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An inactivated H5N2 vaccine reduces transmission of highly pathogenic H5N1 avian influenza virus among native chickens

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ABSTRACT

Vaccination against H5N1 highly pathogenic avian influenza in endemically affected areas is a potentially attractive option for local prevention and control. In Indonesia the majority of local outbreaks have occurred in back yard flocks with native chickens, and it is therefore of interest to determine whether these birds can be protected against infection by vaccination. To this end two transmission experiments were carried out with H5N1 virus (A/chicken/Legok/2003) in vaccinated and unvaccinated native chickens. The vaccine contained an inactivated heterologous H5N2 strain (A/turkey/England/N28/73 H5N2). Birds were vaccinated at 4 and 7 weeks of age and challenged at 10 weeks of age. During 10 days post-challenge tracheal and cloacal swabs were taken for virus isolation, and serum blood was collected regularly to measure haemaglutinin inhibiting (HI) antibody responses. The results show that transmission of H5N1 virus was rapid and efficient in unvaccinated birds, that infection and transmission were completely prevented in vaccinated birds, and that vaccinated birds that were exposed to unvaccinated inoculated birds were still protected from infection. These findings indicate that vaccination with a heterologous H5N2 vaccine is able to prevent virus transmission in flocks of native chickens.

1. Introduction

Avian influenza (AI) is a highly contagious viral disease caused by type A influenza virus which is not only able to infect humans, but also a wide variety of avian species [1–5]. Strains of the H5 and H7 subtypes are notifiable to the World Organisation of Animal Health [6], and highly pathogenic strains of these subtypes can cause severe clinical signs in poultry which may result in mortality that ranges up to 100% [7–9].

An epidemic with a highly pathogenic H5N1 strain started in Hong Kong in 1997. Subsequently, the virus spread to several other countries in Asia, Europe, and Africa [6,10]. One of the countries severely hit is Indonesia, where the poultry industry faced outbreaks since 2003 [6]. Infections with H5N1 strains not only resulted in production losses and high mortality in poultry but the virus also infected humans [11]. Up to now most human fatal cases occurred in this country: until January 2009, 141 human AI cases were reported, 115 of which were fatal [11].

The virus is considered to be endemically present in the poultry population in Indonesia, and control efforts mainly make use of vaccination [12,13]. Commercial farmers with high biosecurity standards (sectors 1 and 2 [14]) are using various vaccination and monitoring programs for their flocks, but the results of these programs remain largely unknown. The majority of the outbreaks are currently only reported as result of the participatory disease surveillance and reporting (PDS/R) system implemented by FAO in 2006 [14]. Most of these outbreaks occur in small back yard flocks with native chicken, classified as sector 4 [15]. Native chickens in back yard flocks are generally considered to be at high risk for AI virus introduction from migratory birds [16], not only in Indonesia, but in other Asian countries as well [17]. Moreover, many human cases in Indonesia are linked to contact with these native chickens [11].

Vaccination, already applied on a wide scale in the commercial poultry industry, has also been applied on a small scale in native chicken flocks. Monitoring results, however, suggest that the effective vaccine coverage, i.e. the percentage of native chickens in a vaccinated population with a protective haemagglutination inhibition antibody titer of $\geq 1{:}2^5$ (1:32) is low [19]. It has been suggested that native chickens are low responders by nature [13,20].

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The question arose as to whether native chicken could be protected against transmission of H5N1 virus by vaccination at all. Field studies are difficult to interpret since they may be confounded by various sources of bias. Moreover, a natural challenge might not occur [19]. Therefore, we carried out transmission experiments in which we investigated the efficacy of an AI vaccine in groups of native chicken under well-defined experimental conditions. The efficacy of vaccination was determined for an experimental heterologous vaccine (AI H5N2 strain) by measuring HI antibody titers, virus shedding, and reduction of horizontal virus transmission in a group of vaccinated chickens compared to a group of unvaccinated chickens.

2. Materials and methods

2.1. Animals

The experiments were carried out in the high containment unit at PT. Vaksindo Satwa Nusantara, Cileungsi, Indonesia. Embryonated eggs were purchased from a commercial native chicken breeder, and hatched at the facilities of Vaksindo. The day-old chicks were housed in one experimental unit for the duration of the experiment. They were fed with a commercial ration, and had tap water ad libitum.

2.2. Vaccine

A commercially available oil-emulsion adjuvanted vaccine (Vaksiflu N2®, PT. Vaksindo) was used containing an inactivated low pathogenic H5N2 strain isolated from turkeys (A/turkey/England/N28/73(H5N2)), kindly provided by the OIE reference laboratory VLA (Weybridge, UK) in 2007. The vaccine dosage was 0.5 ml per bird, containing 256 HAU per dose, and was administered intramuscularly in the breast muscle. Chickens were vaccinated at the age of 4 weeks and received a booster vaccination 3 weeks later.

2.3. Inoculum

H5N1 strain (A/chicken/Legok/2003), provided by PT. Vaksindo Indonesia, was used as challenge virus. It has been used in many experiments carried out by PT. Medion Indonesia, and was able to induce infection of SPF layer chickens, which resulted in typical AI signs and high mortality (up to 100%) [21]. The inoculum contained 10^6 EID_{50} per ml, a dose that had previously shown to induce 100% mortality of SPF chickens [21]. The protein homology of the haemagglutinin (HA1) of the vaccine strain and the challenge strain was 92%. Virus titers were confirmed before and after inoculation by titration on embryonated SPF eggs according to standard operating procedures [6]. Each inoculated bird received 0.1 ml inoculum which was administered intratracheally. Inoculation was done when the birds were 10 weeks old.

2.4. Experimental design

Two transmission experiments were carried out with groups of native chickens: Experiment 1 with two groups of chickens, and Experiment 2 with one group.

In Experiment 1, one group consisted of 20 unvaccinated birds, and the other group contained 20 vaccinated chickens. The aim of the Experiment 1 was to quantify virus transmission in homogenous groups of chickens, homogenous with respect to vaccination status. The birds within each group could mingle freely (the groups were separated by a corridor of approximately 0.5 m width). The density of chicks was approximately 5.4 m^{-2}. Five unvaccinated sentinel layer type chickens from PT. Vaksindo were housed in the same room between the two experimental groups (physically separated from the vaccinated and unvaccinated groups) to detect whether virus transmission between the two groups of native chickens occurred. This was done to demonstrate independence of the experimental units (i.e. group).

The design of the experiment was as follows. Three weeks after the second vaccination, 10 unvaccinated and 10 vaccinated chickens were removed from their group and placed in two cages in a separate room; the vaccinated ones in one and the unvaccinated ones in another cage. These 20 birds were inoculated with H5N1 AI virus strain. After 24 h, these inoculated (*I*) birds were re-united with the birds from their original group. The non-inoculated birds (*C* birds) were subsequently exposed to the inoculated birds. Thus each group, vaccinated and unvaccinated, consisted of 10 inoculated birds and 10 contact-exposed birds, thus homogeneous with respect to vaccination status.

Experiment 2 was carried out in which 10 vaccinated birds were housed together with 10 unvaccinated birds. The unvaccinated birds were inoculated (*I*) according to the method described in the previous section, and the vaccinated birds were contact-exposed (*C*) to these inoculated ones. The aim of this experiment was to determine whether vaccinated birds would be protected against virus transmission when exposed to a high virus load, excreted by unvaccinated pen mates.

After inoculation, birds were kept and observed for 4 weeks. The surviving birds were then killed by cervical dislocation.

2.5. Sampling

Clinical signs were recorded during 10 days after challenge (dpc). Tracheal and cloacal swabs were gathered daily during 10 days after inoculation to monitor virus shedding and the occurrence of virus transmission. Each sampling day, the vaccine group was sampled first, followed by the control group; per group the contact birds were sampled first, followed by the inoculated birds. When sampling the birds, animal caretakers used a new pair of gloves for each subgroup (*I*, *C*, vaccinated or unvaccinated). Sentinel birds were not sampled regularly, only if they showed signs of AI or were found dead. Swabs were brought to the laboratory immediately and incubated for 1 h in 1 ml of PBS medium containing penicillin–streptomycin and nystatin. The medium was harvested and subsequently stored at −70 °C until testing. Serum blood samples were taken from all birds by puncturing the wing vein at day of vaccination, day of challenge and at the end of the experiment. Serum samples were stored at −20 °C until testing.

2.6. Tests

The presence of AI virus in swabs was determined qualitatively by virus isolation. Three SPF embryonated chicken eggs, incubated for 9 days, were inoculated with 0.2 ml swab medium per egg. After 72 h, or when the embryo had died before that time, the allantoic fluid was harvested. A haemagglutination assay (HA) was performed following standard procedure [6]. When at least one of the eggs was positive in the HA, the sample was considered to be positive. The test results were recorded as positive for AI virus or negative.

Serum was tested in a haemagglutination inhibition (HI) test using chicken erythrocytes from SPF chickens according to standard procedures [6]. Tests were carried out in duplo using 4 HAU of the H5N1 (A/chicken/Legok/2003(H5N1)) and the H5N2 virus strain ((A/turkey/England/N28/73 (H5N2)). Twofold dilutions of the serum samples were made, and titers were expressed as the serum dilution that caused complete inhibition of agglutination [6].

Table 1
Overview of virus isolation data of the experiment with unvaccinated native chickens (Experiment 1). Cells show the result of virus isolation from tracheal and cloacal swabs.

	Bird	Exposure	Days after inoculation					
			1	2	3	4	5	6
Unvaccinated birds	1	Inoculated	+/+	[a]				
	2	Inoculated	+/+	[a]				
	3	Inoculated	+/+	+/+	[a]			
	4	Inoculated	−/−	+/−	[a]			
	5	Inoculated	−/−	[a]				
	6	Inoculated	+/+	[a]				
	7	Inoculated	−/−	[a]				
	8	Inoculated	+/+	[a]				
	9	Inoculated	−/−	+/+	[a]			
	10	Inoculated	+/+	+/+	[a]			
	11	Contact	−/−	−/−	+/−	+/+	[a]	
	12	Contact	−/−	−/−	+/+	[a]		
	13	Contact	−/−	−/−	+/+	+/+	[a]	
	14	Contact	−/−	−/−	+/+	[a]		
	15	Contact	−/−	−/−	+/+	[a]		
	16	Contact	−/−	−/−	+/+	+/+	[a]	
	17	Contact	−/−	−/−	−/−	[a]		
	18	Contact	−/−	+/−	[a]			
	19	Contact	−/−	+/−	+/−	+/+	+/+	[a]
	20	Contact	−/−	−/−	+/+	[a]		

[a] The bird died.

2.7. Quantification of transmission

A stochastic SEIR (susceptible-exposed-infected and infectious-removed) model formed the basis for the estimation of the epidemiological parameters of interest. The gist of the statistical analyses is given by Van der Goot et al. [22,23]. Key parameters are the duration of the infectious period (denoted by T_I; unit: day), the transmission rate parameter which determines the expected number of new infections that are caused by one infectious bird per unit of time (denoted by β; unit: day^{-1}), and the reproduction number which is defined as the expected number of infections caused by one typical infectious bird over its entire infectious period in a large susceptible population (denoted by R; unit: dimensionless). In our experimental setting the reproduction number is given by the product of the transmission rate parameter and infectious period: $R = \beta T_I$. The reproduction number is of particular interest because only if its value exceeds the threshold value of 1 it is possible that a chain reaction of infections leading to an epidemic can occur [24]. Estimates of the transmission rate parameter and infectious period were obtained by maximum likelihood, assuming a period of latency of 1 day [22]. Assuming independence of the transmission rate parameter and infectious period, and assuming that the infectious period is normally distributed, confidence bounds of the parameters of interest were calculated by using the profile likelihood [25].

3. Results

3.1. Transmission in unvaccinated birds (Experiment 1)

In the experiment with unvaccinated birds (Table 1), the inoculated birds died 2–3 days after inoculation. Most but not all of these birds were positive for virus isolation from both the trachea and cloaca for 1 or 2 days. The virus was transmitted rapidly and efficiently to the contact birds. In fact, all contact birds died 3–6 days after infection of the inoculated birds, indicating a short generation interval of approximately 2 days. The formal analyses yield estimates of the transmission rate parameter of 8.0 day^{-1} (95%CI: 3.6–17 day^{-1}), and an infectious period of the contact infected birds of 1.5 day (95%CI: 0.82–2.2 day). Hence, the reproduction number is estimated to be 12 (4.7–28.7), which is substantially higher than the threshold value of 1. A graphical overview of the analyses is given in Fig. 1.

3.2. Transmission in vaccinated birds (Experiment 1)

The course of the experiment with vaccinated birds was completely different from what was observed in the experiment with unvaccinated chickens. Specifically, none of the inoculated birds and none of the contact birds were positive on any day in the virus isolation, and none of the birds showed any clinical signs of disease. This indicates that vaccination is able to effectively prevent a productive infection, disease, and transmission.The antibody response of birds vaccinated with the H5N2 vaccine is shown in Table 2. Anti-

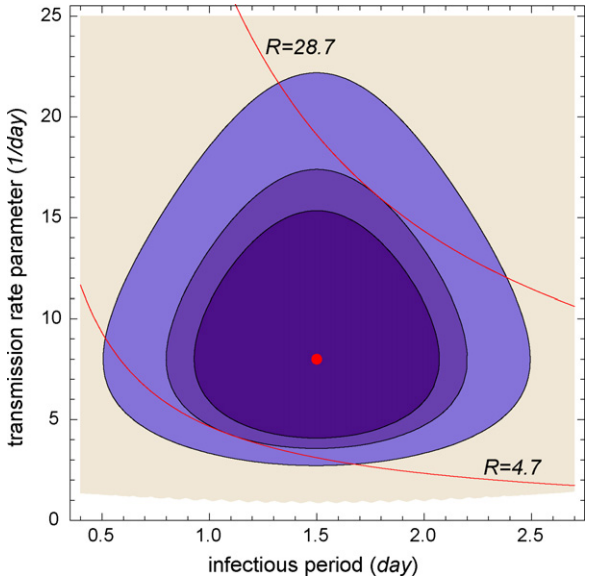

Fig. 1. Estimates of the infectious period and transmission rate parameter (red dot) with associated 90%, 95%, and 99% confidence regions. The reproduction number is given by the product of the infectious period and transmission rate parameter. Lines indicate the lower and upper bound of the 95% confidence interval of the reproduction number. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 2
Serological response of vaccinated chickens (Experiment 1). Blood samples were collected at moment of challenge and at the end of the experiment. The birds were contact-exposed or inoculated with H5N1 A/chicken/Legok/2003. The titer is expressed as twofold dilution that caused inhibition of haemagglutinin (HA) (2×). The strain used in the HI test was either identical to the challenge virus (H5N1 A/chicken/Legok/2003) or to the vaccine virus (H5N2 A/turkey/England/N28/73). The number of HAU in each test was 4.

Treatment	Bird	At challenge (2×)		End of the experiment (2×)	
		H5N1[a]	H5N2[a]	H5N1[a]	H5N2[a]
Inoculated birds	1	7	10	7	14
	2	7	10	7	12
	3	7	10	9	14
	4	3	10	7	11
	5	10	10	mv	mv
	6	7	10	7	12
	7	7	8	9	15
	8	7	10	7	14
	9	4	10	6	15
	10	6	10	6	13
Contact birds	11	5	>10	6	mv
	12	6	>10	6	13
	13	6	10	7	14
	14	7	10	7	13
	15	6	8	8	11
	16	8	10	10	11
	17	4	10	7	13
	18	5	10	6	12
	19	4	10	8	11
	20	6	10	7	14

mv: missing value.
[a] Antigen used in the HI test.

bodies directed against H5N2 and H5N1 were detected by the HI assay in all birds. Not surprisingly, titers directed against the vaccine strain were higher (range: 2^8 to $>2^{12}$) than titers directed against the challenge strain (range: 2^3–2^{10}). The HI titers in serum samples taken from inoculated birds at challenge and at the end of the trial did not show a fourfold increase. Therefore, increase in HI titers was not used to determine infection of contact-exposed birds.

3.3. Transmission from unvaccinated to vaccinated birds (Experiment 2)

To determine whether vaccinated birds would still be protected against infection and disease when confronted with highly infectious unvaccinated birds, an experiment was carried out in which vaccinated contact birds were exposed to unvaccinated inoculated birds. All unvaccinated inoculated birds were productively infected (Table 3), and died within days (range: days 2–6 after inoculation). Interestingly, however, none of the vaccinated contact birds were infected, even though they had been in contact with infectious birds and housed in an area contaminated with H5N1 virus. Table 4 shows the antibody titers of the contact-exposed birds. The contact-exposed birds had no H5N1-specific fourfold increase in HI antibody titers.

The formal analyses yield a maximum likelihood estimate of the transmission rate parameter of 0, with a 95% upper confidence limit of 0.96 day^{-1}. This indicates that the transmission rate of 8.0 day^{-1}

Table 3
Overview of virus isolation data of the experiment with unvaccinated inoculated chickens and vaccinated contact chickens (Experiment 2). Cells show the result of virus isolation from tracheal and cloacal swabs.

	Bird	Exposure	Days after inoculation					
			1	2	3	4	5	6
Unvaccinated birds	1	Inoculated	–	[a]				
	2	Inoculated	–	–	[a]			
	3	Inoculated	–	–	+/+	+/–	[a]	
	4	Inoculated	–	+/+	+/+	[a]		
	5	Inoculated	–	[a]				
	6	Inoculated	–	+/–	+/–	[a]		
	7	Inoculated	–	[a]				
	8	Inoculated	–	[a]				
	9	Inoculated	–	+/–	+/+	–	–	[a]
	10	Inoculated	+/+	[a]				
Vaccinated birds	11	Contact	–/–	–/–	–/–	–/–	–/–	–/–
	12	Contact	–/–	–/–	–/–	–/–	–/–	–/–
	13	Contact	–/–	–/–	–/–	–/–	–/–	–/–
	14	Contact	–/–	–/–	–/–	–/–	–/–	–/–
	15	Contact	–/–	–/–	–/–	–/–	–/–	–/–
	16	Contact	–/–	–/–	–/–	–/–	–/–	–/–
	17	Contact	–/–	–/–	–/–	–/–	–/–	–/–
	18	Contact	–/–	–/–	–/–	–/–	–/–	–/–
	19	Contact	–/–	–/–	–/–	–/–	–/–	–/–
	20	Contact	–/–	–/–	–/–	–/–	–/–	–/–

[a] The bird died.

Table 4
Serological response of vaccinated contact-exposed chickens after two vaccinations (Experiment 2). Blood samples were gathered at moment of challenge and at the end of the experiment. The titer is expressed as twofold dilution that caused inhibition of haemagglutinin (HA) (2×). See Table 2 for details.

Bird	At challenge		End of the experiment	
	H5N1	H5N2	H5N1	H5N2
1	7	10	7	10
2	5	10	mv	mv
3	7	10	8	10
4	7	10	8	10
5	7	10	6	9
6	8	10	10	10
7	9	10	7	7
8	8	9	8	8
9	5	9	6	6
10	4	9	8	8

mv: missing value.

from unvaccinated to unvaccinated birds has decreased to at least 0.96 day^{-1} from unvaccinated to vaccinated birds. Hence, we may conclude that the vaccine reduces the susceptibility of birds at least $(1 - 0.96/8.0) \times 100\% = 88\%$.

4. Discussion

The aim of this study was to determine whether vaccination of native chickens with H5N2 vaccine could reduce disease rates and transmission of H5N1 virus. The main results of this study indicate that native chickens develop substantial HI antibody titers directed against H5N1 upon vaccination with a heterologous H5N2 vaccine, and that this provides a level of protection that is generally sufficient to prevent a productive H5N1 infection. Moreover, our results provide a proof-of-principle that vaccination with a heterologous vaccine can reduce transmission levels of H5N1 influenza virus to the extent that no epidemics can occur. In addition, the unvaccinated native chickens were not resistant to infection, and showed signs of infection that are comparable to layer chickens infected with H5N1 virus [21,26]. Finally, our results reaffirm that H5N1 virus spreads both rapidly and extensively among unvaccinated chickens.

The experiments in this paper were conducted under standardized conditions; the chickens were hatched and raised under laboratory conditions, and the vaccines could be applied under optimal conditions. Conditions in the field are almost surely less favourable. This could negatively affect the results, although there are reports of successful vaccination campaigns [27]. HI titers obtained under experimental conditions are usually higher than in the field, which has not only been observed for AI in native chicken, but also for AI in commercial flocks [19], and also for other viral diseases like Newcastle disease [28]. Various explanations have been given for these observations, such as inadequate vaccination practices, suboptimal storage conditions for vaccines, and concurrent diseases [29,30]. As it is rather difficult to locate and catch all birds in a village, let alone apply vaccination more than once, a suboptimal vaccination program seems the most likely explanation for the low vaccination coverage found in Indonesia.

Although we demonstrated that vaccination with an inactivated heterologous H5N2 vaccine is able to reduce transmission to the extent that no epidemics can occur, it is also true that we have tested only one challenge strain and one experimental vaccine. It is conceivable that other virus/vaccine combinations may be less effective, especially if the match between virus and vaccine is antigenically poor [12,31]. In this respect it is interesting to notice that the vaccine used in the current study was already substantially different from the Legok strain used for challenge with a protein homology between the two of only 92% [32]. This indicates that cross-protection even between different subtypes may be expected. This seems to be consistent with findings of others [23,26,33,34] who demonstrated protection against H5N1 after vaccination with heterologous vaccine with respect to the neuraminidase.

HI titers were determined using the homologous H5N2 antigen as well as a heterologous H5N1 antigen of the circulating virus. Not surprisingly, after vaccination antibody titers were higher with the H5N2 antigen as compared to the H5N1 antigen. Nevertheless, H5N1 antibodies titers formed were sufficient to give protection against disease and transmission. Similar results were reported by Van der Goot et al. [23]. In addition, native birds developed HI titers similar to those of SPF layer chickens after two vaccinations [21]. Of course, these experiments had not been carried out simultaneously, as the aim of the current study was to determine vaccine efficacy in groups of native chicken. Nevertheless, our findings are an indication that native chicken are able to respond to vaccination as the HI antibody titers were higher than 1:25, which are believed to be sufficient for protection [18], and can be protected to transmission of H5N1 in an experimental setting comparable to layer type chickens.

We carried out two experiments, of which the first was carried out according to a standard protocol and consisted of groups of birds which were homogeneous with respect to vaccination status [35,36]. The second experiment contained a group of birds that was heterogeneous with respect to the treatment, as the inoculated birds were unvaccinated and the contact birds were vaccinated. This experiment also provided useful information as the vaccinated contact birds did not become infected despite exposure to the high amounts of virus shed by the unvaccinated birds. This finding is relevant for monitoring programs that are based on the use of unvaccinated sentinel birds [6]. Farmers are often reluctant to accept unvaccinated sentinel birds in their flocks, as they are considered to be a risk for virus introduction and propagation [19]. Our findings suggest that the risk of sentinels acquiring and spreading the infection in a properly vaccinated flock is low.

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Human care of animals: This study contributes to a better control strategy for HPAI in Indonesia and possibly other countries in Asia. Although the infection with the HPAI H5N1 strain caused symptoms of AI and high mortality rates in unvaccinated birds used as controls in this vaccine trial, we were of the opinion that the trial was justified.

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