SELECTIVE PRESSURES DRIVING THE EMERGENCE OF HIGHLY PATHOGENIC INFLUENZA VIRUS IN AVIAN HOSTS

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I Introduction

- Highly pathogenic avian influenza viruses (HPAIV) of the H5 and H7 subtypes emerge from low pathogenic (LPAIV) precursors upon transmission from wild waterfowl (reservoir hosts) to poultry.
- The HPAIV phenotype results from the acquisition of basic amino acids (multibasic cleavage site, MBCS) in the hemagglutinin cleavage site. The monobasic cleavage site is cleaved by trypsin-like proteases expressed in the respiratory and intestinal tract. The MBCS is cleaved by ubiquitously expressed furin-like proteases which promotes severe systemic disease in poultry (Fig. 1), but not necessarily in reservoir hosts.
- It is currently unknown why and how HPAIV exclusively emerge in poultry and not in reservoir hosts.

Objective: to understand what drives the selection of a minority of HPAIV once they have emerged from a majority of LPAIV precursors in both poultry (chicken) and reservoir host (duck).

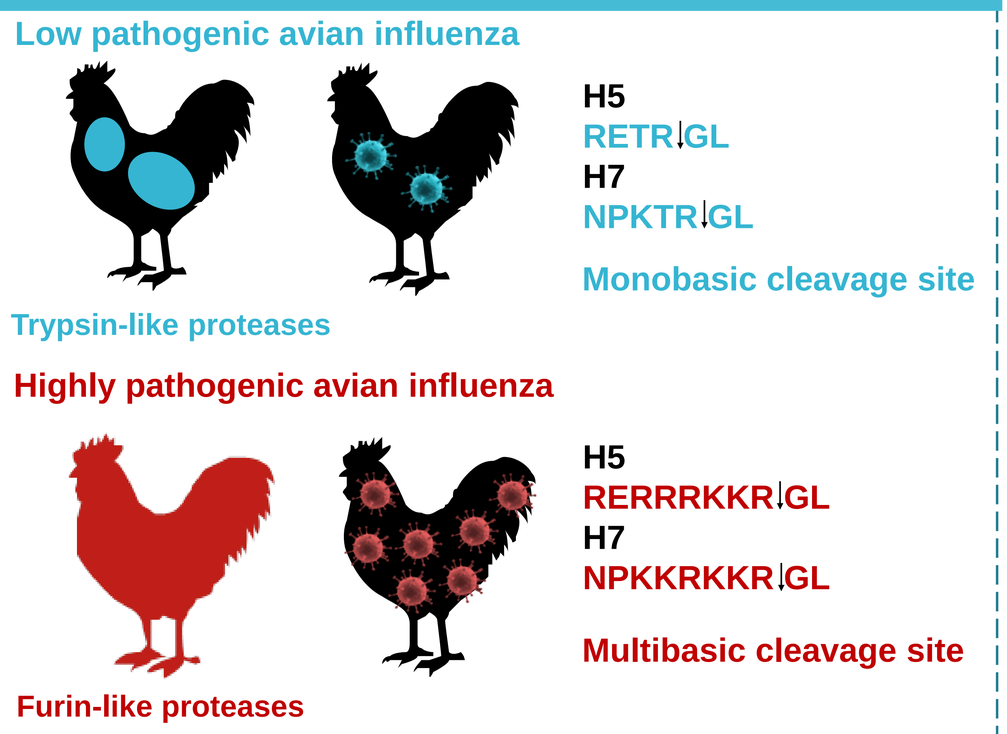

Figure 1. Cleavage and activation of HPAIV and LPAIV hemagglutinins by differentially expressed proteases determines systemic spread of the virus in poultry.

II Materials and Methods

De novo HPAIV generation from LPAIV precursors was mimicked by inoculating embryonated eggs, respiratory explants, and chickens with a mixture of **HPAIV (wild-type; 1%)** and **LPAIV (HPAIV ΔMBCS; 99%)**. The proportions of HPAIV and LPAIV during infection were determined by RT-qPCR (Fig. 2).

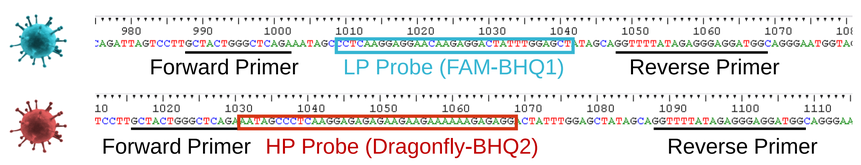

Figure 2. HPAIV and LPAIV hemagglutinin RNAs were differentially recognized by RT-qPCR using different probes.

III Results

***In ovo*:**
The HPAIV outgrew the LPAIV which was productively infecting the allantoic cavity in 14-day old chicken eggs and both young and old duck eggs. In all occasions HPAIV replicated to high viral RNA levels in embryonic tissues (Fig. 3).

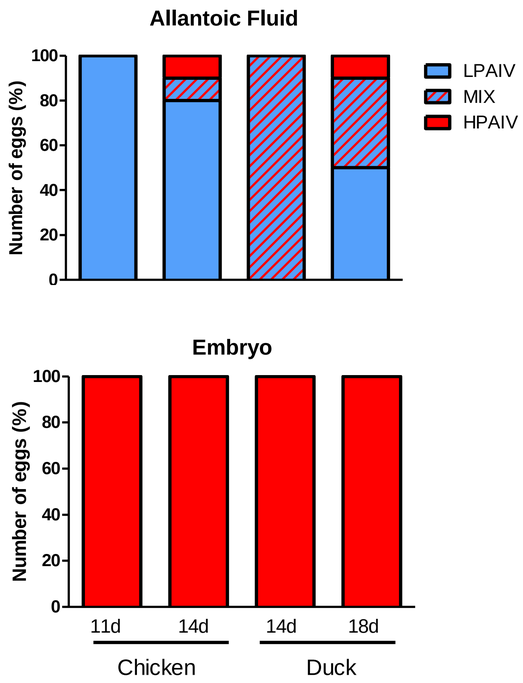

Figure 3. *In ovo* competition experiment. Chicken (11- and 14-day old) and duck (14- and 18-day old) embryonated eggs were inoculated via the allantoic cavity with an H5N1 HPAIV(1%)/LPAIV(99%) pair. Presence and nature of viral RNA in allantoic fluid and embryo was determined by RT-qPCR at 48hpi (10 eggs per group).

***Ex vivo*:**
In explants, the HPAIV did not outcompete the LPAIV (Fig. 4). In the explants inoculated with only HPAIV, the virus load reached high RNA levels in all four explant models with a mean ΔCt between 15 and 26 (data not shown).

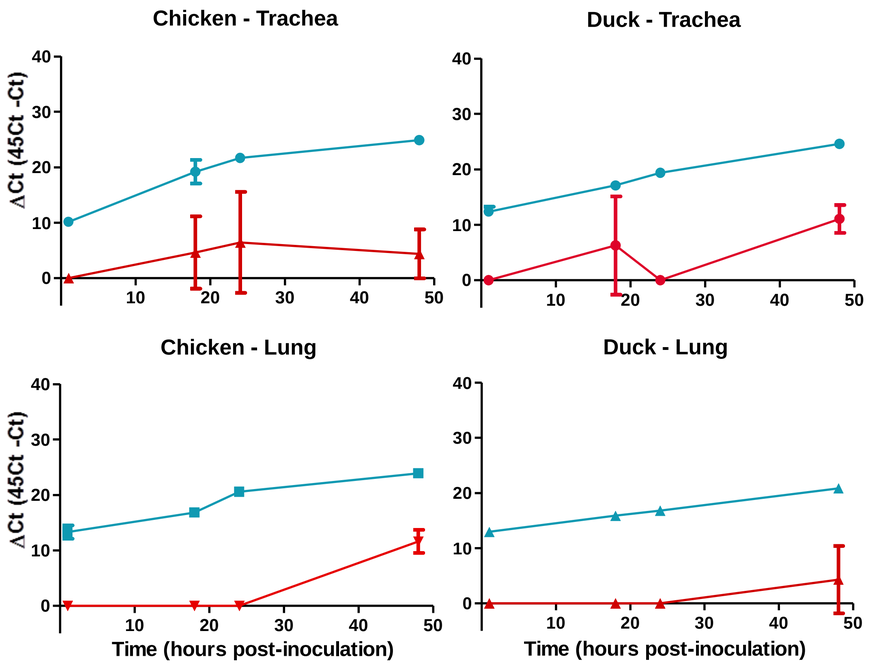

Figure 4. *Ex vivo* competition experiment. Trachea and lung explants were prepared from chicken (17-day old) and duck (23-day old) embryos and inoculated with an H5N1 HPAIV(1%)/LPAIV(99%) pair. Viral RNA in supernatant from 0-48hpi was analyzed by RT-qPCR (LPAIV, blue; HPAIV, red). Error bars represent SD. n=2.

***In vivo*:**
To further understand the selection of HPAIV *in vivo*, three chickens were inoculated with an H7N7 HPAIV/LPAIV pair. Two chickens showed clinical signs consistent with an HPAIV infection at 2dpi and were euthanized at 4dpi. HPAIV viral RNA (Fig. 5) and infectious virus (data not shown) was detected in all organs. Chicken #3 withheld from clinical symptoms up to 8dpi and presented negative for HPAIV in most organs (Fig. 5).

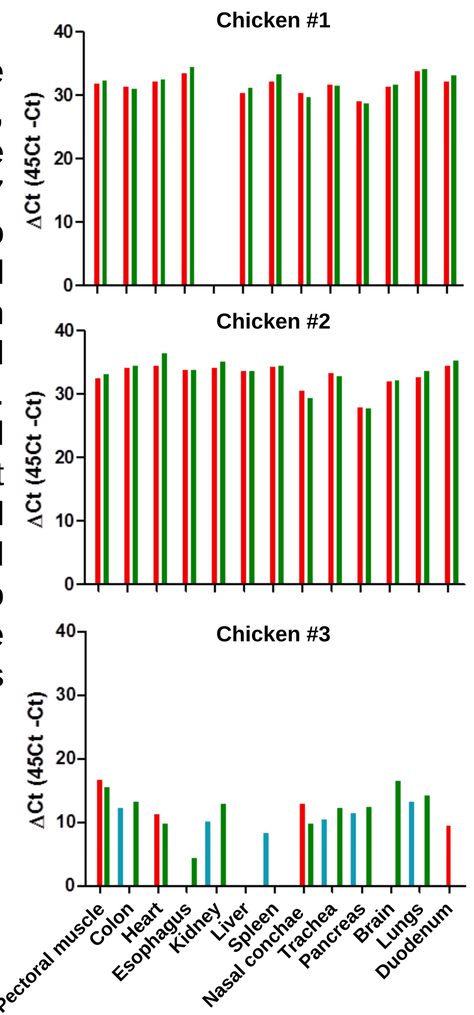

Figure 5. *In vivo* competition experiment. Three 4-week old chickens were inoculated with an H7N7 pair of HPAIV(0,2%)/LPAIV(99,8%) in the eyes, nose, trachea, and esophagus. Clinical signs and weight were monitored daily. Necropsies were performed at 4dpi (chicken #1 and #2) or 8dpi (chicken #3). Viral RNA in the organs was analyzed by RT-qPCR (LPAIV, blue; HPAIV, red; influenza matrix segment, green).

IV Conclusion and Discussion

We demonstrate that HPAIV outcompeted a productive LPAIV infection in embryos and *in vivo,* but not *ex vivo*. HPAIV was selected in embryos and *in vivo* as it had the ability to replicate where the LPAIV virus could not (i.e. embryonic, extra-respiratory and intestinal tissues). However, HPAIV infected embryonic tissues of both chickens and ducks. *In vivo* duck experiments and the study of tropism of HPAIV in embryos will give additional insight in the mechanisms behind HPAIV selection from LPAIV precursors and the species-specific emergence of HPAIV.

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