Efficacy of a commercial inactivated H5 influenza vaccine against highly pathogenic avian influenza H5N1 in waterfowl evaluated under field conditions

M. Rudolf (1), M. Pöppel (2), A. Fröhlich (3), T. Mettenleiter (4), M. Beer (1) & T. Harder (1)*

(1) World Organisation for Animal Health (OIE) and National Reference Laboratory for Avian Influenza, Institute for Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, D-17493 Greifswald-Insel Riems, Germany
(2) Veterinary Practice, Poultry Specialisation, Delbrück, Germany
(3) Institute of Epidemiology, Friedrich-Loeffler-Institut, Wusterhausen, Germany
(4) Institute of Molecular Biology, Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany
*Corresponding author: timm.harder@fli.bund.de

Summary
Highly pathogenic avian influenza virus (HPAIV) can cause devastating losses in the poultry industry. In addition, several HPAIV exhibit a zoonanthropootic potential and can cause fatal infections in humans. These attributes particularly apply to HPAIV H5N1 of Asian origin. Due to the absence of overt clinical symptoms, introduction and subsequent spread of HPAIV H5N1 in domestic waterfowl (especially ducks) may occur undetected, which increases the risk of transspecies transmissions to highly vulnerable gallinaceous poultry and mammals, including humans. Humans may also become infected with HPAIV H5N1 by food products from slaughtered, silently infected ducks. Vaccination against HPAIV can raise a protective barrier against an incursion of HPAIV since, at least under experimental conditions, the reproduction factor $R_0$ is lowered to <1, which ensures eradication of the virus. The objective of this study was to analyse whether these results can also be obtained under free-ranging field conditions in commercially reared flocks of goose parents and fattening ducks injected with a licensed, adjuvanted inactivated H5N2 vaccine. The time and labour required for the vaccination of these geese and duck flocks exceeded expected values, mainly due to animal sorting according to foot ring labels. No adverse effects directly associated with vaccination were observed. Serologically, a homogenous H5-specific antibody response was induced. Titres varied with temporal distance from the last application of vaccine. Geese parents were clinically protected against challenge with HPAIV A/Cygnus cygnus/Germany/R65/06 (H5N1), but still could be infected and spread HPAIV H5N1, albeit at lower levels and for shorter periods compared to unvaccinated controls. Fattening Pekin ducks proved to be clinically resistant against challenge virus infection and shed very little virus.

Keywords
Introduction

Highly pathogenic avian influenza virus (HPAIV) can cause devastating losses in poultry. In several developing countries where small-scale, so-called ‘sector four’ or backyard poultry production is a significant source of food for the population, continuing HPAIV outbreaks may even lead to protein deprivation and malnutrition (2, 23). In addition, several HPAI viruses (HPAIV) exhibit a zoonanthroponotic potential and may cause fatal infections in humans (5). All of these attributes particularly apply to HPAIV H5N1 of Asian origin. This virus, whose precursor surfaced in 1996 in the Chinese province Guangdong, spread over large parts of Asia and Europe and, in 2005, also reached the African continent (6). In several Southeast Asian countries, as well as in parts of Africa and the Middle East, representatives of different lineages of this virus have established endemic infections with year-round viral activity detectable in poultry (11).

There are several ways in which highly pathogenic infectious agents can establish endemicity. The clinical picture of an infection with HPAIV depends, among other factors, on the bird species affected (1). In gallinaceous poultry, after a short time of incubation, a severely depressed general condition becomes apparent, and the birds die rapidly within days. In domestic waterfowl, however, substantial differences in clinical characteristics following an HPAIV infection have been noticed. This has been analysed in greater detail for HPAIV H5N1 of Asian origin (21, 29, 30). Factors influencing the clinical course relate to species, age of animals and the virus strain (19, 18). Introduction and subsequent spread of HPAIV H5N1 in duck flocks may, therefore, be clinically silent, as observed in live bird markets in Southeast Asia, where HPAIV H5N1 can be isolated year-round, mainly from clinically inconspicuous domestic waterfowl, particularly ducks (11, 26). Recent strains isolated from such outbreaks induce no clinical symptoms in ducks, but remain highly pathogenic for chickens and turkeys. Thus, during syndrome surveillance, infection will only be detected after spread into highly susceptible gallinaceous species (11). This situation is not necessarily confined to backyard and rice-paddy rearing systems: industrial duck holdings with high biosecurity standards may also be affected, as demonstrated by clinically silent infections in two German holdings of fattening ducks in 2007 (12). Furthermore, silently infected ducks living in close contact with humans increase the risk that HPAIV will cross the species barrier (17). Human contacts with HPAIV are also conceivable via food products from silently infected ducks.

Therefore, measures to prevent, control or eradicate outbreaks of these viruses form an integral part of legislation worldwide (4, 22). Prevention is principally based on biosecurity to prohibit virus incursion into poultry holdings. However, the level of success depends on the structure of the poultry industry and on HPAIV epidemiology. Biosecurity measures are easier to implement in regions with a high percentage of well-controlled industrial poultry holdings. Sector four holdings, spatially highly clustered and inhomogeneous concerning age and species of poultry, pose a more severe problem in this respect.

Vaccination against HPAIV can increase barriers against an incursion of HPAIV. Successful vaccination is determined by:

- absence of symptoms after infection
- an increased ability of vaccinees to resist infection
- an effective reduction of virus shedding after infection.

Transmission of field virus will be interrupted when the reproduction factor \( R_0 \) drops below 1, thus ensuring eradication of the virus (32). However, in a modelling approach, Savill et al. (24) demonstrated that even 90% vaccination coverage with currently available vaccines does not prevent clinically ‘silent’ field virus infections and further virus spread. A field vaccination study of chickens in Hong Kong resulted in 81.7% successfully vaccinated animals (7). Today, in the European Union (EU), three inactivated oil emulsion vaccines are licensed and commercially available. Two of them are specific for subtype H5 (8). Inactivated vaccines have to be administered individually via injection, causing significant logistical challenges. While it has been experimentally shown that \( R_0 \) can drop below 1 by using these vaccines (3, 31, 32), it remains to be elucidated whether these results, obtained in a laboratory setting with very limited numbers of animals, can be extrapolated to conditions in commercial poultry production. Besides questions about the protective efficacy of the available vaccine in a field situation, there are also apprehensions that widespread and sustained, but uncontrolled, vaccination may facilitate the development by antigenic drift of variants which escape immunity induced by the vaccines (16, 26).

This study evaluated the time and labour required for the vaccination of geese and duck flocks against HPAIV H5 under commercial conditions. Vaccine efficacy was investigated by the induction of antibodies, and by challenge experiments. The authors show that vaccinated, commercially reared geese parents are protected from disease but still can be infected and shed HPAIV H5N1 albeit at reduced levels and for shorter periods compared to non-vaccinated controls. Pekin ducks in this study proved to be clinically resistant against challenge virus infection and shed only very little virus, if any at all.
Material and methods

Study design and supervision of flocks

Two commercial, professionally reared free-range flocks were selected: one of goose parents (n = 1,200) and one of fattening Pekin ducks (n = 1,500). The holdings were operated under fully commercial conditions in Northwestern Germany; however, neither products nor animals from these flocks were allowed to be marketed during the trial. The geese flock was kept from November 2006 onwards on 7.5 ha pastures but had an open stable available. During times of reduced meadow regeneration (November 2006 to January 2007 and September 2007 to January 2008) the birds received supplements of carrots, corn and wheat. During laying periods special breeding forage (loose forage, supplied by Goldott-Entenzucht p, Germany) was provided.

Six-week-old fattening ducks were kept under free-range conditions with sheds available. The geese and duck flocks were monitored for 20 and 5 months, respectively, for productivity determinants (daily feed consumption, loss rate, production rate), clinical status and avian influenza virus (AIV)-specific virological and serological parameters. The study received full legal approval as an animal experiment (authorisation no. LALLF M-V/TSD/7221.3-1.1-040/05). Since AIV vaccination is legally prohibited in Germany and in the EU, derogation was granted by the European Commission (EC) as laid down in decision 2006/705/EC.

Vaccine and immunisation schemes

An inactivated and adjuvanted vaccine based on low pathogenic avian influenza virus (LPAIV) strain A/duck/Potsdam/1402/86 (H5N2) was used to immunise a subpopulation of the flocks. A total of 800 of 1,200 geese, and 1,000 of 1,500 ducks were vaccinated according to the manufacturer’s protocols (1.0 ml vaccine per animal). Primary AIV H5 vaccination of geese was performed at 17 weeks of age (Fig. 1a, squares). Ducks received the primary AIV H5 vaccination at seven weeks of age (Fig. 1b, squares). Standard vaccinations against parvoviruses and

![Fig. 1](image-url)

Vaccination and sampling scheme of geese (a) and duck flocks (b)

The geese were vaccinated at the age of 17 weeks (V1), 21 weeks (V2), 48 weeks (V3) and 77 weeks (V4). Sampling (●, swabs and blood) was done at week 17 (S1), 21 (S2), 25 (S3), 47 (S4), 50 (S5), 76 (S6) and 81 (S7). Challenge experiments (△) were conducted in week 21 (C1), 25 (C2), 47 (C3) and 77 (C4).

The ducks were vaccinated at the age of 7 weeks (V1) and 11 weeks (V2). Sampling (●, swabs and blood) was done in week 7 (S1), 10 (S2), 14 (S3) and 25 (S4). Challenge experiments (△) were conducted in week 10 (C1), 14 (C2) and 26 (C3).
salmonellosis were carried out twice before the laying period in geese, and Pasteurella anatisfetifer vaccine, based on a strain isolated from this herd, was applied at 12 days of age in ducks. All vaccines were given by intramuscular injection. The second AI H5 vaccination (‘booster’) was performed four weeks after primary vaccination. The geese were revaccinated twice, after six and 12 months, following the booster vaccination. Avian influenza virus-vaccinated animals were identified by a coloured plastic foot-ring imprinted ‘AIV’.

Flock sampling schedules

Combined oropharyngeal and cloacal swabs (Virocult® MW 950, Medical Wire & Equipment Co. Ltd, Corsham, Wiltshire, England) were taken from 60 animals of each group (vaccinated, non-vaccinated) in each flock at time points indicated by the circular symbols (S) in Figures 1a and 1b. Heparinised blood samples (lithium-heparin tubes, Kabe Labortechnik, Germany) were taken from the same birds in parallel to swabbing. Swabs and blood samples were kept cooled until arrival in the laboratory within 48 h.

Challenge experiments

Vaccine efficacy as defined by protection against disease, infection, and excretion of HPAIV, was evaluated by challenge experiments using HPAIV A/Cygnus cygnus/Germany/R65/06 (H5N1). This strain had been isolated from a naturally infected whooper swan dying from the infection in Germany in spring 2006 (34). At the time points (C) indicated by triangles in Figures 1a and 1b, eight vaccinated and eight non-vaccinated birds were withdrawn from each of the flocks and transported to the high containment facilities of the Friedrich-Loeffler-Institut, Insel Riems, Germany. Ducks and geese were transported separately and had no other contact. For challenge infection, each vaccinated animal was inoculated by the oculo-oronasal route with 106 50% egg infective dose (EID50) of HPAIV H5N1 diluted in cell culture medium containing 10% foetal calf serum. Three non-vaccinated animals were inoculated with the same amount of inoculum to verify the presence of AIV. Animals that died or had to be euthanised in a moribund state were referred for pathological investigations. Blood samples were obtained from all animals before inoculation and from all surviving birds at the end of the observation period.

Virus detection by real-time reverse transcriptase polymerase chain reaction

Ribonucleic acid was isolated from swabs either manually using the Viral RNA kit of Qiagen (Hilden, Germany) or automatically with the Nucleospin 96 Virus Extraction Kit (Macherey & Nagel, Germany) on a Tecan Freedom Evo® pipetting platform. One-step real-time reverse transcriptase polymerase chain reaction (rRT-PCR) for detection of an M-gene fragment (27, modified by 33) was carried out for swabs taken from the flocks. Five of these swabs were pooled for rRT-PCR analysis. Single swabs from positive pools were re-tested. Single positive swab samples were then tested by rRT-PCR analysis. Single swabs from positive pools were re-tested. Single positive swab samples were then tested by rRT-PCR analysis. Single swabs from positive pools were re-tested. Single positive swab samples were then tested by rRT-PCR analysis. Single swabs from positive pools were re-tested. Single positive swab samples were then tested by rRT-PCR analysis. Single swabs from positive pools were re-tested. Single positive swab samples were then tested by rRT-PCR analysis. Single swabs from positive pools were re-tested. Single positive swab samples were then tested by rRT-PCR analysis.

Molecular characterisation of non-H5/H7 avian influenza virus

Swabs taken from birds during the challenge experiments were processed individually and examined by the H5-specific rRT-PCR only.

Virus isolation in embryonated chicken eggs

Swabs were processed and supernatants inoculated into embryonated chicken eggs according to the EU diagnostic manual (9). Two passages each of five days were carried out after inoculation of nine- to 11 day old embryonated eggs. Detection of haemagglutinating activity in amnio-allantoic fluids prompted further molecular investigation, as described above, to verify the presence of AIV.

Virus titration in cell culture

Serial tenfold dilutions were made from swab fluids or challenge virus inocula in cell culture medium containing 10% foetal calf serum, and used to infect MV1Lu cells (ATCC CCL 64) during seeding into microtitre plates (100 µl per well, four replicas per dilution). MV1Lu cultures were grown in Dulbecco’s modified Eagle’s medium containing 10% foetal calf serum. The cultures were incubated at 37°C in 5% CO2 for three days, after
which infected cells were identified by an immune peroxidase monolayer assay (IPMA) targeting the viral nucleocapsid protein (NP). To this end, cell monolayers in microtitre plates were fixed using a 1/1 (vol/vol) mixture of methanol and acetone at −20°C after removing the medium and rinsing the monolayer once with phosphate-buffered saline (PBS). Fixed cells were rehydrated with PBS, and then overlaid with appropriately diluted anti-NP monoclonal antibody (ATCC HB 65) for one hour at room temperature. After two washes with PBS containing 0.05% (vol/vol) Tween 20, the cells were overlaid with anti-Mouse IgG peroxidase conjugate (Sigma, Saint Louis, Missouri, United States of America) for 1 h. Following another wash, cells were stained with 3-aminoethylcarbazole and H2O2. Coarse intracellular granular reddish precipitates were used to identify antigen-positive cells by light microscopy at a magnification of 200×. Infectivity titres were estimated according to Karber (13).

Detection of avian influenza virus nucleocapsid protein-specific antibodies by competitive enzyme-linked immunosorbent assay

Heparinised blood samples were screened for AIV-specific antibodies using the Al A blocking enzyme-linked immunosorbent assay (ELISA) (Institut Pourquier, Montpellier, France), or the ID Screen® Influenza A NP Antibody Competition ELISA (ID VET, Montpellier, France). These tests enable a qualitative detection of antibodies directed against the influenza A virus NP protein of all subtypes. Both assays appeared to have comparable performance according to the authors’ validation data (not shown) and were therefore used interchangeably. The instructions of the suppliers were followed exactly. Positive samples were further tested for subtype-specific reactivity by haemagglutination inhibition (HI).

Detection of avian influenza virus subtype-specific antibodies by haemagglutination inhibition

Hemagglutination inhibition antigen was prepared from inactivated allantoic fluids of embryonated chicken eggs inoculated with either the vaccine (A/duck/Potsdam/1402/86 [H5N2]) or the challenge virus (A/Cygnus cygnus/Germany/R65/06 [H5N1]). In addition, duck sera were also tested against a recent H10 field virus isolate from Germany (A/mallard/NVP/Wv1015/04, H10N7). Haemagglutination inhibition was performed as outlined in the World Organisation for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (35).

Statistical analysis

Statistical analyses were performed using the open-source software package R 2.1.1 (The R Foundation for Statistical Computing [2005]; www.r-project.org) and STATISTICA für Windows (Software-System für Datenanalyse) Version 6.0 (StatSoft, Inc. [2001]; www.statsoft.com) to assess statistical differences of clinical signs or serological titres.

Depending on the small sample size of different groups, Fisher’s exact test was used to verify differences in the distributions of data measured. Survival analysis was performed by the log rank test. The sign-test was performed to compare connected samples of values on an ordinal scale, e.g. titres. For result interpretation a strictly defined significance level at p-value 0.05 was used. Multiple tests on the same data according to the critical significance levels were not corrected.

Results

Monitoring trial flocks reveals undisturbed general health but suboptimal production parameters

During the whole study period the health status of the geese and duck flocks was very good. Gross-pathological investigations of dead or randomly selected diseased birds revealed that these cases were largely the result of injuries accidentally caused by manipulation during sampling and vaccination (particularly manipulation to obtain blood samples). After blood sampling, a few (4 to 6) birds per flock died, due to uncontrolled bleeding from the injection site, or had to be euthanised because of broken wings or leg injuries incurred when the birds panicked during capture.

Geese

During the first and second laying periods, 82 and 61 animals, respectively, died or were selected for pathological examination for various reasons. These figures include 13 losses by predators (foxes). A more frequent cause of loss was oviduct-peritonitis in laying geese, probably also provoked or aggravated by capture. Sixty-four geese were removed for challenge infections. The birds produced on average 32 eggs each. This value is at the lower range of normal production. It could well be that frequent handling for sampling and vaccination had a negative influence on egg production. It could well be that frequent handling for sampling and vaccination had a negative influence on egg production. It could well be that frequent handling for sampling and vaccination had a negative influence on egg production. It could well be that frequent handling for sampling and vaccination had a negative influence on egg production. It could well be that frequent handling for sampling and vaccination had a negative influence on egg production. It could well be that frequent handling for sampling and vaccination had a negative influence on egg production. It could well be that frequent handling for sampling and vaccination had a negative influence on egg production. It could well be that frequent handling for sampling and vaccination had a negative influence on egg production.
Ducks

In the duck flock, 52 birds died during the 126 day observation period due to accidents during handling and a further 48 birds were used for challenge experiments. The average animal weight at the end of the fattening period was 3,680 g (a gain of 880 g). Usually, a fattening period for ducks does not exceed 49 days; however, in this experiment other conditions prevailed according to the objectives of the study. Consequently, no values for forage consumption are indicated.

Avian influenza virus vaccination by injection was logistically demanding but prompted no significant vaccine-induced side-effects in the birds

No adverse reactions during and after application of the vaccine were observed in geese or ducks. During capture and manipulation of the animals for vaccination considerable care had to be taken to prevent injuries, overheating and suffocation of the birds, particularly in layers (see above). Precautionary measures demanded up to two additional technical assistants than usually considered for vaccination of gallinaceous poultry. For vaccination of 800 geese within three hours five persons were required. This included additional work to separate vaccinated geese from non-vaccinated controls during revaccination runs. Two thirds of the ducks (1,000 animals) were vaccinated within three hours by a total of five people.

Avian influenza virus-specific antibody titres in flocks decreased with temporal distance from revaccination

All blood samples taken prior to vaccination tested negative for AIV NP-specific antibodies. Considering the performance of the ELISAs used, the AIV seroprevalence in the herds prior to vaccination was estimated to be <4.87%, and the herds were classified to be seronegative and fully susceptible for AIV infections.

Geese

Only one AIV NP and H5 seropositive sample was detected throughout the observation period (n = 420 samples) among the non-vaccinated birds. Most likely this sample was from a vaccinated bird which had lost its foot ring. Generally, 89.54% ELISA-positive samples turned out to be positive (≥ 4 log₂) in HI-test. In vaccinated geese, there was a gradual increase of H5-specific mean titres over the course of the observation period (Fig. 2a). After the second vaccination (S3), 92% of the vaccinated geese showed H5 vaccine-specific antibody titres ≥ 5 log₂. In the

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observation period that followed titres declined with temporal distance from the last vaccination to below 4 log₂, and were boosted after each revaccination (Fig. 2a). Titres against the challenge virus were 3 to 7 log₂ steps lower.

**Ducks**

Development of AIV H5-specific antibodies in vaccinated ducks (Fig. 2b) took a similar course and all tested birds showed AIV-specific antibody titres ≥5 log₂, after booster vaccination (S3), which only marginally decreased after 11 weeks (S4). Surprisingly, and in contrast to the geese flock, AIV NP-specific antibodies were also detected in non-vaccinated ducks (Fig. 2b) from week 10 onwards (S2). According to virological investigations (see below), this was most likely due to a natural infection with an AIV field isolate of subtype H10. Using a recent H10N7 isolate originating from wild birds in Germany, development and rapid decline of H10-specific mean HI antibody titres was evident in non-vaccinated ducks (Fig. 2b, triangles). Also, some vaccinated ducks developed H10-specific antibodies, albeit at lower frequency and titres compared to non-vaccinated animals.

**Virological monitoring revealed a natural avian influenza virus infection in the free-range duck herd but not in the geese flock**

**Geese**

By generic AIV rRT-PCR specific for an M gene fragment no evidence for naturally occurring influenza A virus infections was obtained during the study period, in either the vaccinated or non-vaccinated subpopulation.

**Ducks**

Prior to vaccination (S1) all swabs tested negative for AIV RNA. However, at three weeks after the first vaccination (S2), 40 of 120 swabs (20/60 from non-vaccinated and 20/60 from vaccinated birds) were positive in M-gene specific rRT-PCR. Cycle threshold values (Ct-values) ranged between 39 and 30. No viruses were isolated from any of the PCR positive swabs. By conventional RT-PCR for the HA2 encoding part of the HA gene, specific amplification products were obtained from two animals and sequenced (sequences available in GenBank under accession number EU883664). The AIV A/duck/Shimane/45/1997 (H10N7) proved to be the closest relative of these sequences in a BlastN2 database search. At the monitoring time points that followed (S3, S4) no influenza A virus RNA was detectable in the swabs.

**Immunologically naïve geese and ducks were susceptible to challenge virus infection but only geese developed significant central nervous symptoms and mortality**

**Geese**

In the first two challenge experiments (C1, C2), three non-vaccinated animals were each inoculated with 10⁶ EID₅₀ of HPAIV H5N1 as controls. All of these animals fell sick, and five out of six died. Clinically, central nervous symptoms such as altered behaviour, phases of circular motion and severe torticollis were observed from 4 days post inoculation (dpi). The geese died or were euthanised in moribund status between dpi 6 and 10. Pathological alterations found in dead animals were non-specific, consisting primarily of mucosal haemorrhages in proventriculus or pancreatitis. For animal welfare reasons no non-vaccinated controls were used in challenge experiments C3 and C4 since the pathogenicity of the challenge strain for susceptible geese had been demonstrated adequately.

**Ducks**

During each of the three challenge experiments three non-vaccinated ducks were inoculated with 10⁶ EID₅₀ of HPAIV H5N1 as controls. All of these animals had low titres of H10-specific antibodies (mean titre C1: 4 log₂; C2: 4 log₂; C3: 2.7 log₂). None of these ducks developed any clinical signs.

**Absence of clinical signs in challenged vaccinated geese and ducks**

With the single exception of one animal which died during challenge at C4, vaccinated geese fully resisted an inoculation of 10⁶ EID₅₀ of HPAIV H5N1 with respect to clinical symptoms, even after a single vaccination (Fig. 3). The one dead animal shed virus up to a Ct-value of 25 and showed a mild rhinitis and pancreatitis. As expected, vaccinated animals did not show clinical symptoms upon exposure to 10⁶ EID₅₀ of HPAIV H5N1 (not shown).

Like the non-vaccinated ducks, the vaccinated ducks displayed no clinical signs.

**Vaccinated geese and ducks transmitted challenge virus to non-vaccinated and vaccinated sentinels, although reduced challenge virus excretion in level and duration was evident**

**Geese**

During challenge trial C1, three weeks after the first vaccination, all five non-vaccinated in-contact animals
died on dpi 10 due to HPAIV H5N1 infection, while all vaccinated animals survived without developing clinical symptoms. At C2, three weeks after the second vaccination, two of five non-vaccinated in-contact animals died on dpi 10. At C3, one of five non-vaccinated animals died on dpi 8. C4 was conducted six months after the first revaccination. In contrast to previous challenge experiments, the in-contact birds had also been vaccinated. While none of the vaccinated contact animals died, one of eight vaccinated and inoculated geese fell severely ill and died due to challenge virus infection.

Intermittent virus shedding started at dpi 1 in inoculated geese (Table I). Until dpi 10, episodic excretion from the oropharynx and/or the cloaca was evident for vaccinated geese in all challenge experiments. Non-vaccinated in-contact animals started virus shedding after two days of contact. Ct-values of up to 24 were reached between days 7 and 9 post challenge. Shedding from the oropharynx was significantly more pronounced than shedding from the cloaca (geese: 127 rRT-PCR-positive oropharyngeal swabs out of 383 [127/383] versus 34/381 positive cloacal swabs; ducks: 36/329 positive oropharyngeal swabs versus 3/329 positive cloacal swabs; p < 0.0001, Fisher’s exact test). In cell culture, HPAIV H5N1 was re-isolated only from swabs obtained from non-vaccinated in-contact birds. The 50% tissue culture infectious dose (TCID₅₀) values ranged between 10⁻⁶ and 10⁻⁵.₁ per ml swab fluid. It was not possible to re-isolate challenge virus from swabs taken from vaccinated in-contact animals (C4), except for two vaccinated and inoculated geese of C4, one of which succumbed to the infection. These birds excreted

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**Fig. 3**
Kaplan-Meier curves of the geese challenge experiments C1 to C4
Vaccinated animals are plotted as grey squares, controls as black circles and associated in-contact birds as white lozenges. For animal welfare reasons, no controls were included in C3 and C4. In-contact birds of C1 and C2 were taken from the non-vaccinated subpopulation, in C3 and C4 vaccinated in-contact birds were chosen.
infectious HPAIV H5N1 detectable in cell culture with a TCID₅₀ of 10⁻¹ to 10⁻²⁰ per ml. Two of five vaccinated contact birds seroconverted against the challenge virus, indicating that they had become infected.

**Ducks**

In Table II the results of the duck challenge trials are summarised. Only low loads (Ct-values >30) of H5-specific AIV RNA were detected in a few cases scattered across the observation period. The HPAIV H5N1 was re-isolated from only two cases: a vaccinated duck (C1, dpi 1), and one non-vaccinated in-contact animal (C3, dpi 4). Vaccinated birds revealed increased avian influenza-specific haemagglutination inhibition titres after challenge experiments.

As shown in Figure 4, in each challenge experiment with HPAIV H5N1 a booster effect on the vaccine-induced H5-specific antibody titres (>4 log₂ steps) was evident in the vaccinated birds. The increase in antibody titre was most significant for antibodies against the challenge virus,
but an increase against vaccine antigen was also evident in most cases.

Discussion

An efficacy assessment of an inactivated adjuvanted vaccine against HPAIV H5N1 infection in commercially reared flocks of geese parents and fattening ducks was carried out. Feasibility of application, protection against disease, and protection against infection with, and excretion of, HPAIV field virus were investigated.

Feasibility of vaccine application

Intramuscular application of the vaccine posed no particular problems in 16-week-old geese or in 7-week-old fattening ducks. About 200 birds were injected per hour by one person with the help of three or four assistants. This included time required to identify the bird’s vaccination status by reading the leg ring. It should be mentioned that remnants of oil from the absorbed vaccine were detected in breast muscles of fattening ducks for up to 50 days after vaccination. This may cause problems in marketing meat products from these animals; a subcutaneous route of application would be preferable.
Protection against disease

A considerable host- and virus-strain-related variability in the clinical outcome of HPAIV infections in waterfowl has been documented (18, 19, 21, 30). The challenge strain chosen for this study originated from an outbreak in wild birds in Northern Germany in 2006. It exhibited an intravenous pathogenicity index of 2.97 in chickens (34) and caused significant mortality, though not a 100%, in susceptible adult geese within ten days. This strain, like others, shows an age-dependent pathogenicity in Pekin ducks, causing 100% mortality in four week-old animals (Rudolf et al., in preparation), but did not induce any detectable clinical signs in adult Pekin ducks. Moreover, virus replication seemed to be limited in adult ducks in line with the restricted virus excretion. Nevertheless, successful challenge virus infection of all animals was indicated by seroconversion at dpi 10 (Fig 4). Thus, concerning protection from disease, no effect of vaccination could be assessed.

A possible co-protective role of heterosubtypic immunity induced by the accidental, natural infection with an AIV of subtype H10 in the free ranging duck flock is unclear. Even if, at C3, H10-specific antibodies were no longer detectable, cellular immunological memory functions may have been still active.

In contrast, the protective effects of the vaccination of geese were statistically verified (Table III): vaccination protected successfully against illness and death. (In C1 protection could not be formally verified, as one out of three non-vaccinated control animals survived as well.) A Kaplan-Meier Curve (Fig. 3) shows that there is a 100% probability that vaccinated animals will stay healthy, only in C4 did one vaccinated animal die. Analysis by Fisher's exact test shows significant (p<0.05) differences between healthy and diseased/dead animals in vaccinated and non-vaccinated groups.

Protection against infection with, and excretion of, highly pathogenic avian influenza field virus

One of the gravest concerns about vaccination against HPAIV is the risk of clinically silent HPAIV infections

### Table III

**Efficacy of protection of vaccinated geese**

<table>
<thead>
<tr>
<th>Challenge trial</th>
<th>Disease *</th>
<th>Death **</th>
<th>Clinically apparent transmission to non-vaccinated animals ***</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1</td>
<td>0/8 (0.00606)</td>
<td>0/8 (0.05455)</td>
<td>5/5 (0.00077)</td>
</tr>
<tr>
<td>C2</td>
<td>0/8 (0.00606)</td>
<td>0/8 (0.00606)</td>
<td>4/5 (0.00699)</td>
</tr>
<tr>
<td>C3</td>
<td>0/8 (no controls)</td>
<td>0/8 (no controls)</td>
<td>2/5 (0.1282)</td>
</tr>
<tr>
<td>C4</td>
<td>0/8 (no controls)</td>
<td>1/8 (no controls)</td>
<td>0/5 (1)</td>
</tr>
</tbody>
</table>

* number of diseased vaccinated animals after challenge

** number of dead vaccinated animals after challenge

*** number of diseased non-vaccinated animals after nine days of contact

(a) figures in parentheses indicate the efficacy of protection of vaccinated geese against disease (Fisher’s exact test, p-values under null-hypotheses)

(b) figures in parentheses indicate efficacy of protection of vaccinated geese against death (Fisher’s exact test, p-values under null-hypotheses)

(c) figures in parentheses indicate efficacy of protection of vaccinated geese against virus transmission leading to clinically apparent disease in non-vaccinated control birds (Fisher’s exact test, p-values under null-hypotheses)

(d) vaccinated contact animals were used in the fourth trial

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**Fig. 4**

*Average HI-titres against the vaccine antigen (A/duck/Potsdam/1402/86 [H5N2], light grey) and the challenge virus antigen (A/Cygnus cygnus/Germany/R65/06 [H5N1], dark grey) in vaccinated geese (a) and ducks (b) at dpi 0 and dpi 10 of the challenge experiments*

The median titre and 25% and 75% quartiles are depicted in box plots. Outliers are marked by black dots. The dotted line represents a titre of 5 log₂ indicative of a solid vaccine-induced immunity which was shown to correlate with protection against disease in chickens (15)
under immune coverage. According to mathematical modelling (24) very high levels of population immunity are required to completely suppress both infection and spread. However, there are no accurate data available on immunisation rates of different poultry species under field conditions, since all data were obtained from trials run under experimental conditions. The HI-specific antibody titres correlate with clinical protection and, in chickens, an HI-titre of ≥ 5 log₂ was associated with good protection (15). However, a similar correlation has not been made in other avian species.

In the two waterfowl species examined here the development of specific humoral immunity was evaluated by NP-specific antibody ELISA and by strain-specific HI. In line with previous experiences, ducks and geese in our study reached, on average, lower antibody titres compared with chickens. In addition, in geese, these titres declined more rapidly (14), so that only 40% of vaccines had titres of 5 log₂ six months after the basic immunisation. Therefore, bi-annual revaccinations of geese were required to keep the average HI titre against the homologous (vaccine virus) antigen at ≥5 log₂, in at least 90% of vaccines. In contrast, basic immunisation of ducks was sufficient to maintain this level during the observation period. Antibody titres against the heterologous HPAIV H5N1 challenge virus were significantly lower, which was expected due to the comparatively large differences in homology of HA sequences between the two viruses (homology of HA sequences 92%).

However, in the author’s study no vaccinated geese showed overt signs of disease upon challenge, not even animals with an average HI titre of 2.4 log₂ (±1.4) against the homologous vaccine virus antigen at C3, which is considerably lower than anticipated for solid clinical protection in chickens (15). Similar findings were seen in the other challenge experiments. The one goose which died in C4 had no detectable antibodies against the vaccine antigen prior to challenge. Interestingly, in the same experiment, two other animals without detectable antibodies survived the challenge infection. Therefore, the predictive value of specific HI antibody titres as an indicator of the protective efficacy of vaccination in geese seems to be questionable. This may also point towards a substantial contribution to protection of the cellular immune response.

In order to evaluate protection from infection with, and excretion of, HPAIV, vaccinated birds were challenged with a recent H5N1 HPAIV of Asian origin. In addition to evaluation of challenge virus excretion by molecular and virological methods, non-vaccinated in-contact birds (sentinels) were chosen to closely mimic a field situation in a flock with a heterogenous H5-specific immune status (32), but it was chosen since it more likely resembles a realistic situation in the field. However, for the final challenge experiment (C4) vaccinated in-contact geese were used.

In the geese experiments, challenged vaccinated birds became infected and excreted challenge virus albeit at lower titres and intermittently compared to non-vaccinated control geese which received the same inoculation dose. Non-vaccinated in-contact geese acquired the infection after challenge virus transmission from vaccinated birds and developed clinical disease in experiments C1 and C2. Only one of five non-vaccinated in-contact geese succumbed to the infection in the third trial (C3), although all in-contact birds became infected (TCID₅₀ ranged between 10¹⁶ and 10¹³). The authors were not able to clearly demonstrate transmission of challenge virus to vaccinated in-contact animals (C4), as only one out of five contact animals revealed an rRT-PCR positive swab and on only one occasion. Nevertheless, seroconversion against the challenge virus indicates infection of at least two of the vaccinated in-contact geese of C4 (Table IV).

Successful re-isolation of virus from comparatively few of the PCR-positive animals (Tables I and II) indicates that the cell system used is not as sensitive as live sentinel birds. Thus, despite the inability to re-isolate challenge virus, transmission of virus from vaccinated animals to non-vaccinated sentinels did occur.

For reasons detailed above, no proper assessment of the protective efficacy of the vaccination was possible for the ducks. We only detected low levels of virus shedding, and virus re-isolation was only possible in isolated cases even in non-vaccinated animals. Nevertheless, we found significantly higher numbers of rRT-PCR positive swabs in the non-vaccinated animals than in the vaccinated birds (7 out of 384 swabs from vaccinated ducks versus 28 out of 144 swabs from non-vaccinated ducks; p< 0.0001, Fisher’s exact test). This indicates that vaccination led to a detectable reduction of virus shedding, even if none of the

<table>
<thead>
<tr>
<th>Table IV</th>
<th>Serological response (log₂) of vaccinated in-contact geese in challenge experiment C4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal number</td>
<td>H5N2 (a)</td>
</tr>
<tr>
<td></td>
<td>0 dpc</td>
</tr>
<tr>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>7</td>
</tr>
</tbody>
</table>

(a) vaccine antigen
(b) challenge virus
dpc: days post challenge
animals showed any sign of illness. Beato et al. (3) provided data on the efficacy of a two-dose vaccination programme in the first 30 days of life of Pekin ducks. The same vaccine was used as in the authors’ experiments, but the challenge virus was HPAIV A/duck/Vietnam/12/05 (H5N1). The animals were challenged at four weeks of age. Only non-vaccinated animals fell sick and died. Furthermore, viral shedding by vaccinated animals was marginal. Apparently, the older age of the ducks used in our experiment prevented the development of serious clinical signs.

Conclusions

Vaccination of geese under field conditions was successful in protection against illness and death. However, our experimental evidence demonstrates that geese vaccinated under field conditions can become infected with and transmit challenge virus to non-vaccinated geese. These contact animals fell ill, shed virus at higher titres than the vaccinated animals, and served as amplification hosts for the virus. However, when vaccinated in-contact geese were used (C4), transmission was greatly reduced. This indicates that even under field conditions vaccination is a means to limit and, ideally, abolish virus transmission if a high level immunity can be achieved within the population (flock).

Both vaccinated and non-vaccinated ducks were able to resist infection with HPAIV H5N1 and virus excretion was found to be intermittent and with low titres. Susceptible four-week-old ducklings, in contrast, died within a few days after challenge with the same dose of challenge virus HPAIV A/Cygnus cygnus/Germany/R65/06 (H5N1) (Rudolf et al., in preparation). An age-dependent susceptibility of ducks for different strains of HPAIV has been demonstrated in other studies (18). These findings, along with results of the study by Beato et al. (3), suggest that vaccination of ducks at as young an age as possible could avoid losses of animals caused by HPAIV. It might also help reduce virus shedding in adult vaccinated animals. Ducks have been identified as a major reservoir of endemic HPAIV H5N1 infections in Southeast Asia, since syndrome surveillance fails as an early warning system in ducks. Vaccination of ducks against HPAIV H5N1 may help reduce infection rates and excretion levels of HPAIV-exposed vaccinated birds. More importantly, vaccination in this species would not interfere with syndrome-based early warning systems since even without vaccination, older animals do not exhibit clinical symptoms in contrast to, for example, chickens or geese. Since clinical observation will often not detect infection of ducks by HPAIV, other means of surveillance, including targeted sampling and laboratory diagnosis, are required to eliminate this important virus reservoir.

Acknowledgements

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Évaluation de l’efficacité sur le terrain d’un vaccin commercial à virus H5 inactivé contre l’influenza aviaire hautement pathogène H5N1 destiné aux oiseaux aquatiques

M. Rudolf, M. Pöppel, A. Fröhlich, T. Mettenleiter, M. Beer & T. Harder

Résumé
Le virus de l’influenza aviaire hautement pathogène (IAHP) est responsable de pertes économiques colossales dans le secteur de l’aviculture. En outre, plusieurs virus de l’IAHP ont un potentiel anthropozoonotique et peuvent provoquer une infection léthale chez l’homme. Ces caractéristiques s’appliquent en particulier au sous-type H5N1 du virus de l’IAHP, d’origine asiatique. Parce qu’il ne provoque pas de signes cliniques manifestes, le virus de l’IAHP de sous-type H5N1 peut s’introduire et se propager parmi les populations d’oiseaux aquatiques domestiques (en particulier les canards) sans être détecté, ce qui accroît le risque de transmission trans-spécifique aux espèces extrêmement vulnérables de gallinacés et de mammifères (dont l’homme). L’homme peut également contracter l’infection par le virus H5N1 de l’IAHP par l’intermédiaire de produits alimentaires dérivés de canards porteurs silencieux du virus au moment de l’abattage. La vaccination contre le virus de l’IAHP permet de dresser une barrière protectrice contre les nouvelles incursions du virus : en effet, dans des conditions expérimentales, elle fait passer le facteur de reproduction $R_0$ en dessous de 1, ce qui suffit pour éradiquer le virus. La présente étude a pour objet de déterminer si des résultats similaires peuvent être obtenus sur le terrain chez des troupeaux vivant en liberté d’oies reproductrices commerciales et de canards gras, après injection d’un vaccin autorisé à virus H5N2 inactivé comprenant un adjuvant. Le temps de travail et la main d’œuvre nécessaires pour procéder à la vaccination de ces troupeaux d’oies et de canards ont été plus importants que prévu, en raison de la nécessité de trier les volailles en tenant compte de leurs bagues d’identification. Aucun effet indésirable directement imputable à la vaccination n’a été constaté. La vaccination a induit une réaction sérologique comportant l’apparition d’anticorps homogènes spécifiques pour H5. Les variations des titres d’anticorps constatées reflétaient le temps écoulé depuis la dernière application du vaccin. Le vaccin protège cliniquement les oies reproductrices contre une inoculation d’épreuve avec la souche A/Cygnus cygnus/Allemagne/R65/06 du virus H5N1 de l’IAHP, mais ne les empêche pas de contracter l’infection ni de disséminer ce virus, bien qu’à des niveaux moindres et sur des périodes plus courtes que chez le groupe témoin non vacciné. Les canards de Pékin étaient protégés cliniquement contre l’inoculation d’épreuve et excrètaient le virus en très faibles quantités.

Mots-clés
Efficacité protectrice – Influenza aviaire – Oiseaux aquatiques – Vaccination – Validation sur le terrain.
Evaluación de la eficacia en aves acuáticas y en condiciones naturales de una vacuna comercial contra la influenza aviar altamente patógena por H5N1 a base de virus H5 inactivados

M. Rudolf, M. Pöppel, A. Fröhlich, T. Mettenleiter, M. Beer & T. Harder

Resumen
Los virus de la influenza aviar altamente patógena (virus IAAP) pueden causar pérdidas devastadoras para el sector avícola. Además, varios de ellos presentan potencial zoonotónico y pueden provocar infecciones mortales en el ser humano. Estas características se aplican especialmente a la cepa H5N1 del virus IAAP, de origen asiático. Debido a la ausencia de síntomas clínicos ostensibles, la penetración y subsiguiente propagación de dicha cepa en aves acuáticas domésticas (en especial el pato) pueden pasar desapercibidas, lo que incrementa el riesgo de transmisión a otras especies muy vulnerables de gallináceas o mamíferos, comprendido el ser humano. Las personas también pueden resultar infectadas por la cepa H5N1 al ingerir alimentos procedentes de patos que portaban una infección silente al ser sacrificados. La vacunación contra el virus puede conferir protección contra una incursión vírica, por cuanto, al menos en condiciones experimentales, hace descender el factor de reproducción (factor \(R_0\)) por debajo de 1, lo que garantiza la eliminación del virus. Los autores describen un estudio encaminado a determinar si es posible obtener resultados similares en condiciones naturales tras administrar una vacuna patentada (a base de virus H5N2 inactivados con adyuvante) a bandadas industriales de ocas reproductoras y patos de engorde criadas en libertad. La vacunación de esas bandadas de ocas y patos fue más larga y laboriosa de lo previsto, sobre todo por la necesidad de clasificar a los animales según sus anillas identificativas. No se observó ningún efecto adverso relacionado directamente con la vacunación. Por lo que respecta a la serología, se indujo una respuesta homogénea de anticuerpos específicos de las cepas H5. Los títulos de anticuerpos variaban según el tiempo transcurrido desde la última administración de vacuna. Las ocas reproductoras quedaron clínicamente protegidas contra la infección experimental por virus IAAP A/Cygnus cygnus/Germany/R65/06 (H5N1), pero aún podían resultar infectadas por la cepa vírica H5N1 y a su vez transmitirla, aunque en niveles menores y por períodos más cortos que los animales testigo no vacunados. Los patos de Pekín, en cambio, resultaron ser clínicamente resistentes a la inoculación experimental y excretar muy pocos virus.

Palabras clave
Aves acuáticas — Eficacia de la protección — Influenza aviar — Vacunación — Validación en condiciones naturales.
References


