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Transmission of highly pathogenic avian influenza H5N1 virus in Pekin ducks is significantly reduced by a genetically distant H5N2 vaccine

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ABSTRACT

Domestic ducks play an important role in the epidemiology of H5N1 avian influenza. Although it is known that vaccines that have a high homology with the challenge virus are able to prevent infection in ducks, little is yet known about the ability of genetically more distant vaccines in preventing infection, disease, and transmission. Here we study the effect of a widely used H5N2 vaccine (A/Chicken/Mexico/232/94/CPA) on the transmission of H5N1 virus (A/Chicken/China/1204/04) in ducks. The quantitative analyses show that despite the low level of homology between the virus and vaccine strain transmission was significantly reduced two weeks after a single or double vaccination. Mortality and disease rates were reduced markedly already one week after a single vaccination.

Introduction

Wild aquatic birds are the natural host species for avian influenza (Webster et al., 1992; Alexander, 2000). Most highly pathogenic avian influenza (HPAI) viruses replicate but do not cause disease symptoms in ducks (Alexander et al., 1978, 1986; Westbury et al., 1979; Wood et al., 1985, 1995). In accordance with this finding, early Asian HPAI H5N1 viruses were non-pathogenic to ducks. However, since 2002 several H5N1 strains emerged that induce severe disease and mortality, while other H5N1 strains still cause asymptomatic infections (Sturm-Ramirez et al., 2004, 2005; Hulse-Post et al., 2005; Kishida et al., 2005; Tian et al., 2005; Webster et al., 2006b; Beato et al., 2007; Middleton et al., 2007). Most HPAI viruses can be transmitted from infected to uninfected ducks, as has been shown in experimental studies (Alexander et al., 1978, 1986; Westbury et al., 1979; Chen et al., 2004; Sturm-Ramirez et al., 2004, 2005; Beato et al., 2007; Pantin-Jackwood et al., 2007). In the group of Asian H5N1 viruses there does not seem to be a clear correlation between the pathogenicity and the ability to spread from duck to duck. In fact, transmission is observed in strains that cause subclinical infections as well as in strains that cause severe disease and mortality (Sturm-Ramirez et al., 2004, 2005; Hulse-Post et al., 2005; Beato et al., 2007; Pantin-Jackwood et al., 2007).

Domestic ducks play an important role in the epidemiology of HPAI H5N1 viruses. It has been shown that free-ranging ducks act as a reservoir of H5N1 avian influenza viruses (Songserm et al., 2006), and are a risk factor for the presence of H5N1 virus infections of domestic poultry (Gilbert et al., 2006, 2008). Since not all H5N1 infections are symptomatic, the chance that infections in adult ducks are not detected is substantial. This was illustrated by Kwon et al. (2005), who showed that an H5N1 infection in adult breeder ducks was only detected after the 9 day old offspring showed signs of infection.

Vaccination is a potentially attractive tool for the prevention and control of avian influenza outbreaks. At present, vaccination against HPAI H5N1 is practiced in several countries e.g. Indonesia, People's Republic of China and Vietnam (OIE, 2008b). Vaccines based on different seed viruses are used, with different antigenic homology with the circulating field strains. In chickens it has been shown that the homology between the hemagglutinin of the vaccine strain and the challenge strain is an important factor in the reduction of virus shedding (Swayne et al., 1999, 2000a).

Experimental studies investigating the effect of vaccination in ducks show that virus excretion is significantly reduced after vaccination (Tian et al., 2005; Webster et al., 2006a; Beato et al., 2007; Middleton et al., 2007). In fact, in most studies no virus could be isolated from the

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Table 1
Transmission of H5N1 in unvaccinated ducks

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	+/-	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	a
I	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
I	+/+	+/+	+/+	+/+	†						b
I	+/-	+/-	+/+	+/+	+/-	-/+	-/-	-/-	-/-	-/-	a,c
S	nd	+/-	+/-	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	a
S	nd	+/-	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	+/+	+/+	+/-	+/-	+/-	-/-	-/-	-/-	a
S	nd	+/-	+/-	+/+	+/-	+/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/+	+/+	+/+	+/-	-/-	-/-	-/-	-/-	a
I	+/+	+/+	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-
I	+/+	+/+	+/+	+/-	+/+	†					b
I	+/-	+/+	+/+	+/+	+/-	+/-	-/-	-/-	-/-	-/-	a
I	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	a,c
S	nd	+/-	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-
S	nd	+/+	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	a
S	nd	+/-	+/+	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus.
Two duplicate experiments were performed.
[a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; †, duck died; *x*/*y*, results of the virus isolation in ECE's of the tracheal swab (*x*) and the cloacal swab (*y*); grey box, trachea and/or cloacal swab was positive in the virus isolation; a, conjunctivitis; b, bird died; c, depressed; –, no symptoms were observed.

Table 3
Transmission of H5N1 in ducks two weeks after a single vaccination

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	-/-	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	d
I	-/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	+/-	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	a
I	+/-	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	a,d
S	nd	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	+/-	+/+	+/+	-/-	-/-	-/-	-/-	-/-	-

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus, two weeks after a single vaccination with an inactivated H5N2 vaccine (A/Chicken/Mexico/232/94/CPA). Two duplicate experiments were performed.
[a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; *x*/*y*, results of the virus isolation in ECE's of the tracheal swab (*x*) and the cloacal swab (*y*); grey box, trachea and/or cloacal swab was positive in the virus isolation; a, conjunctivitis; d, swollen oropharynx; –, no symptoms were observed.

swabs at all. But it should be noticed that in most of these studies the homology between vaccine and virus strains is high, and that not all currently used vaccines have this high homology with the recent Asian H5N1 viruses. For this reason we are interested in the effectiveness of vaccines that are currently used and that have a larger genetic and antigenic distance from the Asian H5N1 viruses.

In this paper we report results of transmission experiments that were carried out to quantify the effectiveness of vaccination with a widely used H5N2 vaccine (A/Chicken/Mexico/232/94/CPA) in reducing disease and transmission of H5N1 virus (A/Chicken/China/1204/04) in Pekin ducks. In a transmission experiment a number of infected ducks is housed together with a number of uninfected ducks, and the infection chain is monitored on a regular basis. In our experiments disease symptoms and excretion of virus were monitored daily, while the antibody response was determined weekly. The aim of transmission experiments is to obtain estimates of the basic reproduction ratio (R), which is defined as the number of secondary infections that would

Table 2
Transmission of H5N1 in ducks one week after a single vaccination

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	-/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	-/-	-/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
I	+/+	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	a
I	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	-/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
I	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/-	-/-	+/-	+/-	-/-	-/-	-/-	-/-	-/-	-
S	nd	+/+	+/+	+/-	-/-	-/-	-/-	-/-	-/-	-/-	a

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus, one week after a single vaccination with an inactivated H5N2 vaccine (A/Chicken/Mexico/232/94/CPA). Two duplicate experiments were performed.
[a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; *x*/*y*, results of the virus isolation in ECE's of the tracheal swab (*x*) and the cloacal swab (*y*); grey box, trachea and/or cloacal swab was positive in the virus isolation; a, conjunctivitis; –, no symptoms were observed.

Table 4
Transmission of H5N1 in ducks two weeks after a double vaccination

	D1[a]	D2	D3	D4	D5	D6	D7	D8	D9	D10	Symptoms
I	-	-	-	-	-	-	-	-	-	-	-'
I	-	-	-	+/-	-	-	-	-	-	-	-'
I	-	-	-	-	+/-	-	-	-	-	-	-'
I	-	-	-	-	-	-	-	-	-	-	-'
S	nd	+/-	+/-	+/+	+/-	+/-	-	-	-	-	a
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	a
I	-	-	-	-	-	-	-	-	-	-	-'
I	+/-	+/-	+/-	+/-	+/-	-	-	-	-	-	-'
I	-	-	-	-	-	-	-	-	-	-	-'
I	-	-	-	-	-	-	-	-	-	-	-'
I	+/-	+/-	+/-	+/-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'
S	nd	-	-	-	-	-	-	-	-	-	-'

Pekin ducks were inoculated with A/Chicken/China/1204/04 H5N1 virus, two weeks after a double vaccination with an inactivated H5N2 vaccine (A/Chicken/Mexico/232/94/CPA). Two duplicate experiments were performed.
[a]Day after challenge; I, inoculated bird; S, contact bird; nd, not determined; –, swab was negative in the RRT-PCR; *x*/*y*, results of the virus isolation in ECE's of the tracheal swab (*x*) and the cloacal swab (*y*); grey box, trachea and/or cloacal swab was positive in the virus isolation; a, conjunctivitis; –', no symptoms were observed.

Table 5
Overview of the statistical analysis of the transmission experiments

Treatment group	Final size	R (final size) (95% CI)	H_0: $R \geq 1$	H_0: $R_v = R_c$	Infectious period (day) (95% CI)	Transmission rate parameter (day^{-1}) (95% CI)	R (GLM)
Unvaccinated	5,5	>1.5	ns		4.3 (3.8–4.8)	4.7 (2.3–9.4)	20
Single vaccination (challenge: 1 week)	5,5	>1.5	ns	ns	3.4 (2.9–3.9)	2.7 (0.87–8.6)	9.2
Single vaccination (challenge: 2 weeks)	1,2	0.6 (0.1–2.2)	ns	0.01	3.3 (1.9–4.8)	0.23 (0.09–0.55)	0.76
Double vaccination (challenge: 2 weeks)	1,0	0.2 (0.005–1.5)	0.08	0.002	5 ($n=1$)	na	na

CI, confidence interval; R_v, reproduction ratio amongst vaccinated ducks; R_c, reproduction ratio amongst unvaccinated ducks; GLM, generalized linear model; ns, not significant; na, not applicable.

be caused by one primary infected individual in a large population of susceptible individuals. If R exceeds 1, an infected animal infects on average more than one susceptible animal, and a chain reaction of infections may occur. If R is smaller than 1, a prolonged chain reaction of infections is not possible, and an epidemic cannot occur.

We show that a vaccine that is genetically and antigenically distant from the HPAI H5N1 virus protects the ducks against severe illness and mortality and significantly reduces the transmission of HPAI H5N1 in Pekin ducks.

Results

Clinical symptoms

In the unvaccinated groups several birds showed clinical symptoms (Table 1). Two inoculated ducks died, and eight other birds showed depression and/or conjunctivitis. In all vaccinated groups there was a marked reduction in the number of birds that showed symptoms. In the groups that were challenged one week after a single vaccination only two ducks showed conjunctivitis (Table 2). In the groups that were challenged two weeks after a single vaccination three ducks showed symptoms: one duck showed conjunctivitis, one duck had a swollen oropharynx (possibly due to the swabbing), and one duck showed conjunctivitis and a swollen oropharynx (Table 3). In the groups that received a double vaccination two ducks showed conjunctivitis (Table 4).

Effect of vaccination on virus excretion and transmission

In the unvaccinated groups virus was isolated from all inoculated and all contact ducks (Table 1). The reproduction ratio based on the final size method is $R > 1.5$ with 95% confidence. The transmission rate parameter calculated using the Generalized Linear Model (GLM) is 4.7 (day^{-1}) (95% CI: 2.3–9.4), and the infectious period is 4.3 days (95% CI: 3.8–4.8) (Table 5).

In the groups that were challenged one week after a single vaccination virus was also isolated from all inoculated and all contact ducks (Table 2). Again, the estimate of the reproduction ratio based on the final size method is $R > 1.5$ with 95% confidence. The transmission rate parameter calculated using the GLM is 2.7 (day^{-1}) (95% CI: 0.87–8.6), and the infectious period is 3.4 days (95% CI: 2.9–3.9) (Table 5).

In the groups that were challenged two weeks after a single vaccination virus was isolated from 9 of the 10 inoculated ducks and from 3 contact ducks (Table 3). The estimate of the reproduction ratio R based on the final size method is 0.6 (95% CI: 0.1–2.2). The transmission rate parameter calculated using the GLM is 0.23 (day^{-1}) (95% CI: 0.09–0.55), and the infectious period is 3.3 days (95% CI: 1.9–4.8) (Table 5). There is a significant difference ($p=0.01$) between the reproduction ratios R of the unvaccinated groups and the groups that were challenged two weeks after a single vaccination.

In the groups that were challenged two weeks after a double vaccination virus was isolated from 4 of the 9 inoculated ducks and from one contact duck (Table 4). In one group one duck died before the start of the experiment, therefore 4 instead of 5 ducks were inoculated. The estimate of the reproduction ratio based on the final size method is 0.2 (95% CI: 0.005–1.5) (Table 5). The GLM could not be applied because one contact duck became positive before inoculated ducks became positive in the virus isolation. Here we also see that there is a significant difference ($p=0.002$) between the reproduction ratios R of the groups that received a double vaccination and the unvaccinated groups (Table 5).

The results of the RRT-PCR are shown in Fig. 1, and in Supplementary tables of the supplementary data. The mean cycle threshold (C_t) values of the tracheal swabs of the inoculated ducks of each group are given, and it shows that vaccination reduces virus excretion of the ducks already at one week after a single vaccination (Fig. 1). Logistic regression was applied to the results of the virus isolation (positive or negative) and the RRT-PCR (C_t value) of the tracheal swabs of the experiments with the unvaccinated ducks and the ducks that received a single vaccination ($n=617$). The estimates for the intercept (a) and slope (b) parameters are: $a=19.34$ (SE:2.17), $b=-27.87$ (SE:3.14). The analyses show that when the C_t value of the swab is ≥36.2 the probability of a positive result in the virus isolation is smaller than 5%, and this can be used as a cut-off value for the RRT-PCR. When the result of the RRT-PCR is negative, the probability of a positive result in the virus isolation is 0.0002. This implies that the RRT-PCR can be used as a pre-screening, and we decided that only swabs with a positive RRT-PCR result from the experiment with a double vaccination were inoculated in ECEs.

Serology of the ducks after vaccination

The hemagglutination inhibition (HI) assay was performed with the challenge virus (H5N1) or the vaccine virus (H5N2) as antigen. In the groups that were challenged one week after a single vaccination only 2/20 ducks had a measurable HI titer with the H5N1 antigen ($\log_2$ titers: 1.0 and 1.7) at the moment of challenge (Fig. 2). When the HI test was performed with the H5N2 antigen 11/20 ducks had a measurable titer with a $\log_2$ geometric mean titer (GMT) of 1.5 (95% CI: 0.8–2.1) (Fig. 2). In the groups that were challenged two weeks after a

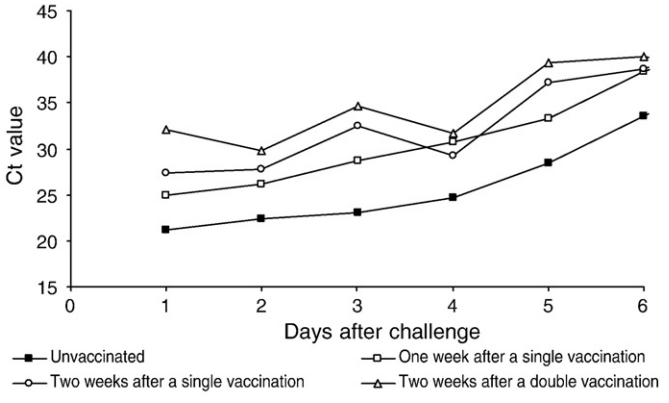

Fig. 1. C_t values of the RRT-PCR of the tracheal swabs of the inoculated ducks. The mean C_t values of the groups are given.

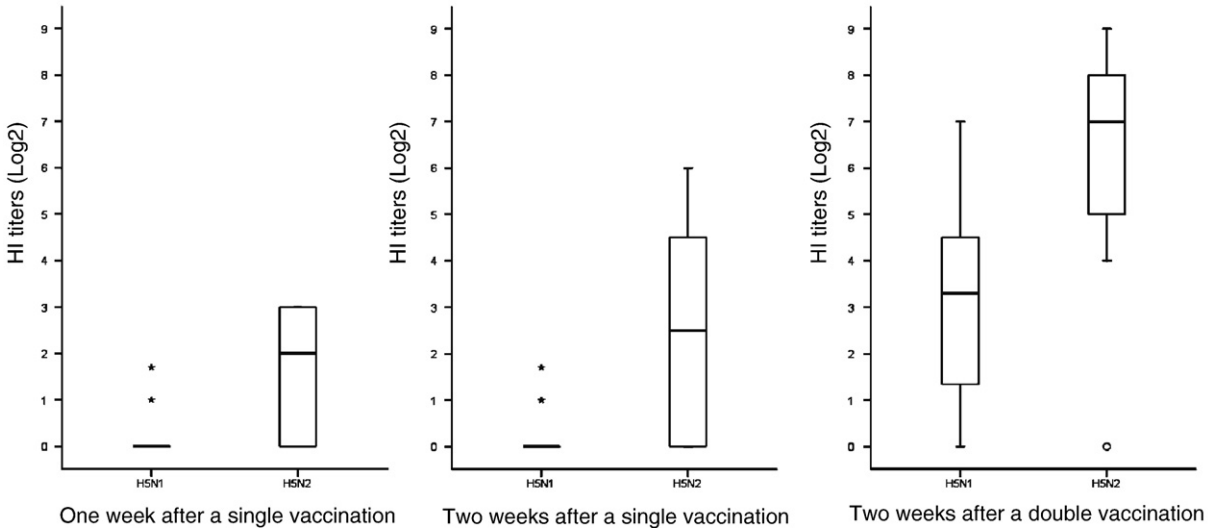

Fig. 2. Hemagglutination inhibition titers of the ducks prior to challenge. The HI test is performed with the challenge strain (H5N1) or the vaccine strain (H5N2). Each box shows the median value of the HI titers (black line), the interquartile range (upper and lower boundaries of the box), and the range of the HI titers (whiskers). ○, outlier, defined as cases with values between 1.5 and 3 box lengths from the upper or lower edge of the box; *, extreme value, defined as cases with values more than 3 box lengths from the upper or lower edge of the box.

single vaccination 3/20 ducks had a $\log_2$ titer of 1.0 or 1.7 with the H5N1 antigen at the moment of challenge. When the HI test was performed with the H5N2 antigen 12/20 ducks had a measurable HI titer, with a $\log_2$ GMT of 2.5 (95% CI:1.4–3.6) (Fig. 2). In the groups that received a double vaccination 17/19 ducks had a measurable HI titer with the H5N1 antigen with a $\log_2$ GMT of 3.1 (95% CI: 2.1–4.1) and 18/19 ducks had a measurable HI titer with the H5N2 antigen with a $\log_2$ GMT of 6.6 (95% CI: 5.5–7.7) at the moment of challenge (Fig. 2).

Discussion

The effect of the protein homology of the HA1 of the vaccine and challenge strain on virus shedding after challenge has been studied systematically in chickens (Swayne et al., 1999, 2000a,b; Lee et al., 2004). When the protein homology of the HA1 varied from 96.8 to 100% no positive correlation was found between the sequence identity of the HA1 from the vaccine strain and challenge virus, and the ability to reduce the quantity of challenge virus shed from the cloaca or oropharynx (Swayne et al., 1999). When a fowl pox vectored vaccine containing the hemagglutinin (HA) of H5 and a variety of challenge strains was used with a HA1 protein homology varying from 87.3 to 100% there was a significant positive correlation in hemagglutinin sequence similarity between challenge viruses and vaccine, and the ability to reduce titers of challenge virus isolated from the oropharynx (Swayne et al., 2000a). In a study where the percentage of amino acid similarities of the HA1 ranged from 84.6 to 99.7% it was also found that the level of virus shedding in the trachea was correlated with the antigenic differences of vaccine and challenge strains (Lee et al., 2004). Transmission in chickens was completely stopped after a single vaccination with vaccine strains that had a protein homology of the HA1 of 92 and 98% with the challenge strain (Van der Goot et al., 2005).

Vaccination against HPAI virus in ducks has been studied less systematically. Previous studies in ducks generally used vaccines that had a high homology with the challenge virus. In most experiments the hemagglutinin of the vaccine and challenge strains both belonged to the Eurasian H5N1 subtype, and these vaccines were able to completely prevent virus excretion (Tian et al., 2005; Webster et al., 2006a; Middleton et al., 2007). Two studies used viruses and vaccines that were genetically more distant (Beato et al., 2007; Middleton et al., 2007). Middleton et al. used a vaccine with an H5 virus (A/Chicken/Italy/22A/98 H5N9) with a protein homology of the HA1 of ~89% with the H5N1 challenge virus, and did find some virus shedding. Beato et al. used a vaccine based on the A/Duck/Potsdam/1402/86 H5N2 virus with a protein homology of the HA1 of 89% with the H5N1 challenge strain and did not isolate any virus. When the effect of vaccination on transmission was studied, no transmission from inoculated to contact ducks was detected (Webster et al., 2006b; Beato et al., 2007).

In these previous studies vaccines that had a high degree of homology (89–100%) with the HA1 of the challenge strain were able to completely protect ducks against morbidity and mortality, virus excretion and transmission. However, not all currently used vaccines have such a high degree of homology with the circulating H5N1 field viruses. Reasons to choose a genetically more distant vaccine may be the availability of vaccines or the choice for a vaccine with a different neuraminidase to be able to adopt a DIVA (Differentiating Infected from Vaccinated Animals) strategy (Capua et al., 2002). In ducks little is known about the effectiveness of genetically more distant vaccines in preventing infection, disease, and transmission. In this paper we show that a widely used H5N2 vaccine strain that has a HA1 protein homology of 84% with the H5N1 challenge virus not only prevented severe morbidity and mortality but also significantly reduced virus excretion and transmission of H5N1 in ducks two weeks after vaccination.

Viruses and vaccines can be compared based on protein sequences of the HA1. It should be kept in mind, however, that although there is a correlation between genetic and antigenic distance, it is also possible that specific genetic differences of only one amino acid lead to a substantial difference in antigenic distance (Smith et al., 2004). For influenza it has been observed that antigenic distance is linearly related to the logarithm of the HI measurement. This principle is the basis for the construction of antigenic maps in which antigenic distances are visualized (Smith et al., 2004). In our study the low level of genetic homology between virus and vaccine is confirmed antigenically in the HI assay. Fig. 2 shows that with the use of the H5N2 antigen in the HI test titers are substantially higher than if it is carried out with the H5N1 challenge antigen.

Although there is a clear relation between HI titers and antigenic distance it is difficult to relate antigenic distance to the level of protection and reduction of transmission. We find a substantial difference (3.5 twofold dilutions) in the HI assay between the H5N1 and the H5N2 viruses, but the vaccine is still able to reduce transmission and virus excretion and to protect the birds against

mortality and severe morbidity. Human influenza vaccines are updated when there is an antigenic difference of at least 2 twofold dilutions in the HI assay (Smith et al., 2004). It would be a challenge to develop corresponding criteria for avian vaccines. However it should be borne in mind that there are various differences between avian and human vaccination such as host differences, vaccination schedules, and the use of different adjuvants.

The experimental set up could potentially influence the outcome of transmission experiments. One of the factors that are of importance is the moment of adding the contact birds to the inoculated birds. In our experiments contact ducks were added at 1 day after inoculation while in previous studies, where no transmission was found, the contact animals were added at day 3 after inoculation (Webster et al., 2006a; Beato et al., 2007). Since the dynamics of avian influenza virus transmission in birds occur on a fast time scale of a few days (Van der Goot et al., 2003, 2005, 2007), it is important that the contact birds are added quickly after inoculation. Tables 1–4 indicate that the infectious periods of the inoculated birds are short (1–5 days). Hence, if the contact birds are added late, this may result in an underestimation of the overall transmission level.

Two ducks in our experiments remained negative in both HI tests and in the Np-antibody ELISA after vaccination, which makes the fraction of non responders in our experiments 0.034 (95% CI: 0–0.08). Both ducks were infected, shed virus and seroconverted in the experiments. One of these birds suffered from conjunctivitis while the other showed no symptoms. These facts may seem of little importance but if the reproduction ratio in unvaccinated birds is high enough, even a low fraction of non responders may turn attempts to prevent outbreaks by vaccination futile. For instance, epidemiological theory states that the critical fraction of the population that needs to be immune to obtain herd immunity, p_c, is related to the reproduction ratio R of an unvaccinated population through: $p_c = 1 - 1/R$. Our point estimate of the reproduction ratio of the unvaccinated group is 20. This implies that at least 95% of the ducks should be fully protected by vaccination to obtain herd immunity. This may be difficult to achieve in an experimental setting, let alone under field conditions.

Overall, our results demonstrate that a widely used H5N2 vaccine strain with a low level of genetic and antigenic homology with the H5N1 challenge virus is able to reduce transmission in ducks significantly. Whether this is true in general remains to be investigated, and therefore it is important to explore the effectiveness of avian vaccines related to their genetic and antigenic distance to circulating field viruses.

Materials and methods

Ducks

Pekin ducks (*Anas platyrhynchos*) obtained from a duck farm were used in all experiments. The ducks were tested in the ELISA to detect antibodies against influenza prior to the experiments, and all were negative. An overview of the ages of the ducks is given in Table 6. The experiments were undertaken in a high containment unit under BSL3+ conditions at the Central Veterinary Institute of Wageningen UR. The experiments comply with the Dutch law on animal experiments and were reviewed by an ethical committee.

Table 6
Overview of the experiments

Treatment groups[a]	Age at vaccination	Age at challenge
Unvaccinated	na	8 weeks
Single vaccination, one week[b]	7 weeks	8 weeks
Single vaccination, two weeks[c]	6 weeks	8 weeks
Double vaccination, two weeks[d]	6 and 9 weeks	11 weeks

na, vaccination was not applied.
[a] Every treatment group consisted of two groups of 10 ducks.
[b] Challenge one week after vaccination.
[c] Challenge two weeks after vaccination.
[d] Challenge two weeks after the second vaccination.

Virus and vaccine

The virus used in this study was A/Chicken/China/1204/04 H5N1. The virus was obtained from the Veterinary Laboratories Agency in Weybridge, is deposited in GenBank (accession number CAJ75442), and belongs to clade 2.4. The virus is also known under the name: A/Chicken/GxLA/1204/04, but since this is not a standard designation we did not use this name. For the transmission experiments ducks were inoculated both intranasally and intratracheally with 0.1 ml diluted allantoic fluid containing $10^{6.0}$ (experiments with a single vaccination) or $10^{6.5}$ (experiments with a double vaccination) median egg-infectious dose (EID_{50}) virus per ml. The inoculation dose per duck was $10^{5.3}$ and $10^{5.8}$ EID_{50} respectively.

An inactivated oil emulsion vaccine was used based on the strain A/Chicken/Mexico/232/94/CPA H5N2 (Intervet Schering-Plough Animal Health, The Netherlands). The vaccine is commercially available, and widely used. A dosage of 0.5 ml vaccine was injected subcutaneously in the lower part of the neck, as recommended by the manufacturer. The protein homology of the genetically most variable region of the hemagglutinin (HA1) between the vaccine strain and the challenge strain is 84%.

Virus isolation

Swabs were put in 2 ml 2.95% tryptose phosphate buffer with 5×10^3 IU of penicillin-sodium and 5 mg streptomycin per ml. The swabs were stored at −70 °C until analyzed. Three embryonated chicken eggs (ECEs) incubated for 9 days were injected with 0.2 ml of the swab fluid per egg. After three days the allantoic fluid was harvested and a standard hemagglutination assay (HA) with chicken red blood cells was performed (OIE, 2008a). When at least one of the eggs was positive in the HA the swab was considered to be positive.

RNA extraction and real time RT-PCR

Viral RNA was extracted using the MagNA Pure LC Total Nucleic Acid Isolation Kit and the MagNA Pure LC Instrument (Roche®). Fluid from the swabs (200 µl) was mixed with 300 µl lysis buffer. The rest of the procedure was followed as recommended by the manufacturer. Five µl of the extracted RNA was used in the RRT-PCR.

A one-tube RRT-PCR was performed to detect the matrix gene of the influenza virus. The Qiagen one-step RT-PCR kit was used with a 25 µl reaction mixture containing 1 µl of kit-supplied enzyme mixture, 1 µl dNTP mix, 4 U of RNase inhibitor (Promega, Madison, WI), 0.5 µM of each primer M-Fw (5′-CTTCTAACCGAGGTCGAAACGTA-3′), M-Rev (5′-CACTGGGCACGGTGAGC-3′), and 0.3 µM of probe M (5′-FAM-CTCAAAGCCGAGATCGCGCAGA-3′-TAMRA). The RRT-PCR was performed with the MX4000 (Stratagene®, Texas). The RT-PCR program consisted of 30 min at 50 °C and 15 min at 95 °C. A three-step cycling protocol was used as follows: 95 °C for 5 s, 58 °C for 15 s, and of 72 °C for 20 s for 45 cycles.

Logistic regression

The relation between virus isolation (y) and the RRT-PCR (x) was modeled by logistic regression:

$$\log\left(\frac{P(y=+)}{1-P(y=+)}\right) = a + b^{*}\frac{x-1}{44}$$

Here, $x=1$ was the lowest and $x=45$ was the highest C_t value (for technical reasons a negative RRT-PCR result was given a C_t value of 45). The intercept a is associated with the highest probability for positive virus isolation (for C_t value $x=1$), and $(a+b)$ is associated with the lowest probability for positive virus isolation (for C_t value $x=45$). The logistic regression model was fitted with the statistical programming language GenStat (2007).

Antibody assays

The sera were incubated for 30 min at 56 °C, and subsequently incubated with 20% chicken erythrocytes at 4 °C overnight. The hemagglutination inhibition assay was performed by standard methods (2008a). Briefly, the test was performed in V-bottom 96 well microtiter plates with 8 hemagglutinating units of the H5N1 challenge virus or the H5N2 vaccine virus and 1% v/v specific-pathogen-free chicken erythrocytes. A competitive ELISA that detects antibodies against the nucleoprotein of influenza A was performed, based on the ELISA that has been described before (De Boer et al., 1990).

Transmission experiments and quantification of transmission

An overview of the experiments is given in Table 6. Within every experimental group all ducks were given the same treatment, and all experiments were performed in duplicate. The design of the experiments is as follows: five ducks are inoculated with A/Chicken/China/1204/04 H5N1 HPAI virus, and 24 h later these five ducks are housed together with 5 uninfected contact birds. To monitor the infection chain tracheal and cloacal swabs are taken daily during the first ten days and twice a week for the next 11 days. The experiments were terminated 21 days after challenge.

The statistical analyses are based on a stochastic SEIR epidemic model in which individuals are susceptible (S), latently infected (i.e. infected but not yet infectious) (E), infected and infectious (I), and recovered and immune or dead (R) (Anderson and May, 1991). The aim is to obtain estimates of the basic reproduction ratio (R), as well as the infectious period and the transmission rate parameter (β). Here we rely on a final size method and a Generalized Linear Model (GLM). Technical details can be found in previous papers (Becker, 1989; De Jong and Kimman, 1994; Van der Goot et al., 2003, 2005, 2007; Velthuis et al., 2007). The analyses are based on the results of the virus isolation in ECEs. For the final size method we took an exponentially distributed infectious period. For the GLM we have assumed a latent period of 1 day, and the infectious periods are directly observed from the infected contact birds. The analyses were carried out in Mathematica 6.0 (final size analysis) and SPSS 15.0 (GLM).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2008.08.037.

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