Heterologous post-infection immunity against Egyptian avian influenza virus (AIV) H9N2 modulates the course of subsequent infection by highly pathogenic AIV H5N1, but vaccination immunity does not

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Abstract

In Egypt, zoonotic A/goose/Guangdong/1/96 (gs/GD-like) highly pathogenic avian influenza virus (HPAIV) H5N1 of clade 2.2.1.2 is entrenched in poultry populations and has co-circulated with low-pathogenic avian influenza virus H9N2 of the G1 lineage since 2010. Here, the impact of H9N2 infection or vaccination on the course of consecutive infection with a lethal Egyptian HPAIV H5N1 is studied. Three-week-old chickens were infected with H9N2 or vaccinated with inactivated H9N2 or H5N1 antigens and challenged three weeks later by an HPAIV H5N1. Interestingly, pre-infection of chickens with H9N2 decreased the oral excretion of H5N1 to levels that were comparable to those of H5N1-immunized chickens, but vaccination with inactivated H9N2 did not. H9N2 pre-infection modulated but did not conceal clinical disease by HPAIV H5N1. By contrast, homologous H5 vaccination abolished clinical syndromic surveillance, although vaccinated clinical healthy birds were capable of spreading the virus.

Avian influenza virus (AIV) is a member of the family Orthomyxoviridae genus Influenza virus A, which, according to the antigenic differences of the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA), is classified into 16 HA and 9 NA subtypes [1, 2]. Two further subtypes, H17N10 and H18N11, have previously been reported from bats, but they have not been detected in avian hosts [3]. AIVs infect both domestic and wild birds. In addition, sporadic natural transmission of AIV to marine and terrestrial mammals, including swine, equines, carnivores, seals and humans, has been reported [4–6]. In poultry, AIVs are phenotypically distinguished into low-pathogenic avian influenza virus (LPAIV) or highly pathogenic avian influenza (HPAIV), based on the amino acid sequence spanning the cleavage site of the HA protein, where LPAIVs encode a monobasic amino acid motif, while HPAIVs harbour a polybasic, subtilisin-like cleavage site. Due to their processability by subtilisin-like host proteases, which are ubiquitously expressed in the avian host, HPAIVs are able to spread and replicate systematically in all organs and tissues, while LPAIVs are usually restricted to the gastrointestinal and respiratory epithelia [7]. LPAIVs are frequently reported from apparently healthy migrating waterfowl and shorebirds [8]. In domestic poultry, LPAIV may cause subclinical infections, mild respiratory symptoms, or a drop in egg production [9]. HPAIV causes devastating epidemics among gallinaceous poultry, but, following spill-over infections from poultry, it also affects wild-bird populations [10]. Since 2003, HPAIV H5N1 of the goose/Guangdong (gs/GD) lineage has emerged, diversified and spread worldwide, causing continuous threats, mainly to poultry production in the Middle East, West Africa and Asian countries [11, 12]. Novel AIV genotypes were reported over recent years in different countries as a result of frequent reassortment between LPAIV and gs/GD HPAIV [13–15].

Reports in recent decades have shown that natural co-infections and consecutive infections of LPAIV and HPAIV occur frequently in countries that vaccinate against HPAIV H5 [16, 17], and the clinical effects of a pre-LPAIV infection on consecutive HPAIV infection have been a focus of interest. Notably, some LPAIV strains were shown in experimental infection studies to induce heterologous partial protection against lethal challenge for some HPAIV strains [18–20]. The effect of prior LPAIV infection on the
outcome of a sequential HPAIV infection ranged from full protection to partial or no masking of clinical signs of subsequent HPAIV infection. The protective efficacy was dependent on the subtypes and even the clade and lineage-specific characteristics of the associated viruses. Two of these studies used pairs of LPAIV H9N2 and HPAIV H5N1 [19, 20]. In Egypt, G1-like LPAIV H9N2 and HPAIV H5N1 continue to co-circulate intensively in poultry [16, 21, 22] and vaccination of poultry against both viruses using inactivated monovalent or bivalent H5/H9 vaccines is common [23]. This study attempted to experimentally evaluate the influence of heterologous immunity raised by Egyptian H9N2 on the course of consecutive infection with a currently circulating HPAIV H5N1 of clade 2.2.1.2.

The viruses used in this study were obtained from the repository of the Friedrich-Loeffler-Institute (FLI) and had previously been provided by the National Laboratory for Veterinary Quality Control on Poultry Production, Animal Health Research Institute, Giza, Egypt: LPAIV H9N2 (A/chicken/Egypt/AR755-14/2014) and HPAIV H5N1 (A/chicken/Egypt/AR236-15/2015). The viruses were purified through two consecutive rounds of plaque purification using Madin–Darby canine kidney (MDCK) cell cultures. The viruses were then propagated in specific-pathogen-free (SPF) embryonating chicken eggs (ECE). Virus-containing allantoic fluids were diluted using PBS in order to obtain an inoculum with titres of $10^{5.0}$ mean tissue culture infectious dose (TCID$_{50}$) in MDCK cells per 0.5 ml. A mock inoculum was prepared using allantoic fluid from non-infected SPF ECE. White leghorn chickens were hatched from SPF ECE purchased from Lohmann Animal Health and kept at FLI until they were 3 weeks old. The birds were housed in isolation units with feed and water provided ad libitum. All animal experiments were carried out in accordance with a protocol legally approved by an ethics commission of the Ministry of Agriculture of the Federal State of Mecklenburg-Vorpommern, Germany (MV-LALLF-7221.3-1-019/16). Infection and immunization experiments were performed in biosafety level-3 (BSL-3) animal facilities. Three-week-old SPF chickens were separated into four groups (twelve each). Ten birds of group 1 were inoculated conjunctivally and intranasally with 0.5 ml containing $10^{5.0}$ TCID$_{50}$ of the H9N2 virus; two birds were kept as sentinels and were added to the infected birds 1 day post-inoculation (p.i.). In addition, an adjuvanted antigen preparation of each of the H9N2 and H5N1 viruses was produced by mixing equal amounts of undiluted inactivated allantoic fluids with an HA titre of 128–256 with Freund's complete adjuvant. The allantoic fluids were inactivated using beta-propiolactone (0.05 % v/v, 2 h at 37 °C) and tested for virus sterility through three consecutive blind passages in ECE. All of the allantoic fluids used for virus inoculum or antigen preparation were also tested for bacterial sterility. Freund's complete adjuvant was used because good practical experience with this adjuvant has been achieved in this laboratory. In contrast to mammalian hosts, avian species do not develop alterations at the injection site and no adverse effects following injection were observed in any of the chickens. Twelve birds each of groups 2 and 3 were injected subcutaneously with 0.5 ml of either H9N2 or H5N1 antigens, respectively. The control group (group 4) was injected subcutaneously with 0.5 ml of the mock inoculum prepared as described above, but using the allantoic fluid of uninfected SPF eggs instead. At day 21 post-H9N2-infection or post-immunization, respectively, at 6 weeks of age, the four groups were challenged via the conjunctival–nasal route with $10^{5.0}$ TCID$_{50}$ of HPAI H5N1 virus. Two birds of each group served as sentinel controls and were housed with the challenged birds from day 1 after the H5N1 challenge infection (days post-challenge, d.p.c.). Clinical signs were scored daily through the following 14 days: 0=healthy (no abnormal signs); 1=sick (showing one of the following symptoms: ruffled feathers, respiratory manifestations, depression, facial oedema, cyanosis of comb and wattles or diarrhoea); 2=severely sick (showing two signs or more); 3=dead.

Blood samples were collected before the start of the animal experiment, at day 21 before challenge infection and from surviving birds at the end of the experiment. Serum samples were investigated for AIV-specific antibodies using an indirect influenza A antibody ELISA kit (BioCheck) according to the manufacturer's instructions. Positive samples were further examined by a haemagglutination-inhibition (HI) assay using the homologous H9N2 and H5N1 viruses and applying the standard protocols described by the World Organization for Animal Health (OIE) [24]. In order to compare the virus shedding patterns via the respiratory or digestive tracts, viral RNA loads in oropharyngeal and cloacal swabs were determined at indicated time points post-H5N1 challenge. Briefly, individual oropharyngeal and cloacal swabs were collected from the surviving birds at d.p.c. 2, 4, 7 and 10. Viral RNA was extracted using the NucleoMag RNA extraction kit (Machery-Nagel) following the manufacturer's instructions. Virus shedding was detected by amplification of the extracted RNA materials by reverse transcription quantitative PCR (RT-qPCR), targeting the matrix (M) gene [25] against standard quantified genomic RNA. To relate M-specific Cq values to viral infectivity, viral RNA was extracted from diluted HPAIV H5N1 suspensions with a known infectivity titre; Cq values from these extracts were used to generate a standard curve linking infectivity with Cq values based on M-gene-specific RT-qPCR. The resulting values were labelled the 'infectivity equivalent titre' (e.g. Fig. 3).

The variation in virus shedding patterns was evaluated using the Kruskal–Wallis rank sum test [R Studio software, version 0.99.903, from the R Foundation for Statistical Computing (www.r-project.org)]. $P$ values of less than 0.05 were considered to be significant.

Before H5N1 challenge infection, neither vaccination (groups 2 and 3) nor H9N2 infection (group 1) induced any clinical signs, and all chickens remained healthy until 21 d.p.i. Viral RNA specific for LPAIV H9 was detected in both oropharyngeal and cloacal swabs obtained from inoculated birds of group 1 at 2 d.p.i., but not at 10 d.p.i. (not shown). Sentinel birds of the LPAIV H9N2 infected group, associated at
specific HI antibodies were observed exclusively in the serum compared to the H9N2-vaccinated (*P=0.02) H9-specific HI antibody titres than the virus-infected group. H5-specific HI antibodies were observed exclusively in the serum samples collected from the chickens in group 3 vaccinated with H5 antigen. No influenza-A-specific antibodies were detected in the mock-vaccinated group.

All chickens of groups 2 and 4 developed severe clinical signs from 2 d.p.c. onwards, and all chickens were dead by 5 (group 2) or 4 (group 4) d.p.c. (Fig. 2). The clinical score of these two groups was 2.55 and 2.65, respectively. In chickens that had previously been infected by H9N2 virus, the onset of H5N1-induced disease was delayed, with only two sick birds at 2 d.p.c. Nevertheless, all chickens of this group developed terminal disease and were dead by 7 d.p.c. (scoring, 2.38) (Fig. 2). In the H5N1-vaccinated chickens in group 3 neither clinical signs nor mortality were observed (scoring, 0.0) (Fig. 2). A significant difference between different groups was recorded for virus shedding in oropharyngeal and cloacal swabs at day 2 post H5N1- challenge: all of the chickens that went through an H9N2 infection at 3 weeks of age (group 1) shed significantly lower HPAIV titres in the oropharyngeal swabs at 2 d.p.c. (Fig. 3a, b) as compared to the H9N2-vaccinated (*P<0.001) and mock groups (P<0.001). Comparisons for other days were not possible, since the birds of other groups had already died. Notably, homologous H5N1 vaccination (group 3) did not induce sterile immunity, and viral excretion through the oral and cloacal routes was detected until 10 d.p.c. Virus excretion in this group peaked at 7 d.p.c., with Cq values equivalent to 10^5 TCID_{50} in the oropharyngeal swabs, and Cq values equivalent to up to 10^2 TCID_{50} in the cloacal swabs, respectively (Fig. 3c, d). Even at 10 d.p.c., excretions of viral RNA were detectable in five birds in this group.

HPAIV H5N1 is endemic in poultry in Egypt and has posed a public health hazard since 2006. Moreover, the widespread occurrence of H9N2 in commercial chickens was reported to be accompanied by a decreased incidence of H5N1 outbreaks [16]. The detection of HPAIV H5N1 in poultry flocks previously infected with H9N2 in Egypt under natural conditions has been reported [21]. However, data on the impact of prior infection or the vaccination of chickens with H9N2 on the virulence and excretion of HPAIV H5N1 are still lacking. In this study, prior infection of chickens with H9N2 virus modulated clinical signs and the birds survived 2 days longer than the sham-vaccinated chickens, and 1 day longer than the H9N2-vaccinated chickens. Partial protection against lethal challenge for some HPAIVs due to prior infection with some LPAIVs has previously been described [18–20].

Interestingly, although H9N2-vaccinated birds responded with a significantly higher (P=0.02) humoral immune response than H9N2-infected chickens, prior-infection – but not vaccination – of chickens with H9N2 decreased the oral excretion of H5N1 to levels that were comparable to those of H5N1-immunized chickens. The immunological mechanisms mediating the delayed development of clinical signs and decreased virus excretion are likely based on

![Fig. 1. Humoral immune response after infection or vaccination with AIVs of subtypes H9N2 or H5N1. Comparison of the serologic reactions using (a) indirect ELISA (OD_{450}, all sera were tested in one run) and (b) haemagglutination inhibition (log titre) against homologous H9 and H5 antigens among four groups of ten chickens each sampled at day 21 after H9N2 infection (H9N2 virus), or following immunization using inactivated adjuvanted antigen (AG) of H9N2, HPAIV H5N1, or allantoinic fluid of SPF eggs (mock). *Indicates a significant difference (P<0.05) between the H9N2 infected group and the group vaccinated with adjuvanted H9N2 antigen.](http://www.microbiologyresearch.org/doi/10.1173/jgv.1730026)
cross-reactive cellular immunity, possibly induced by the internal proteins of the heterologous LPAIV [20]. Khalenkov et al. [19] suggested that sustained LPAIV H9N2 replication to high titres and consistent transmission are necessary for the potential induction of cross-reactive cellular immunity mechanisms. In this study, which attempted to mimic the Egyptian situation as closely as possible, a single LPAIV H9N2 isolate was used, which obviously replicated intensively enough to induce such cross-protection. Nevertheless, the possibility that using other LPAIV/HPAIV H5N1 pairs might result in different protection rates, higher or lower, cannot currently be excluded.

In addition, despite vaccination with the H5N1 antigen, H5N1-infected birds excreted viruses for up to 10 d.p.c., although at markedly lower titres compared to the non-vaccinated mock group. In Egypt, where currently no 'stamping out' policy is known to be applied, the prolonged survival and virus shedding of HPAIV H5N1 in chickens, either after prior LPAIV H9N2 infection or following homologous vaccination with inactivated H5 vaccines, may foster the endemic status of the virus and its continuous evolution in partially immunized birds.

In conclusion, chickens previously infected with an Egyptian LPAIV H9N2 developed a delayed course of infection, with prolonged viral shedding following challenge with a lethal dose of Egyptian HPAIV H5N1 clade 2.2.1.2. In countries with circulating H9 infection but no H5 vaccination policy, H9 pre-infections would not interfere with syndromic surveillance against HPAIV H5N1, since the clinical signs developed in a slightly delayed manner but were not concealed. By contrast, homologous H5 vaccination did conceal the clinical signs, but did not prevent the infection and excretion of HPAIV H5N1. This study helps us to understand the possible effects of prior H9N2 infection or vaccination on the infection of chickens with HPAIV H5N1 in Egypt, where it is endemic in poultry. It is recommended that further studies be conducted to identify the impact of cellular immunity on clearance of and protection against AIV infection and how such components of the immune response can be invoked by improved vaccines.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
All procedures involving animals were carried out in accordance with the legally approved protocol (MV-LALLF-7221.3-1-019/16). All animal infection and immunization experiments were performed in biosafety level-3 (BSL-3) animal facilities.

References

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