission efficiency of this mutant to chickens vaccinated against the parent strain, under sub-optimal vaccination conditions resembling those often found in the field.
*Methods:* In this study we employed reverse genetics techniques and transmission experiments to examine if the HA mutations of our most antigenically-distant mutant affect its efficiency to transmit to vaccinated chickens. In addition, we simulated sub-optimal vaccination conditions in the field, by using a very low vaccine dose.
*Results:* We find that the mutations in the HA protein of our most antigenically-distant mutant are not enough to allow it to evade even low levels of vaccination-induced immunity.
*Discussion:* Our results suggest that – for the antigenic distances we investigated – vaccination can reduce transmission of an antigenically-distant strain compared to the unvaccinated groups, even when low vaccine doses are used, resulting in low levels of immunity.


## 1. Introduction

A particular lineage of highly pathogenic avian influenza (HPAI) H5N1 is unique among other HPAI strains in that since its emergence in Guangdong province in China in 1996, it has spread on a global scale [1]. Its efficiency in transmitting within and between poultry flocks, coupled to severe morbidity and high (up to 100%) case mortality rates, make it a serious threat to the poultry industry. Culling of infected poultry or pre-emptive culling is one of the main means to control infection and results in huge economic losses, as well as ethical dilemmas. In addition, with this HPAI H5N1 lineage, human infections are often reported. To this date, the World Health Organisation (WHO) reports 851 cases of human infections with HPAI H5N1 (mainly among poultry workers and their families), out of which 450 were fatal [2,3]. Although human-to-human transmission is still not reported, many believe that HPAI H5N1 virus is one of the prime candidates for a new pandemic influenza outbreak [4].

One of the most important genes in the influenza genome is the haemagglutinin (HA) gene, encoding for the HA protein. HA is the major surface protein of influenza viruses, outnumbering the neuraminidase (NA) protein by a ratio of 4:1 [5,6]. It is essential for influenza virulence, transmissibility and antigenicity, since it is

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the primary target of neutralising antibodies [5–13]. Indeed, the HA experiences the most intense selection pressure compared to other parts of the genome and is particularly prone to mutations at both the nucleotide and amino acid level due to the error-prone RNA polymerase of the virus [6,9,11,14–16]. As a consequence, mutants that are antigenically different from their parent strains are often selected in the field, and this is thought to be one of the reasons why vaccines are failing to protect against transmission [5,6,17].

Although vaccination against HPAI H5N1 is used in some countries, transmission still takes place and in some instances vaccination has been implicated in the selection of antigenic variants capable of escaping vaccination-induced immunity [4,17–20]. Oftentimes, the failure of vaccination to stop transmission has been attributed to the antigenic distances between vaccine and field strains, thus requiring constant vaccine updating (similar to vaccination against human influenza). This has led many to believe that vaccination of poultry is not cost-effective and should not be used as a means to stop transmission of field strains. However, recent reports suggest that vaccine inefficiency in stopping transmission could be attributed to its failure in inducing sufficiently high levels of immune response even against the vaccine strain itself, to a sufficiently high percentage of the vaccinated population [4,17,18,21–24].

In previous studies we have selected for escape mutants of the HPAI H5N1 A/turkey/Turkey/1/2005 virus (from now on abbreviated to H5N1 t/T) using homologous polyclonal sera and have characterised these mutants genetically and antigenically [25]. We then studied the effect of vaccination-induced immunity and antigenic distance on the transmission dynamics of the parent strains to animals vaccinated with the mutants, by using either a high (128 Haemagglutination Units, HAU) or low (4 HAU) vaccine dose [26]. We concluded that vaccine dose (and consequent immunity) has a much bigger effect on transmission than the antigenic distances between the vaccine and challenge strains we studied. In addition, we calculated the minimum level of immunity ($\geqslant$8 Haemagglutination Inhibition Units, HIU, as measured by HI titres), as well as the minimum percentage of the vaccinated population ($\geqslant$86.5%) necessary to display said immunity, in order for transmission to be prevented ($R < 1$).

In this study, we examine the effect that the mutations found in the HA protein of our latest and most antigenically-distant mutant (t/T-P42) may have on the transmission of this mutant to sub-optimally vaccinated animals. For this reason, we used reverse genetic techniques to insert the HA of t/T-P42 into the backbone of its H5N1 t/T parent strain, thus creating strain rgt/T-P42. We then used rgt/T-P42 to challenge animals vaccinated with the H5N1 t/T parent strain, so as to simulate a field situation in which such an antigenically-distant strain may well be the co-circulating field strain, selected under immune pressure. To examine if there are differences in the transmission efficiency between the parent strain and the rgt/T-P42 mutant, we also included a homologous group, in which animals were similarly sub-optimally vaccinated and then challenged with the parent strain. Sub-optimal field vaccination was simulated by using a very low vaccine dose of 1 HAU, resulting in very low levels of immunity of approximately – but not higher than – 8 HIU ($\leqslant$8 HIU) and high percentages of animals exhibiting said low immunity ($\geqslant$86.5% of vaccinated population). This would theoretically lead to $R < 1$ in the homologous group but a high probability for some contact infections (as a minor outbreak in this group). We analysed the results from the transmission experiments, using Generalised Linear Models (GLM) in order to see whether the HA mutations result in a higher transmission rate parameter of the mutant compared to the same parameter estimated for the parent strain in a vaccinated population.

## 2. Materials and methods

### 2.1. Construction of reverse genetics viruses

The HA genes from the parent strain H5N1 t/T and the t/T-P42 mutant were synthesised by GenScript® (Piscataway, NJ) and inserted into the pHW2000 bi-directional transcription plasmid using BsmBI cloning sites [27]. The sequence of the HA gene constructs was verified by means of sequencing the HA gene as previously described [25]. The sequence of the HA gene of the t/T-P42 mutant is submitted to GenBank (GenBank: KF042153).

The purified pHW2000-derived plasmids containing the HA of the H5N1 t/T or t/T-P42 mutants and the remaining 7 genes of the H5N1 t/T parent strain were used to rescue viruses using reverse genetics. The detailed protocol is described in the supporting information file.

### 2.2. Pilot vaccination experiment to determine optimum vaccination dose

All animals used in this experiment were specific pathogen-free (SPF) white leghorn chickens, derived from SPF embryonated chicken eggs (ECEs) (Charles River Avian Vaccine Services) that were hatched and raised in our HCU facilities. Inactivation of viruses for vaccine construction took place as previously described [25,26].

In previous work [26] we have calculated and described the threshold level of immunity – as expressed in HI titres – necessary to stop transmission of HPAI H5N1 strains in vaccinated chickens (under experimental conditions). This threshold was calculated to be $\geqslant$8 HI units (HIU) to be reached by at least 86.5% of animals at three weeks post-vaccination (p.v.). In this study, we aim to use a sub-optimal vaccination dose that would lead to levels of immunity of $\leqslant$8 HIU, as measured with the homologous (vaccine) virus, thus maximising the chance that HI titres would be much lower in the heterologous case.

In order to ensure that vaccinated animals would have HI titres of $\leqslant$8 HIU at three weeks p.v., we performed a pilot experiment, in which we vaccinated three groups, each comprising 7 SPF 3-week-old chickens, with three different vaccine doses (1, 2 and 4 HAU) of the inactivated reverse genetics parent strain (rgH5N1 t/T) in the presence of adjuvant (Stimune, Prionics), at a 4:5 volume/volume (v/v) inactivated virus-to-Stimune ratio. The vaccine was delivered intra-muscularly (i.m.) in the leg muscle with 0.5 mL of the respective vaccine dose. The animals were housed in the same room but in separate cages for each group, and were checked twice a day for mortality and signs of disease. At 2 and 3 weeks p.v., 2 mL of blood were taken from each chicken, sera were prepared and inactivated at 56 °C for 50 mins. The sera were used in HI assays, in which their HI titre against the vaccine strain was quantified. HI assays were performed in duplicate as previously described [25,26,28,29].

### 2.3. Transmission experiment

For the transmission experiment, seventy new SPF chickens were vaccinated i.m. in the leg muscle at 3 weeks of age with 0.5 mL of 1 HAU of inactivated rgH5N1 t/T in the presence of adjuvant (Stimune, Prionics) at a 4:5 (v/v) inactivated virus-to-Stimune ratio. The same procedure was followed for another 20 chickens belonging to the two unvaccinated control groups (groups 3 and 4), only these animals were injected i.m. with 0.5 mL of allantoic fluid from SPF ECEs in the presence of adjuvant as described above. One animal belonging to one of the unvaccinated groups (Group 3) died as a result of a shock while the mock vaccine was being administered and was removed from the group. All animals were