TOWARDS AN IMPROVED VACCINATION PROGRAMME AGAINST HIGHLY PATHOGENIC AVIAN INFLUENZA IN INDONESIA

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Towards an improved vaccination programme against highly pathogenic avian influenza in indonesia

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Towards an improved vaccination programme against highly pathogenic avian influenza in Indonesia

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Contents

CHAPTER 1

General Introduction

Avian Influenza

Avian influenza (AI) is a highly contagious disease caused by influenza A virus, a member of the *Orthomyxoviridae* family (Lupiani & Reddy 2009, Alexander & Brown 2009). AI virus strains can infect several bird species, but wild water fowl are considered to be a reservoir. The virus is characterized by surface glycoproteins, of which seventeen haemagglutinin (HA1-HA17) and ten neuraminidase (NA1-NA10) subtypes are distinguished (Alexander 2007, Yee *et al* 2009, Tong *et al* 2012). Based on the ability to cause clinical signs and mortality in domestic poultry species (chicken, quail, and turkeys), AI virus is divided into two different pathotypes: low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) (Alexander 2000, Swayne 2007, Yee *et al* 2009). HPAI strains can be distinguished from LPAI strains based on the intra-venous pathogenicity index (IVPI) of > 1.2, and on the sequence of amino-acids at the cleavage site (Alexander 2000, Swayne 2007, Capua 2007, Alexander 2007, OIE 2012).

All HA and N subtypes can be found amongst LPAI strains, but currently known HPAI viruses belong to the H5 and H7 subtypes (De Jong & Hien 2006, Capua & Alexander 2009), although some other subtypes, among others H6, H9 and H10 (Alexander 2003), have been demonstrated to cause substantial clinical signs as well (Bano *et al* 2003). None of these strains (H6, 9 or 10), however, cause high mortality rates in poultry flocks or have an IVPI indices of > 1.2 (OIE 2012). It is generally assumed that HPAI strains originate from a LPAI virus strain, after mutation of amino-acids at the cleavage site (Alexander 2000, Banks *et al* 2001, Capua & Alexander 2009)

In poultry, LPAI infections are associated with localized infection in the upper respiratory tract, usually characterized by mild clinical signs, and/or production losses (Alexander 2000, Capua & Marangon 2007). Infection of poultry with a HPAI strain, however, is associated with devastating effects, with severe clinical signs, and high mortality rates (Capua & Marangon 2007). Therefore, both LPAI and HPAI caused by H5 and H7 strains are notifiable according to the World Organization for Animal Health (OIE) (OIE 2012). Infection of other bird species with HPAI strains may run a subclinical course (Alexander 2000).

In 1996, a huge epidemic of HPAI virus subtype H5N1 started in Asia. The first isolate came from a goose in Guangdong, China. The virus subsequently caused outbreaks in poultry in Hongkong (Xu *et al* 1999, De Jong & Hien 2006). These outbreaks were controlled by stamping-out infected poultry. The infection re-emerged, however, in 2002, followed by

spread of the virus to other Asian countries like China, Indonesia, Japan, Lao, South Korea, Thailand, Vietnam, and Cambodia, in 2003 and 2004 (Sims *et al* 2003, Eagles *et al* 2009, Yee *et al* 2009). The virus also spread across the continent to some European and African countries (Alexander & Brown 2009, WHO 2006). The virus has been eradicated in Europe, and several countries in Asia, but the virus is still present in Egypt, and some Asian countries like Indonesia, Vietnam and China (Hinrichs *et al* 2010, Swayne *et al* 2011, Sims 2012, OIE 2013).

The outbreaks of HPAI subtype H5N1 has attracted large attention, as this strain appeared to be virulent not only for poultry and other birds species, but also for mammals i.e. domestic cats, ferrets, mice and also wild animals such as tigers and leopards (Keawcharoen *et al* 2004, Webster *et al* 2006, Alexander & Brown 2009). Moreover, also humans became infected, with a case fatality rate (WHO 2011). Up to now, approximately 637 cases have been reported and 378 patients died due to infection with H5N1 (WHO 2013). Although spread between humans has been limited so far, the virus may mutate into a variant that is more transmissible among humans, leading to a pandemic (WHO 2013, Fouchier *et al* 2012, Imai *et al* 2012, Horimoto & Kawaoka 2001).

Highly Pathogenic Avian Influenza H5N1 virus in Indonesia

As mentioned before, the H5N1 virus strain also spread to Indonesia. HPAI H5N1 infections among poultry in Indonesia were first detected in Banten province, Java island in 2003, and subsequently spread to other parts of the country. Outbreaks caused huge economic losses to the poultry industry and since 2003 Indonesia has become endemically infected (OIE 2012, FAO 2011a, Domenech *et al* 2009). In January 2004, HPAI H5N1 outbreaks were officially announced in 11 provinces, causing the death of approximately 20 million birds (Naipospos 2005, Sedyaningsih *et al* 2007, Lam *et al* 2008, Domenech *et al* 2009). Up to 2012, HPAI H5N1 has been detected in poultry in 32 of 33 Indonesian provinces (DGLS 2012). The first human cases of HPAI H5N1 in Indonesia causing death were reported mid 2005 (WHO 2011), and Indonesia has faced the highest incidence of human cases worldwide. Until June 2013, 193 human cases have been reported, of which 161 died (DGLS 2013, WHO 2013).

The poultry industry, producing meat and eggs, is an important branch of the agricultural business. The poultry population in Indonesia is estimated to consist of approximately 1.5 billion birds, including the industrial sector, small scale commercial farms and free-ranging village chickens (Siregar *et al* 2007). Several production types and poultry

species are present in Indonesia: layer chickens, broilers, native chickens, quails, ducks, pigeons, and geese (Ilham & Iqbal 2011).

Table 1. Characteristic of Indonesian poultry production system

Characteristic	Commercial production system			
	Industrial & integrated	Biosafety level		Backyard farming
		High	Low	
	Sector-I	Sector-2	Sector-3	Sector-4
Type of production	Integrated	Contact farmers	Independent	Backyard
Biosafety	High	Moderate-high	Low	Low
Selling products	Export/urban	Urban/rural	Urban/rural	Rural/urban
Dependence for market input	High	High	High	Low
Dependence on good road transport	High	High	High	Very low
Location	Close to large cities	Close to large cities	Small town and rural	Rural
Housing system	Indoor	Indoor	Half open	Open
Type of shed	Closed	Closed/open	Closed/open	Open
Contact with other chickens	No	No	Yes	Yes
Contact with ducks	No	No	Yes	Yes
Contact with wild birds	No	No	Yes	Yes
Veterinary services	Own veterinarian	Fee for service	Fee for service/government	Government
Source of drugs and vaccine	Market	Market/contract farmers	Market/government	Government
Source of technical information	Contract farmers/ company/ associates	Sellers of input	Sellers of input	Government
Poultry population (estimation in percentage)	3.5%	21.1%	11.7%	63.6%

Adapted (Naipospos 2005, FAO 2011a)

Within the poultry industry several levels of biosecurity are distinguished by the Food and Agricultural Organization (FAO): (i) sector-1 are the industrial, integrated farms with high biosecurity levels, birds/products marketed commercially (e.g. farms that are part of an

integrated broiler production enterprise); (ii) sector-2 consists of non-integrated farms with moderate to high biosafety measures implemented, birds/products marketed commercially (e.g. farms with indoor housing to prevent contact with other poultry or wildlife); (iii) sector-3 farms are small and medium scale farms with minimal to low biosafety, birds/products entering live bird markets (e.g. caged layer farm with birds in open shed); and (iv) sector-4 contains backyard poultry with no or low to minimal biosafety, birds/products consumed locally (FAO 2011a). Characteristics of the Indonesian poultry production system are summarized in Table 1.

HPAI control programme in Indonesia

After the first outbreaks in 2003, the Indonesian government implemented veterinary intervention by instigating culling, enhancing biosecurity, and vaccination of all poultry to control the disease (Yupiana *et al* 2010). In 2004, the government established a 'nine points' plan strategy on eradicating HPAI H5N1 including: (1) improvement of biosecurity, (2) selective culling/ infected flock depopulation, (3) vaccination of poultry in infected and high risk areas,(4) restriction of movements of poultry and their products, (5) surveillance and tracking back, (6) public awareness (7) restocking (8) stamping out of poultry in newly infected areas , and (9) monitoring and evaluation (DGLS 2009, Dharmayanti *et al* 2011).

The continuing occurrence of outbreaks of HPAI in poultry indicated, however, that that strategy was not successful, most likely because of insufficient application of the control measures. For example, culling of poultry in infected flocks and in-contact farms was stopped, and ring vaccination around outbreaks was poorly implemented and also discontinued. The reasons for this inconsistent application of measures were insufficient local capacity, a poor veterinary infrastructure, and lack of financial commitment (Azhar *et al* 2010). In addition, the cultural diversity in Indonesia led to ineffective risk communication and to inadequate responses to HPAI outbreaks. Finally, information from sector-1 and -2 farms was very limited, because the competent authority tend to be less assertive or reluctant to acces these farms, implying that control measures on these farms were solely implemented by the farm manager and the efficacy of these measures was rarely evaluated by the government.

From January 2006 to June 2009, the government initiated the participatory disease surveillance and response (PDSR) programme, based on the experience with foot- and mouth disease (FMD) and Rinderpest in other countries (Perry *et al* 2009, FAO 2011b) in an attempt

to control HPAI in sector-4 across Bali, Java, and parts of Sumatra (Azhar *et al* 2010, Loth *et al* 2011). Implementing surveillance systems only, however, will not result in disease control, if control measures are not taken after the report of an outbreak. Therefore, also this programme did not seem to be successful, as the virus is still present in the country, and alternative approaches are required (Sims 2012).

Avian influenza vaccination programme in Indonesia

Vaccination against viral diseases has been an effective tool to control various infectious diseases, such as FMD, but also for the control of outbreaks caused by LPAI virus strains in Italy (Marangon *et al* 2003, Ellis *et al* 2004, Capua 2007). As stated before, the control measures like stamping-out were insufficiently effective in Indonesia, and the disease has become endemic, a situation that would not allow large-scale culling programmes, due to infrastructural (logistical) problems and food security. Therefore, vaccination was included as an additional tool in the control of AI, not only in Indonesia, but also in China and Vietnam (Capua & Marangon 2004, Suarez 2005, Swayne 2006, Sims 2007, Capua & Marangon 2007, Domenech *et al* 2009).

Vaccination was rapidly applied on sector-1 farms with a variety of vaccines, either locally produced or imported. However, this vaccination programme was not coordinated by the Indonesian government (Domenech *et al* 2009). A policy of vaccination was officially introduced by the government in June 2004 as part of the 'nine point' strategy (Siregar *et al* 2007, Indriani *et al* 2011). Vaccine and vaccination services were provided free of charge to owners of backyard poultry and to small-scale sector-3 farmers. Farmers on large commercial sector-1 and -2 farms continued applying their own vaccination programme. Initially, three inactived H5 vaccine strains were used : H5N1, H5N2, and H5N9. The vaccines were locally produced or imported (Peyre *et al* 2009, Indriani *et al* 2011). But most vaccines used in governmental programme were vaccine contained H5N1 or H5N2 strain (Siregar *et al* 2007, Domenech *et al* 2009). The vaccination seem to have reduced morbidity and production losses in the sector-1 farms, although outbreaks still occured in commecial flocks (Domenech *et al* 2009; Bouma *et al* 2008).

Various explanations can be given for the continuing occurrence of outbreaks: e.g. poor biosafety during administration of vaccine resulting in incursion of virus before the vaccine might be effective; improperly applied vaccines or poor or reduced quality of vaccines, low vaccination coverage or low antibody titers (Bouma *et al* 2008, Siregar *et al*

2007, Sims 2007, Swayne *et al* 2011); chicken breed; type of poultry production; the presence of other (immunosuppressive) diseases at time of vaccination, mismatching between vaccine seed strain and field virus due to antigenic variants, and chold chain problems (Peyre *et al* 2009, Domenech *et al* 2009). It has been mentioned rather often that with vaccination alone eradication will not be achieved, and that vaccination should be accompanied by other control measures (Suarez 2005, Alexander 2007, Capua 2007, Swayne 2009, Peyre *et al* 2009). As these are hardly applied in an appropriate manner, it can be expected that the virus will not be eradicated soon.

Since 2003, antigenic variants of H5N1 have emerged (Domenech *et al* 2009, Dharmayanti *et al* 2011, Lam *et al* 2012). These strains may have been circulating unnoticed rather long, as a proper surveillance system to detect and characterize isolates was, and still is, missing in Indonesia. Moreover, due to vaccination, clinical signs may be absent or less severe, which could also cause unnoticed spread of virus within flocks. The emergence of new strains may also explain part of the recently observed suboptimal efficacy of vaccination, as some of the AI vaccines do not give sufficient protection against these recently isolated H5N1 isolates (Swayne 2006, Eagles *et al* 2009, Indriani *et al* 2011). Nowadays, local Indonesian vaccine companies produce inactivated vaccines with four new strains recommended from FAO Network of Expertise on Avian Influenza (OFFLU) project (Asmara 2011).

The control strategy in Indonesia should be improved, especially the vaccination programme, not only to protect poultry against clinical signs and mortality, but also to focus on eradication of the infection and to reduce human health risks. More knowledge about the efficacy of vaccination in Indonesian circumstances should therefore be gained. Epidemiological research provides more insight in this matter. From observational studies, factors that may contribute to the poor vaccination efficacy may be identified, but it cannot be determined from this type of study whether they really contribute or not. Moreover, this type of research appeared to be rather difficult to perform in Indonesia (Bouma *et al* 2008). Experimental studies have the advantage that the effect of one single factor on the outcome can be determined..

The first transmission experiment which was carried out with layer type chickens to determine whether vaccines produced by Indonesian manufacturers were effective at all (Bouma *et al* 2008). Under experimental conditions, the vaccines were indeed effective as they reduced clinical signs and prevented transmission to in-contact chickens. As stated before, outbreaks of AI are being reported in Indonesia, mainly in sectors-3 and -4, but it

should be noted that it cannot be determined what the relative number of outbreaks is in comparison to the number of outbreaks in sectors-1 and -2, due to lack of information. Nevertheless, the efficacy of vaccination in other chicken types should be determined. In addition, the effectiveness of vaccination applied under field conditions should be studied, as vaccines might be applied inappropriately or less adequately in comparison to an experimental setting. Moreover, under field conditions chickens may have maternally derived antibodies, which most likely interfere with the efficacy of vaccination. The focus of this thesis is on transmission experiments in which the efficacy of vaccination was quantified.

Scope of the thesis

Native chickens in backyard flocks are often considered to be at risk for HPAI infection by exposure to wild birds or by spill-over from large commercial flocks nearby, because of a lack of biosecurity measures. Moreover, it has been suggested that native chickens do not respond with a proper immune response upon vaccination (Siregar *et al* 2007). They are also blamed being the source of repeated introductions into commercial poultry flocks, and it was suggested that this bird type is less susceptible subclinically infected. These statements were in conflict with each other and, moreover, no scientific evidence has been provided to substantiate these suggestions. Therefore, it was investigated whether these native chickens were susceptible for infection at all, whether they could be protected against infection by vaccination and whether they might become silent shedders (Chapter 2).

Another hypothesis that has been raised every now and then was that broilers were a source for virus circulation. Broilers are usually not vaccinated, because of short life span of broiler production system, which makes vaccination a relative expensive control tool. Moreover, vaccination was expected to be less effective, as the birds usually have maternally derived antibodies against AI, due to repeated vaccination of the parent stock. Broiler flocks could, once infected, contribute to the persistence of virus in an area. Moreover, as these birds are often transported to collector houses in cities, and live bird markets, they may be a risk for human health (Santhia *et al* 2009, Indriani *et al* 2010). In Chapter 3, the efficacy of vaccination in broilers was therefore determined.

The level of herd immunity is often determined based on the level of haemagglutinin inhibiting antibodies (HI). If the percentage of birds with a high titer is too low, or if the HI titer is too low, revaccination is applied. It is however, unknown which HI titer is protective

against spread of virus. In Chapter 4, the association between HI titer and probability of infection was quantified.

Although the virus is endemic and rapid emergency vaccination is not needed, it might be more efficient to develop simpler application methods to apply vaccination in large flocks, also in Indonesia. Nowadays, vaccines are applied manually by injection of each individual bird. In Chapter 5 it was determined whether spray vaccination was effective, as this might provide a better way to apply mass vaccination.

Outbreaks of AI in vaccinated flocks may also be caused by antigenic drift. Influenza is well known for its rapid genetic changes, and this antigenic drift requires frequent updating of the vaccines. Whether or not 'old' vaccines are still effective against newly emerged virus strains should however be determined. It would be convenient to have an *in vitro* variable that could predict vaccine efficacy against certain wild type strains. In Chapter 6, it was determined whether a particular vaccine strain could protect chickens against a heterologous wild type strain, and whether the *in vitro* determined HI titer was indicative for estimating the level of protection against infection. The results from the various studies described in Chapters 2-6 are discussed in Chapter 7.

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CHAPTER 2

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 three 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 ten 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.

Introduction

Avian Influenza (AI) is a highly contagious viral disease caused by type A influenza virus which are not only able to infect humans, but also a wide variety of avian species (Perdue *et al* 2005, Fouchier *et al* 2005, Park *et al* 2007, Alexander 2000, Alexander 2007). Strains of the H5 and H7 subtypes are notifiable to the World Organization of Animal Health (OIE 2007), and highly pathogenic strains of these subtypes can cause severe clinical signs in poultry which may result in mortality that ranges up to 100% (Alexander 2003, Elbers *et al* 2004, Bos *et al* 2007).

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 (OIE 2007, Alexander 2007). One of the countries severely hit is Indonesia, where the poultry industry faced outbreaks since 2003 (OIE 2007). Infections with H5N1 strains not only resulted in production losses and high mortality in poultry but the virus also infected humans (WHO 2009). 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 (WHO 2009).

The virus is considered to be endemically present in the poultry population in Indonesia, and control efforts mainly make use of vaccination (Capua 2007, Sawitri *et al* 2007). Commercial farmers with high biosecurity standards (sectors 1 and 2 (FAO 2007)) 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 (FAO 2008). Most of these outbreaks occur in small back yard flocks with native chicken, classified as sector 4 (FAO 2008). Native chickens in back yard flocks are generally considered to be at high risk for AI virus introduction from migratory birds (Sedyaningsih *et al* 2007), not only in Indonesia, but in other Asian countries as well (Tensin *et al* 2005). Moreover, many human cases in Indonesia are linked to contact with these native chickens (WHO 2009).

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 $\geq$ 1:25 (1:32) is low (Bouma *et al* 2008). It has been suggested that native chickens are low responders by nature (Sawitri *et al* 2007, Adjid *et al* 2007).

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 (Bouma *et al* 2008). Therefore, we carried out transmission experiments in which 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.

Materials and Methods

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.

Vaccine

A commercially available oil-emulsion adjuvanted vaccine (Vaksiflu N2®, PT. Vaksindo) was used containing 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.

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 and was able to induce infection of SPF layer chickens, which resulted in typical AI signs and high mortality (up to 100%) (Bouma *et al* 2009). The inoculum contained 106 EID50 per ml, a dose that had previously shown to induce 100% mortality of SPF chickens (Bouma *et al* 2009). The protein homology of the haemagglutinin (HA1) of the vaccine strain and the

challenge strain 92%. Virus titers were confirmed before and after inoculation by titration on embryonated SPF eggs according to standard operating procedures (OIE 2007). Each inoculated bird received 0.1 ml inoculum which was administered intratracheally. Inoculation was done when the birds were 10 weeks old.

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 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 meter width). The density of chicks was approximately 5.4/m2. 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, ten unvaccinated and ten 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 hours, these inoculated (*I*) birds were reunited 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 a 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 four weeks. The surviving birds were then killed by cervical dislocation.

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 -70ºC 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 -20ºC until testing.

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 (OIE 2007). 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 (OIE 2007). 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)). Two-fold dilutions of the serum samples were made, and titers were expressed as the serum dilution that caused complete inhibition of agglutination (OIE 2007).

Quantification of transmission

A stochastic SEIR (susceptible-exposed-infected and infectious-removed) model formed the basis for estimation of the epidemiological parameters of interest. The gist of the statistical analyses is given in Van der Goot *et al* (2005, 2008). 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 is it possible that a chain reaction of infections leading to an epidemic can occur (Diekmann *et al* 2000). Estimates of the transmission rate parameter and infectious period were obtained by maximum likelihood, assuming a period of latency of 1 day (Van der Goot *et al* 2005). 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 (Pawitan 2001).

Results

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 one or two 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 (4.2-28.7), which is substantially higher than the threshold value of 1. A graphical overview of the analyses is given in Fig. 1.

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.

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

C, contact chicken; I, inoculated chicken; †, chicken died; +/+ positive tracheal swab/positive cloacal swab; +/-, positive tracheal swab/negative cloacal swab; -/+, negative tracheal swab/positive cloacal swab; -/-, negative tracheal swab/negative cloacal swab.

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. Antibodies 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 four-fold increase. Therefore, increase in HI titers was not used to determine infection of contact-exposed birds.

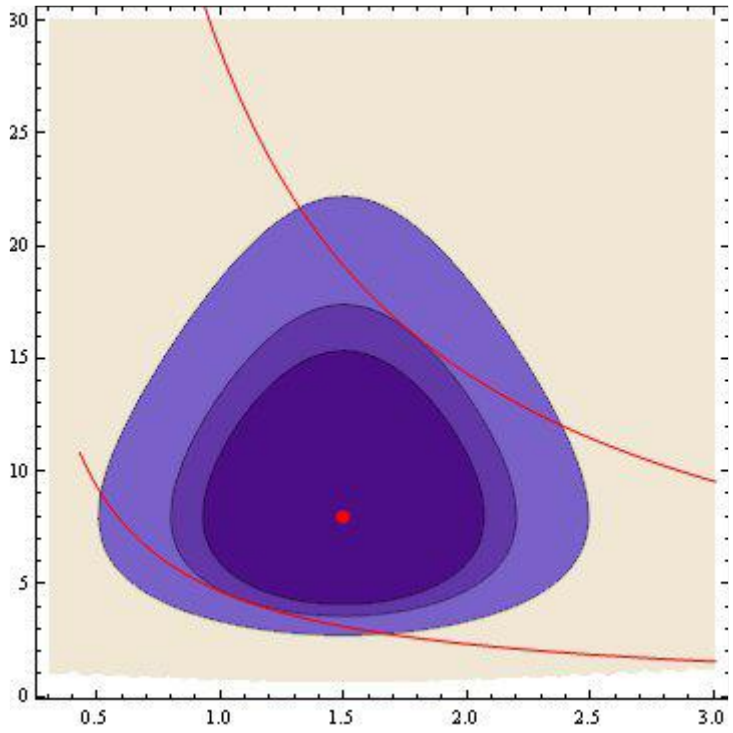

Figure 1. Estimates of the infectious period and transmission rate parameter (red dot) with associated 90%, 95%, and 99% confidence regions. The reproduction number is given by the product of the infectious period and transmission rate parameter. Lines indicate the lower and upper bound of the 95% confidence interval of the reproduction number.

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: day 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}) 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) x 100% = 88%.

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 two-fold dilution that caused inhibition of haemagglutinin (HA) (2x). 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 (2x)		end of the experiment (2x)	
		H5N1a	H5N2a	H5N1a	H5N2a
Inoculated bird	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 bird	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

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.

			Days after inoculation					
	Bird	Exposure	1	2	3	4	5	6
Unvacccinated birds	1	Inoculated	-	†				
	2	Inoculated	-	-	†			
	3	Inoculated	-	-	+/+	+/-	†	
	4	Inoculated	-	+/+	+/+	†		
	5	Inoculated	-	†				
	6	Inoculated	-	+/-	+/-	†		
	7	Inoculated	-	†				
	8	Inoculated	-	†				
	9	Inoculated	-	+/-	+/+	-	-	†
	10	Inoculated	+/+	†				
Vaccinated birds	11	Contact	-/-	-/-	-/-	-/-	-/-	
	12	Contact	-/-	-/-	-/-	-/-	-/-	
	13	Contact	-/-	-/-	-/-	-/-	-/-	-/-
	14	Contact	-/-	-/-	-/-	-/-	-/-	-/-
	15	Contact	-/-	-/-	-/-	-/-	-/-	-/-
	16	Contact	-/-	-/-	-/-	-/-	-/-	-/-
	17	Contact	-/-	-/-	-/-	-/-	-/-	-/-
	18	Contact	-/-	-/-	-/-	-/-	-/-	-/-
	19	Contact	-/-	-/-	-/-	-/-	-/-	-/-
	20	Contact	-/-	-/-	-/-	-/-	-/-	-/-

† 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 two-fold dilution that caused inhibition of haemagglutinin (HA) (2x). See Table 2 for details.

Bird	At challenge		End of experiment	
	H5N1	H5N2	H5N1	H5N2
1	7	10	76	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

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 (Van der Goot *et al* 2005, Swayne *et al* 2006). 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 (Ellis *et al* 2004). 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 (Bouma *et al* 2008), and also for other viral diseases like Newcastle disease (Kapczynski & King 2005). Various explanations have been given for these observations, such as inadequate vaccination practices, suboptimal storage conditions for vaccines, and concurrent diseases (Swayne & Pantin-Jackwood 2008, Swayne & Kapczynski 2008). As it is rather difficult to locate and catch all birds in a village, let alone apply vaccination more than once, a sub-optimal 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 (Capua 2007, Swayne 2007). 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% (NCBI 2008). This indicates that cross-protection even between different subtypes may be expected. This seems to be consistent with findings of others (Van der Goot *et al* 2008, Swayne *et al* 2006, Lee *et al* 2007, Bublot *et al* 2007) 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* (2008). In addition, native birds developed HI titers similar to those of SPF layer chickens after two vaccinations (Bouma *et al* 2009). 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 1:32, which are believed to be

sufficient for protection (Philippa *et al* 2005), 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 (De Jong & Kimman 1994, Velthuis *et al* 2007). 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 (OIE 2007). 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 (Bouma *et a*l 2007). 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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CHAPTER 3

A single vaccination of commercial broilers does not reduce transmission of H5N1 highly pathogenic avian influenza

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Abstract

Vaccination of chickens has become routine practice in Asian countries in which H5N1 highly pathogenic avian influenza (HPAI) is endemically present. This mainly applies to layer and breeder flocks, but broilers are usually left unvaccinated. Here we investigate whether vaccination is able to reduce HPAI H5N1 virus transmission among broiler chickens. Four sets of experiments were carried out, each consisting of 22 replicate trials containing a pair of birds. One chicken in each trial was inoculated with virus. The course of the infection chain was monitored by serological analysis, and by virus isolation performed on tracheal and cloacal swabs. The analyses were based on a stochastic SEIR model using a Bayesian inferential framework. When inoculation was carried out at the 28th day of life, transmission was efficient in unvaccinated birds, and in birds vaccinated at first or tenth day of life. In these experiments estimates of the latent period (~1.0 day), infectious period (~3.3 days), and transmission rate parameter (~1.4 per day) were similar, as were estimates of the reproduction number (~4) and generation interval (~1.4 day). Transmission was significantly less efficient in unvaccinated chickens when inoculation was carried out on the first day of life. These results show that vaccination of broiler chickens does not reduce transmission, and suggest that this may be due to the interference of maternal immunity.

Introduction

Infection of poultry with highly pathogenic avian influenza (HPAI) virus strains invariably results in high mortality rates and substantial economic losses (Bean *et al* 1985, Capua & Marangon 2006, Stegeman *et al* 2010, Swayne *et al* 2006, Swayne & Pantin-Jackwood). It is now almost 15 years ago that the first outbreaks of highly pathogenic H5N1 viruses were reported in South East Asia. Since then, the disease has become endemic in some countries (OIE 2011). Outbreaks caused by infection with H5N1 viruses resulted in the death of millions of birds either from the disease, or by culling. In addition, hundreds of human infections, including 306 fatal ones, have been reported (WHO 2011).

Upon detection of an outbreak in commercial poultry, a set of control measures, including culling of infected flocks, is implemented (Capua & Marangon 2006, Alexander 2006, Stegeman *et al* 2004). Eradication of the H5N1 virus from poultry has been successful in some countries, but in Egypt and Indonesia the virus seems to have become endemic (OIE 2011, Gilbert *et al* 2008, Lee *et al* 2008, Sedyaningsih *et al* 2007). In some of these endemically infected countries vaccination of breeders and layer hens has become a widely used containment strategy that has met with variable success (Capua & Alexander 2008, Peyre *et al* 2009, Sawitri *et al* 2007, Swayne & Kapczynski 2008). In Indonesia, vaccination is widely applied, but it is unclear what the epidemiological situation is, as no official data are available for commercial flocks (FAO 2011). Despite vaccination, it is believed that outbreaks in commercial flocks continue to occur. An indication for this is that on several poultry collecting facility houses in Jakarta, where spent layers and broilers were collected shortly before slaughtering, H5 virus was isolated (WHO 2011, CIVAS 2011). Another indication is the observation of an H5N1 outbreak in a vaccinated commercial layer flock (Bouma *et al* 2008).

The situation has not improved in Indonesia since the incursion of the virus in 2003 (Sims 2007, OIE 2011), and additional control options are urgently needed. Large-scale culling does not seem an option, and, therefore the vaccination strategy in endemically infected countries needs improvement. A large part of the poultry industry in Indonesia consists of broiler flocks, which are generally not vaccinated. The main reasons for the non-vaccination strategy are the costs of vaccination, and the assumed ineffectiveness of vaccination of broilers because of the interference of maternally derived antibodies (MDA) with a vaccine-induced immune response (Swayne & Kapczynski 2008).

To be able to make an informed decision on whether or not vaccination of broilers is useful in the control of HPAI H5N1, and to investigate whether broilers were able to transmit the infection at all, we investigated the effect of vaccination on virus transmission. Focus was on key epidemiological parameters such as the transmission rate parameter, the infectious period, the generation interval, and the reproduction number (De Jong & Kimman 1994, Van der Goot *et al* 2005, Velthuis *et al* 2007). Estimation of these parameters in the field is possible, but difficult even in endemically infected areas (Bouma *et al* 2008). Experimental transmission studies offer the opportunity to quantify these parameters under well-controlled conditions (Stegeman *et al* 2010, De Jong & Bouma 2001). Here we present the results of four sets of experiments with HPAI H5N1 virus strain A/Chicken/Legok/2003 in broiler chickens, to estimates epidemiological parameters, and to determine the effect of a single vaccination on transmission and clinical signs.

Materials and methods

Experiments were carried out in the high containment unit at PT. Medion, Bandung, Indonesia. Four experiments were done each with 22 replicates. Each replicate consisted of one pair of broilers. Experiment 1 was carried out with unvaccinated birds, experiment 2 with birds vaccinated at day 1 of age, and experiment 3 with birds vaccinated at day 10. Experiment 4 consisted of progeny obtained from the same flock, but birds were day-old at time of challenge (see below).

Animal and housing

Approximately 200 18-day-old embryonated eggs were purchased from a commercial breeder farm. The breeders were vaccinated several times against H5N1 with Medivac® (PT Medion, Bandung, Indonesia), containing H5N1 virus strain A/chicken/Legok/2003. At time of purchase of the eggs, no clinical signs of AI in the breeders were reported, indicating the absence of HPAI H5N1 virus at that time.

After hatching at the facilities, day-old chicks were housed in one experimental unit. They were fed with a commercial ration, and had tap water ad libitum. Four groups were formed each consisting of 44 birds for each experiment one. Three groups (experiments 1-3) were of the same age at purchase and challenge and were used to determine the efficacy of vaccination. A fourth group (experiment 4) consisted of day-old chicks (DOC), and were

challenged to determine whether virus could be transmitted amongst DOC. Challenge was done when birds in experiments 1-3 were 28 days old.

One week before challenge birds from experiments 1-3 were moved to the experimental units. Two rooms were available, each with two rows with cages on three levels. Birds in experiments 2 and 3 were housed together in Unit 1 and birds in experiment 1 were housed together with those in experiment 4 in Unit 2: each experimental group at opposite sides of the corridor in each house. In each cage one pair of birds from the same experiment was housed. The cages between each experimental pair were empty. Sentinel birds were placed in empty cages in the middle level below each pair in the upper level. Sentinels were SPF layers, from the SPF unit of PT Medion, and were not older than the experimental birds. Sentinels were used to monitor between-cage virus transmission. The experiments lasted four weeks after inoculation, when the surviving birds in experiments 1-3 were 56 days old.

Vaccine

An inactivated oil-emulsion vaccine was used, which contained the H5N1 strain A/Chicken/Legok/2003 (Medivac®, PT Medion, Bandung, Indonesia) (Bouma *et al* 2009). The vaccine was administrated intramuscularly in one leg using 0.5 mL containing 256 HAU per dose per bird. Chickens in experiments 1 and DOC in experiment 4 remained unvaccinated; chickens in experiment 2 were vaccinated at one day of age; the birds in experiment 3 were vaccinated at 10 days of age.

Inoculation

The HPAI virus strain H5N1 A/Chicken/Legok/2003 was used for challenge. The strain was provided by PT. Medion Bandung Indonesia. The virus has been used in other transmission experiments, and was able to induce clinical signs and transmission (Bouma *et al* 2009, Poetri *et al* 2009). At day of challenge, when the birds in experiments 1-3 were 28 days old and in the DOC experiment (experiment 4) were 1 day old, one bird per pair was inoculated intranasally and intratracheally with 0.2 mL inoculum containing 106/mL median egg infectious dose (EID50) (Bouma *et al* 2009). Before inoculation each bird that had to be inoculated was put in the empty cage near its pen mate. Eight hours after inoculation, they were placed back in their original cage.

Transmission experiments

Throughout, we refer to each experimental pair of chickens as a trial. Each experiment consisted of 22 replicate trials, and in each trial one inoculated bird (*I*) was placed in a cage with an uninfected contact bird (*C*). Both birds had received the same treatment, and were of the same age.

Transmission of virus was monitored by taking daily swab samples from the trachea and cloaca from all birds for 10 days. From birds that survived this sampling period, additional samples were taken at day 14 after challenge. The samples were stored at -70 ºC until further testing. Serum blood samples were taken from surplus birds at day of hatch to determine the level of maternally derived antibodies. From the experimental birds, serum blood samples were taken two days before challenge and four weeks after challenge, at the end of the experiment. Sera were stored at -20 ºC until further testing. Clinical signs were recorded during four weeks after challenge.

The treatment is referred to as: unvaccinated (experiment 1), d1 vaccinated (experiment 2), d10 vaccinated (experiment 3) and DOC (experiment 4). Figure 1 gives an overview of the experimental data from days 0 to 10 after challenge. Additional samples were taken from infectious birds that were still present at day 10 of the experiment (not shown). The complete dataset is available on request from the corresponding author. All experiments were carried out in accordance to article 80 on "Research in Animal Health" of the Indonesian "Law on Livestock and Animal Health UU/18/2009".

Tests

The presence of AI virus in swabs was determined by virus isolation according to standard procedure described by OIE (2009). Briefly, three SPF embryonated chicken eggs, incubated for nine 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. When at least one of the eggs was positive in the HA, the swab was considered to be positive. The test results were recorded as positive for AI virus or negative (Bouma *et al* 2009). A bird was considered infected if at least one sample (either tracheal or cloacal) tested positive at least once.

Serum samples were tested in a haemagglutination inhibition (HI) test according to standard procedures (OIE 2009). It is generally assumed that HI titers $\geq$ 32 are protective against disease, i.e. clinical signs (Philippa *et al* 2005). Tests were carried out in duplo using 4 HAU of the strain A/chicken/Legok/2003(H5N1). Two-fold dilutions of the serum samples

were made, and titers were expressed as the serum dilution that caused complete inhibition of agglutination
(OIE 2009).

Quantification of transmission

A Bayesian inferential framework based on a stochastic SEIR (susceptible-exposedinfected and infectious-removed) epidemic model was used to obtain quantitative estimates of the parameters of interest (Bouma *et al* 2009, Höhle et al 2005, Steftaris & Gibson 2004a, Steftaris & Gibson 2004b). The methods of analysis have been described in detail earlier (Bouma *et al* 2009). Here we give a concise overview.

The main interest is in the transmission rate parameter, and parameters of the latent and infectious period distributions. Together, these parameters determine the basic reproduction number and the generation interval (Bouma *et al* 2009). We parameterize the latent and infectious periods using gamma distributions, and assume uninformative uniform prior distributions (*U(0.0001-100)*) for all parameters. To be precise, we characterize gamma distributions of the latent and infectious periods by their mean and variance (and not the shape and scale parameters), and assume uniform prior distributions for the mean and variance (and not the shape and scale parameters).

In the following β denotes the transmission rate parameter, γ_E and δ_E the parameters determining the latent period probability distribution, and γ_I and δ_I the parameters of the infectious period probability distribution. Specifically, $E[T_E]=\gamma_E$ and $Var[T_E]=\delta_E$, and $E[T_I]=\gamma_I$ and $Var[T_I]=\delta_I$ represent the means and variances of these distributions. The corresponding probability densities are denoted by $f_E(x)$ and $f_I(x)$.

Further, $\mathbf{e}_k$, $\mathbf{i}_k$, and $\mathbf{r}_k$ are *N*-dimensional vectors which contain the time points of the S→E, E→I, and I→R transitions for inoculated ($k=1$) and contact ($k=2$) birds in the *N* trials. Hence, we have $\mathbf{e}_1=(0,\ldots,0)^T$ by definition, and all other transition times are unknown. The unknown transitions are imputed. We adopt the convention that e_{2j} denotes the exact time at which the contact bird in experiment *j* is infected, that i_{1j} denotes the exact time that the inoculated bird in experiment *j* became infectious, etcetera.

As in (OIE 2009), the contribution of trial *j* to the likelihood is given by

$$L^{(j)}=\begin{cases}\lambda^{(j)}(e_{2j})S^{(j)}(e_{2j})f_E(i_{1j})\,f_I(r_{1j}-i_{1j})f_E(i_{2j}-e_{2j})f_I(r_{2j}-i_{2j}) & \text{if the contact bird was infected}\\ S^{(j)}(r_{1j})f_E(i_{1j})f_I(r_{1j}-i_{1j}) & \text{if the contact bird was not infected.}\end{cases} \quad (1)$$

In the above equation $\lambda^{(j)}(t)$ and $S^{(j)}(t)$ denote the infection hazard in trial *j* at time *t* and the probability that the contact bird in trial *j* remains uninfected up to time *t*, respectively. If we let $[\ldots]$ denote the indicator function, the infection hazard is given by

$$\lambda^{(j)}(t) = \frac{\beta}{2}\left[\max\left(t_{add}, i_{1j}\right) \le t < r_{1j}\right], \qquad (2)$$

where the parameter t_{add} represents the delay between the moment of inoculation and the moment that the inoculated birds were placed back in their cages. Hence, the function $\max\left(t_{add}, i_{1j}\right)$ marks the beginning of the at-risk period for the contact bird. In all trials and experiments, the delay is 8 hours, i.e. $t_{add} = 0.33$ (*day*). The probability that the contact bird in trial *j* remains uninfected up to time *t* can be expressed in terms of the infection hazard as follows

$$S^{(j)}(t) = e^{-\int_0^t \lambda^{(j)}(t')dt'}. \qquad (3)$$

With the above preparation at hand, the likelihood function is given by the product of the contributions of the individual trials given in Equation (1). The above equations are furthermore readily generalized to include differences in the epidemiological parameters of inoculated versus contact birds (Bouma *et al* 2009).

The epidemiological parameters and unobserved epidemiological transitions (i.e. S→E, E→I, I→R) were all updated by a random-walk Metropolis algorithm. We used Normal proposal distributions with the current value as mean. After running a number of analyses to explore the posterior distribution and optimize the proposal distributions, we used standard deviations of 0.02 for the epidemiological transitions, and 0.02-0.5 for the epidemiological parameters. The epidemiological parameters and unobserved transitions were updated in blocks, in the order (1) timing of inoculated chickens becoming infectious, (2) timing of removal of inoculated chickens, (3) timing of infection of contact chickens, (4) timing of contact chickens becoming infectious, (5) timing of removal of contact chickens, and (6) updating of the epidemiological parameters (26). Chains were run for 350,000 cycles, of which the first 100,000 cycles were discarded as burn-in. Thinning was applied by taking output from each twentieth cycle, yielding a sample of 12,500.

Below we report not only the basic epidemiological parameters (transmissibility, duration of the latent and infectious periods), but also the generation interval and basic reproduction number. The generation interval is defined as the moment of infection of the

contact bird relative to the moment of infection of the inoculated bird (i.e. it is given by e_{2j} in trial *j* if the contact bird was infected), while the basic reproduction number is defined as the product of the transmission rate parameter (unit: day^{-1}) and infectious period (unit: *day*).

Each of the Experiments 1-4 was analysed separately, assuming a common distribution of the latent period of inoculated and contact birds. Based on the results of the separate experiments, and given the observation that there may be differences between inoculated and contact birds, possibly due to differences in the inoculum size we also analysed the combined data of Experiments 1-3 while relaxing this assumption. Specifically, we allowed the mean of the latent period to differ between inoculated and contact birds, while assuming a fixed common variance (0.001) of the latent periods (Bouma *et al* 2009). Furthermore, the data of Experiments 1-3 were used to explore, by means of logistic regression, whether the probability of infection could be dependent on the immune status (i.e. HI titer) of the birds just prior to the experiments.

Results

Experiment 1 (no vaccination, challenge at day 28)

All inoculated chickens shed virus for 1-7 days (interquartile range: 2-5 days), and all inoculated chickens died 2-8 days post challenge (interquartile range: 3-6 days post challenge) (Table 1, Figure 1). Likewise, all but two of the contact chickens shed virus for 2 to more than 8 days. All virus-positive inoculated chickens showed clinical signs of highly pathogenic avian influenza (data not shown) and died, and 16 of 22 contact chickens died during the course of the experiments. At challenge none of the birds had a HI titer greater than or equal to 32, and only 1 of the surviving contact chickens had developed a significant HI titer (≥ 32) at the end of the experiment. The average HI titers are represented in Table 1.

The estimated transmission rate parameter (i.e. the median of the posterior distribution of the transmission rate parameter) is 1.6 per day (95%CrI: 0.97-2.4) (Table 2). This implies that the estimated per day probability of infection of an uninfected contact chicken by an infected inoculated chicken is $1 - \exp(-1.6) = 0.80$. The estimated mean of the infectious period (i.e. the median of the posterior distribution of the parameter determining the mean of the infectious period) is 3.2 days (95%CrI: 2.5-4.3), while the estimated mean of the latent period is 0.88 days (95%CrI: 0.70-0.94) (Table 2). Based on these estimates, the basic reproduction number and the generation interval are 5.1 (95%CrI:

3.0-8.4) and 1.5 days (95%CrI: 1.3-1.7), respectively. Figure 2 gives a graphical representation of the posterior distribution of the mean versus variance of the latent period, and of the infectious period and the mean infectious period versus the transmission rate parameter.

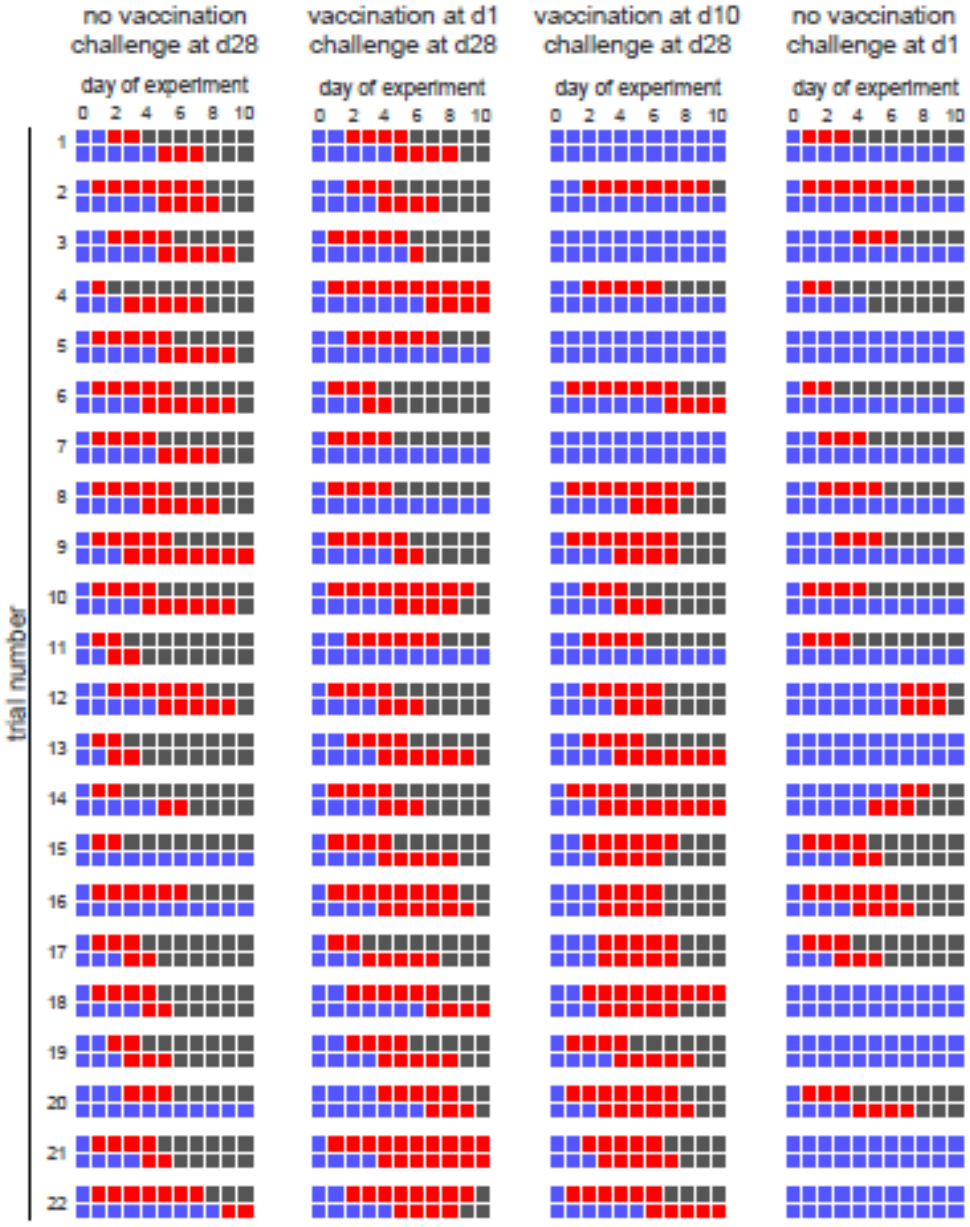


Figure 1. Overview of the transmission studies. Shown are for each of four experiments the experimental data of the 22 replicate trials. The top and bottom rows of each trial refer to the experimentally infected chicken and contact chicken, respectively. Blue squares denote chickens that tested negative, red square represent chickens that tested positive and black squares denote chickens that died or recovered after infection.

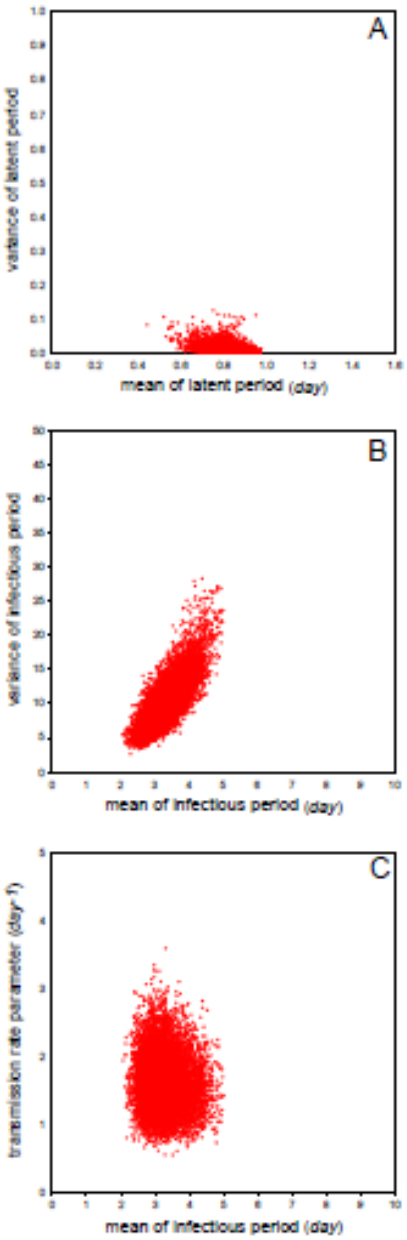


Figure 2. Overview of the analyses of Experiment 1 (no vaccination, challenge at day 28). Shown are samples from the marginal posterior density of the mean versus variance of the latent period (A), the mean versus variance of the infectious period (B), and the mean infectious period versus transmission rate parameter (C).

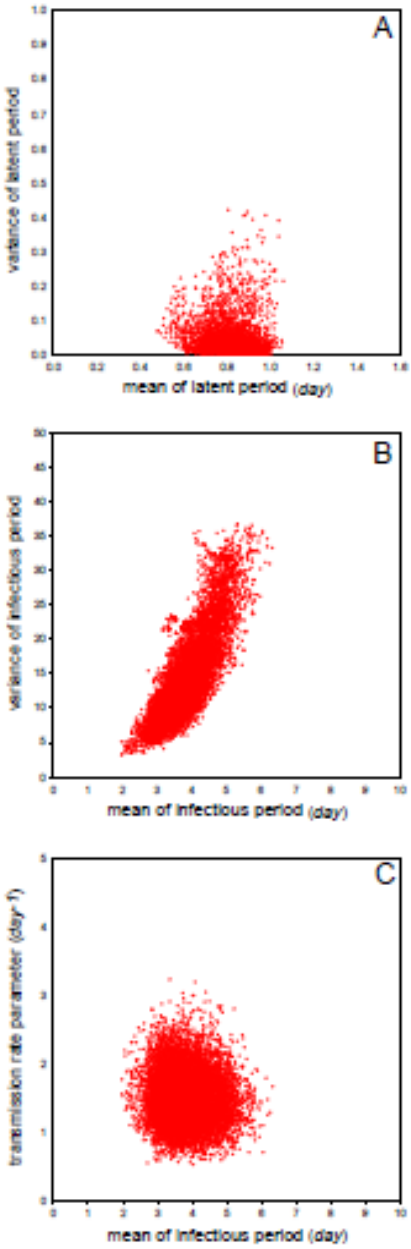


Figure 3. Overview of the analyses of Experiment 2 (vaccination at day 1, challenge at day 28). See Figure 1 for details.

Table 1. Overview of HI titers at challenge, virus isolation data, clinical symptoms, and mortality rates.

Exp.	Treatment	Mean HI titer (absolute (sd))		Number of birds with HI titer ≥ 32 at challenge		shedding virus[a]		with clincial symptoms		that died	
		at challenge	at end[d]	I[b]	C[b]	I	C	I	C	I	C
1	no vaccination challenge at d28 of age	1.6 (1.0)	206 (457)	0	0	22	19	22	22	22	16
2	vaccination at d1 challenge at d28 of age	1.7 (1.3)	166 (381)	0	0	22	18	19	16	16	15
3	vaccination at d10 challenge at d28 of age	1.8 (0.9)	251 (357)	0	0	18	15	16	10	13	7
4	no vaccination challenge at day 1 of age	nd[c]	1.0 (0.4)[f]	nd[c]	nd[c]	16	6	19	19	13	1

[a] total numbers in each group were 22 inoculated chickens and 22 contact-exposed chickens
[b] I: inoculated chickens; C: contact-exposed chickens
[c] not determined
[d] only from surviving and infected birds
e from surplus birds
[f] one bird had a titer of 1024, which is omitted from this average

Experiment 2 (vaccination at day 1, challenge at day 28)

All inoculated chickens shed virus, and 16 of 22 inoculated chickens died before the end of the experiment. Seventeen contact chickens shed virus, and 14 of 22 contact chickens died during the course of the experiments. At challenge none of the birds had a HI titer greater than or equal to 32; 4 of the surviving inoculated and none of the surviving contact chickens developed a significant HI titer (≥ 32) at the end of the experiment. The average HI titers are represented in Table 1.

The basic parameters of interest are very close to those of experiment 1 (no vaccination). Specifically, the estimated transmission parameter is 1.5 per day (95%CrI: 0.87-2.3), the mean of the latent period is 0.86 days (95%CrI: 0.69-0.96), and the mean of the infectious period is 3.7 days (95%CrI: 2.8-5.1) (Table 2). The basic reproduction number and

generation interval are also quite close to the estimates in experiment 1, *viz*. 5.5 (95%CrI: 3.1-9.3) for the reproduction number, and 1.4 days (95%CrI: 1.2-1.5) for the generation interval (Table 2). Figure 3 shows that although there is a striking overall agreement with the results of experiment 1 (Figure 2), there is also some evidence of greater variability in experiment 2 than in experiment 1, especially with respect to the variance of the latent and infectious periods.

Experiment 3 (vaccination at day 10, challenge at day 28)

Eighteen inoculated chickens shed virus, and 13 died of AI (Table 2, Figure 3). Likewise, 15 contact chickens shed virus and 15 of 22 contact chickens survived, including the 7 that did not shed detectable levels of virus. At challenge none of the birds had a HI titer greater than or equal to 32; 5 of the surviving inoculated and 8 of the contact chickens developed a significant HI titer (≥ 32) at the end of the experiment. The average HI titers are represented in Table 1. Three contact chickens and the inoculated one that did not develop HI > 32 did not shed virus.

The results of the analysis of experiment 3 (vaccination on the tenth day of life) are also similar to those of experiments 1 (no vaccination) and 2 (vaccination at the first day of life) (Figure 4). Here, estimates of the key parameters are 1.3 per day (95%CrI: 0.69-2.1) for the transmission rate parameter, 0.89 days (95%CrI: 0.56-1.1) for the mean of the latent period, and 3.5 days (95%CrI: 2.5-5.2) for the mean of the infectious period (Table 2). The reproduction number and generation interval are estimated at 4.4 (95%CrI: 2.3-8.3) and 1.4 days (95%CrI: 1.2-1.5), respectively. Figure 4 shows that, in contrast with experiments 1 and 2, the variance of the infectious period cannot be estimated with high precision anymore, indicating that the experimental data contain little information on the variance of the infectious period (Figure 4).

Experiment 4 (no vaccination, challenge at day 1)

In this experiment, 16 of 22 inoculated birds shed virus, and 13 inoculated birds died (Table 1, Figure 5). Six of the contact birds shed virus, and one died during the course of the experiments. The birds used in the experiment were very young, and could not be tested for the presence of MDA before the experiment. Therefore, eighteen day-old chickens that were not used in the experiments were bled, and tested for the presence of MDA, and 13 of 18 had HI titers ≥ 32, and had an average HI titer of 4.9. The analyses of the experiment show substantial differences in the parameter estimates when compared with those resulting from

the analyses of experiments 1-3 (challenge at four weeks of age). The median of the posterior of the transmission rate parameter is 0.38 per day (95%CrI: 0.17-0.72), and the medians of the posterior distribution of the mean of the latent and infectious periods are 3.3 days (95%CrI: 2.4-4.1) and 2.8 days (95%CrI: 2.1-3.7), respectively (Table 2). The estimates (i.e. the medians of the posterior distribution) of the reproduction number and generation interval are 1.0 (95%CrI: 0.45-2.1) and 1.1 days (95%CrI: 0.77-1.3), respectively.The data contain little information on the variance of the latent period (Figure 5).

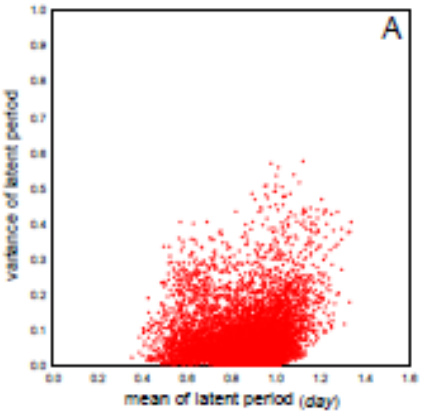
Combined analysis (vaccination at day 28)

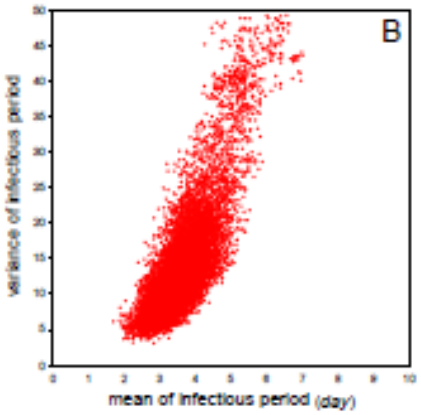
The above analyses indicate that vaccination had no measurable effect on the transmission dynamics when birds were infected and challenged at day 28 (Experiments 1-3; Table 2). We therefore combined and reanalysed the data of these experiments to investigate whether there were differences between inoculated and contact birds in the latent period, and to explore the relation between the HI titer of contact birds prior to the experiment and the probability of infection. Additional file 1, Figure S1 illustrates that the combined analyses enables more precise estimation of the epidemiological parameters of interest (Table 2). The figure furthermore shows that there are no large differences between the means of the latent period of inoculated versus contact birds. The result of the logistic regression of infection outcome as a function of HI titer prior to the experiment was largely inconclusive, as only 4 out of 62 contact birds in trials with a successfully infected inoculated bird had not been infected, and as HI titers prior to the experiment were low and showed little variation (four birds had a HI titer of 4, the remainder had HI titers of 0-2). The analysis indicated that the predicted probability of infection decreased from 95% if the initial HI titer was 0, to 93% if the initial HI titer was 2. However, the associated confidence intervals are wide, and the parameter determining the slope is not significantly different from 0 ($p = 0.63$). HI titers of all birds are given in Additional file 2, Table S1.

Table 2. Overview of the statistical analyses

Exp.	Treatment	Transmission parameter (day^{-1}) (95%CrI)	Latent period (*day*)(95%CrI)	Infectious period (*day*)(95%CrI)	Reproduction number (95%CrI)	Generation interval (*day*)(95%CrI)
1	no vaccination	1.6 (0.97-2.4)	0.88 (0.70-0.94)	3.2 (2.5-4.3)	5.1 (3.0-8.4)	1.5 (1.3-1.7)
2	challenge at day 28 vaccination at day 1	1.5 (0.87-2.3)	0.86 (0.69-0.96)	3.7 (2.8-5.1)	5.5 (3.1-9.3)	1.4 (1.2-1.5)
3	challenge at day 28 vaccination at day 10	1.3 (0.69-2.1)	0.89 (0.56-1.1)	3.5 (2.5-5.2)	4.4 (2.3-8.3)	1.4 (1.2-1.5)
4	no vaccination	0.38 (0.17-0.72)	3.3 (2.4-4.1)	2.8 (2.1-3.7)	1.0 (0.45-2.1)	1.1 (0.77-1.3)
1-3	challenge at day 1 challenge at day 28	1.4 (1.1-1.9)	i: 0.93 (0.88-0.96) c: 0.96 (0.85-1.1)	3.3 (2.7-3.9)	4.6 (3.3-6.4)	1.4 (1.3-1.5)

Parameter estimates are given as posterior medians. Equal-tailed 95% credible intervals are shown between brackets. Estimates of the latent period in the combined analysis of Experiments 1-3 refer to the inoculated (i) and contact (c) birds.





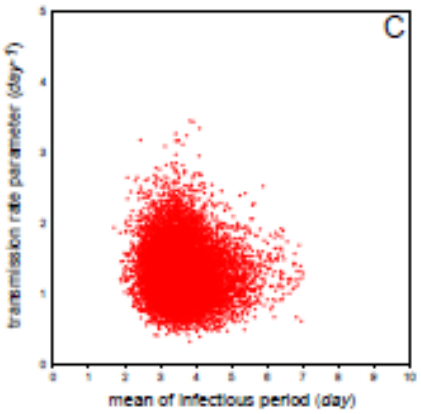
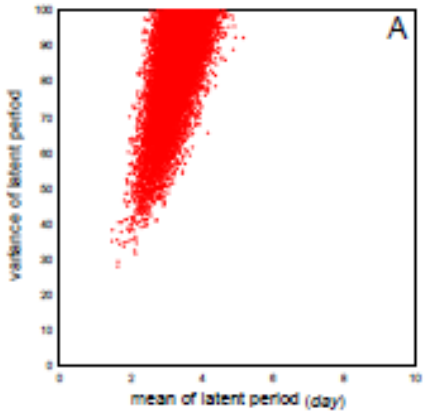
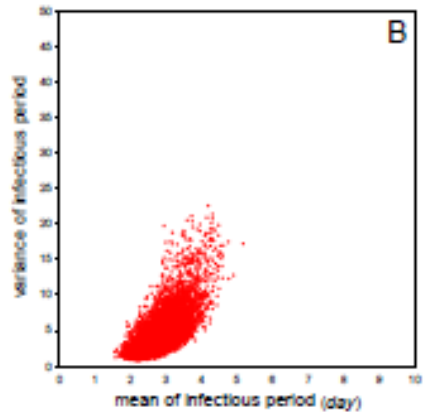


Figure 4. Overview of the analyses of Experiment 3 (vaccination at day 10, challenge at day 28). See Figure 1 for details.





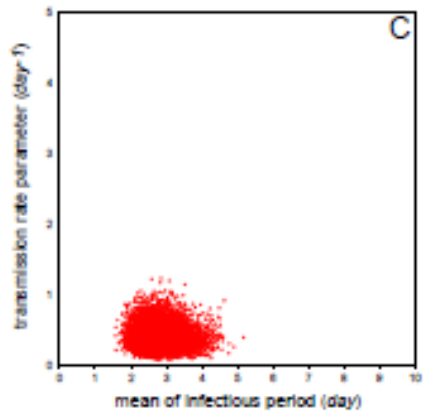


Figure 5. Overview of the analyses of Experiment 4 (no vaccination, challenge at day 1). See Figure 1 for details.

Discussion

The aim of this study was to quantify the effect of a single vaccination of broilers against a HPAI H5N1 on virus transmission, and to which extent virus could spread among broilers and DOC. Vaccination with an inactivated homologous strain did not reduce transmission of H5N1 virus significantly. In both vaccine experiments, in which birds were vaccinated when they were either one or ten days old, the reproduction number was above one indicating that virus could still spread extensively. Moreover vaccination did not prevent the occurrence of clinical signs, although it seemed to reduce mortality slightly. This implies that unnoticed virus spread is unlikely, even in a vaccinated flock, which is often feared by farmers and policy makers as mentioned before (Capua & Alexander 2008, Peyre *et al* 2009, Veits *et al* 2008).

Transmission among day-old chickens, which still had MDA, tended to be reduced compared to chickens of four weeks of age without MDA, as fewer contact birds became infected, but the reproduction number was not significantly below one. Of course the real control group, MDA-free DOC, was lacking, as this was not our research question. In various trials, the clinical signs seemed to be less severe, suggesting that these DOCs may pose a risk in the spread of the infection as they may spread the virus unnoticed. These findings indicate that broilers, including DOC, could play a role in the epidemiology of AI, as virus could spread extensively. It cannot be determined from these experiments, however, to which extent this may occur in the field, as this also depends on the number of virus incursions, and thesubsequent implemented control measures.

Vaccination only induced very low titers of HI antibodies in few birds and a single vaccination was not effective in reducing transmission. The most likely explanation for the reduced efficacy in comparison to layers was the presence of maternally derived antibodies at time of vaccination. MDA in general persist in broilers for approximately 14-21 days after hatch (King *et al* 2012), and for AI it has been demonstrated that MDA titers were low at 7 days after hatching (Ka-Oud *et al* 2008). It has been demonstrated for other viral infections that MDA may interfere with an effective immune response (Klipper *et al* 2004, Ganapathy *et al* 2006), like for example for Newcastle disease (Rauw *et al* 2009) and infectious bronchitis (Terregino *et al* 2008). Whether there was interference between MDA and vaccination could not be demonstrated in this study, as no group of broilers without MDA was included, because this type of broilers is not present in endemically infected areas in

which broiler breeders are vaccinated regularly, and because it was not the research question of this study.

A study on AI vaccination in MDA-positive broilers (Ka-Oud *et al* 2008) demonstrated that HI titers remained high until five weeks post vaccination, indicating that a decrease in immunity was not to be expected within the interval vaccination-challenge applied in our trials. Studies have also shown that HI levels after a single vaccination in birds with MDA reached a peak at six weeks post vaccination (Peyre *et al* 2009, Ka-Oud *et al* 2008), suggesting our interval being too short. We challenged at 28 days of age, however, as this was assumed to be a reasonable interval for field conditions and also considering the duration of the experiments and applied before (Stone 1987).

Another explanation for the failing immune response is that broilers have an immature immune system, as broilers have been bred for growth characteristic, which may have an effect on both the humoral and cellular immune responses (Koenen *et al* 2002). Although the birds in our experiments were not protected, other studies have shown that AI vaccination of broilers at 10 days of age gave satisfactory antibody titers and clinical protection after challenge (Ka-Oud *et al* 2008, De Vriese *et al* 2010, Lebdah & Shanin 2010). A possible explanation for the difference between their observations and ours is the use of different vaccines or adjuvantia.

In our experiment, some birds did survive the infection, although the HI titers at challenge were below 32. In the unvaccinated experiments with 4-week-old birds, for example, all inoculated birds shed virus and showed clinical sign, but some contact-infected chickens survived for more than 10 days. This was also observed in the vaccine experiments and DOC experiment. However, no association could be found between HI titer at moment of challenge and protection against contact-infection and also not between HI titer and infection after inoculation. This phenomenon has also been observed in studies on for example H7N7 in turkeys (Bos *et al* 2008), and on Newcastle disease (Van Boven *et al* 2008). In the latter experiment, vaccinated birds with low or undetectable antibody titres were protected against disease and mortality, but infection and transmission still occurred. One explanation for surviving of contact birds is that these birds had become infected with a low virus dose. As the birds were housed in pairs, the exposure dose could have been low, as some of the inoculated chickens in this experiment died before having shed a large amount of virus. However, Spekreijse *et al* (2011) showed that the case fatality rate of chickens did not differ between dose groups, and in their experiments all birds that were infected died eventually. Another explanation is that other immune responses than antibodies were induced, such as a

cellular immunity, although it is generally assumed that inactivated vaccines usually induce a B cell response only (Chambers *et al* 1988). Another possibility is that the low HI titers may have been sufficiently high to induce some protection.

Of course extrapolation of results from experiments to the field should always be done with caution. Nevertheless, our results indicate that vaccination is not effective in broilers, as early vaccination does not induce a good immune response and if vaccination is applied later, the birds may be protected the moment they are slaughtered. Henning *et al* (2009) demonstratedthat the risk of infection was higher in flocks vaccinated once, in comparison to two vaccinations, also suggesting that vaccination of broilers will not be very effective, but it does not seem to be feasible vaccinating broilers even twice. Although discrepancies between laboratory and field results have been observed more often (Cristalli & Capua 2007), the efficacy of vaccination is usually higher under experimental conditions than under field conditions. Therefore, a proper vaccination scheme with killed vaccines seems useless, although use of other types of vaccines (Swayne & Kapczkinsky 2008, Veits *et al* 2008) could be more successful. Adequate biosecurity measures should therefore be implemented in endemically infected countries to control AI (Capua & Marango 2006, Capua & Alexander 2008, Peyre *et al* 2009, Sawitri *et al* 2007, Swayne & Kapczkinsky 2008, Capua 2007).

Acknowledgements

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Additional Material

Additional file 1: Figure S1. Overview of the combined analyses of Experiments 1-3 (challenge at day 28). The top panel shows the marginal posterior distribution of the mean of the latent period of the inoculated versus contact birds. See Figure 2 for further details.
Additional file 2: Table S1. HI titers at time of challenge and at the end of the trial (4 weeks after inoculation). Titers are expressed as 2 fold dilution (titers of DOC and of other birds at hatch or at time of vaccination could not be determined, because the birds were very small and we did not want to take a risk that birds might die due to blood collection.

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Additional Material

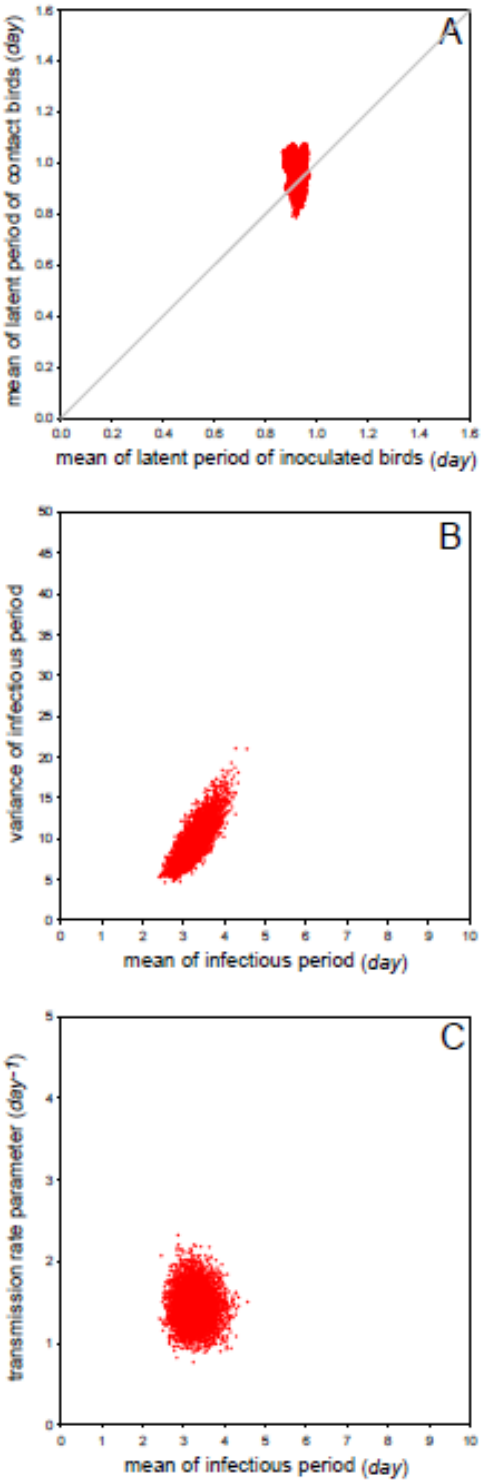

Figure S1. Overview of the combined analyses of Experiment 1-3 (challenge at day 28). The top panel shows the marginal posterior distribution of the mean of the latent period of the inoculated versus contact birds. See Figure 2 for further details.

Table S1. HI titers at time of challenge and at the end of the trial (4 weeks after inoculation). Titers are expressed as 2 fold dilution (titers of DOC and of other birds at hatch or at time of vaccination could not be determined, because the birds were very small and we did not want to take a risk that birds might die due to blood collection.

Treatment group	Pair no.	Inoculated (i) /contact (c)	HI titer at challenge[a]	HI titer at end of experiment[b]	Infected [c]
Unvaccinated	1	I	1	*	1
		C	1	*	1
	2	I	2	*	1
		C	2	*	1
	3	I	2	*	1
		C	2	*	1
	4	I	2	*	1
		c	2	*	1
	5	i	2	*	1
		c	2	*	1
	6	i	1	*	1
		c	1	*	1
	7	i	1	*	1
		c	1	*	1
	8	i	1	1	1
		c	1	*	1
	9	i	1	*	1
		c	1	*	1
	10	i	1	*	1
		c	1	*	1
	11	i	1	*	1
		c	1	*	1
	12	i	4	*	1
		c	2	1024	1
	13	i	1	*	1
		c	1	*	1

Treatment group	Pair no.	Inoculated (i) /contact (c)	HI titer at challenge[a]	HI titer at end of experiment[b]	Infected [c]
	14	i	2	*	1
		c	2	*	1
	15	i	1	*	1
		c	2	*	1
	16	i	1	*	1
		c	4	*	1
	17	i	4	*	1
		c	1	1	1
	18	i	1	*	1
		c	1	1	1
	19	i	4	*	1
		c	1	1	1
	20	i	1	*	1
		c	1	*	1
	21	i	1	*	1
		c	1	1	1
	22	i	2	*	1
		c	4	*	1
Vaccinated at day 1	1	i	1	1	1
		c	1	*	1
	2	i	1	*	1
		c	1	*	1
	3	i	2	*	1
		c	1	*	1
	4	i	2	*	1
		c	2	*	1
	5	i	4	64	1
		c	2	4	0

Treatment group	Pair no.	Inoculated (i) /contact (c)	HI titer at challenge[a]	HI titer at end of experiment[b]	Infected [c]
	6	i	1	*	1
		c	1	*	1
	7	i	1	*	1
		c	1	2	1
	8	i	1	*	1
		c	1	1	0
	9	i	1	*	1
		c	1	*	1
	10	i	1	1024	1
		c	1	*	1
	11	i	1	32	1
		c	1	1	1
	12	i	2	*	1
		c	0	*	1
	13	i	1	*	1
		c	4	1	1
	14	i	1	*	1
		c	1	*	1
	15	i	1	*	1
		c	2	*	1
	16	i	8	1024	1
		c	2	*	1
	17	i	1	*	1
		c	1	*	1
	18	i	4	8	1
		c	1	1	1
	19	i	2	*	1
		c	1	*	1
	20	i	2	2	1
		c	2	1	1

Treatment group	Pair no.	Inoculated (i) /contact (c)	HI titer at challenge[a]	HI titer at end of experiment[b]	Infected [c]
	21	i	2	*	1
		c	2	*	1
	22	i	2	*	1
		c	2	2	1
Vaccinated at day 10	1	i	2	128	0
		c	2	2	0
	2	i	2	*	1
		c	2	128	0
	3	i	2	32	0
		c	2	1	0
	4	i	2	*	1
		c	2	32	1
	5	i	4	16	0
		c	2	4	0
	6	i	1	256	1
		c	1	*	1
	7	i	1	16	0
		c	1	2	0
	8	i	1	*	1
		c	1	*	1
	9	i	1	*	1
		c	1	512	1
	10	i	1	*	1
		c	1	*	1
	11	i	2	*	1
		c	2	128	0
	12	i	4	*	1
		c	1	256	1

Treatment group	Pair no.	Inoculated (i) /contact (c)	HI titer at challenge[a]	HI titer at end of experiment[b]	Infected [c]
	13	i	1	*	1
		c	2	*	1
	14	i	1	*	1
		c	1	1024	1
	15	i	1	*	1
		c	4	*	1
	16	i	4	64	1
		c	2	4	1
	17	i	1	0	1
		c	2	0	1
	18	i	4	128	1
		c	2	128	1
	19	i	1	*	1
		c	1	*	1
	20	i	2	*	1
		c	1	*	1
	21	i	2	64	1
		c	2	16	1
	22	i	1	*	1
		c	1	1024	1
DOC	1	i	nd	*	1
		c	nd	1	0
	2	i	nd	*	1
		c	nd	1	0
	3	i	nd	*	1
		c	nd	1	0
	4	i	nd	*	1
		c	nd	*	1

Treatment group	Pair no.	Inoculated (i) /contact (c)	HI titer at challenge[a]	HI titer at end of experiment[b]	Infected [c]
	5	i	nd	1	1
		c	nd	1	0
	6	i	nd	*	1
		c	nd	1	0
	7	i	nd	*	1
		c	nd	1	0
	8	i	nd	*	1
		c	nd	1	0
	9	i	nd	*	1
		c	nd	*	1
	10	i	nd	*	1
		c	nd	1	0
	11	i	nd	*	1
		c	nd	1	0
	12	i	nd	*	1
		c	nd	1	1
	13	i	nd	2	1
		c	nd	1	1
	14	i	nd	1	1
		c	nd	1	1
	15	i	nd	1	1
		c	nd	1	1
	16	i	nd	1024	1
		c	nd	1	1
	17	i	nd	*	1
		c	nd	1	1
	18	i	nd	1	0
		c	nd	1	0
	19	i	nd	1	0
		c	nd	1	0

Treatment group	Pair no.	Inoculated (i) /contact (c)	HI titer at challenge[a]	HI titer at end of experiment[b]	Infected [c]
	20	i	nd	*	
		c	nd	1	1
	21	i	nd	1	0
		c	nd	1	0
	22	i	nd	0	0
		c	nd	1	0

[a] expressed are the two-fold dilution
[b] * the bird died before the end of the trial and the titer could not be determined
[c] 1 means that the bird was infected
nd not determined

CHAPTER 4

Silent transmission of highly pathogenic avian influenza H5N1 virus amongst vaccinated commercial layers

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Abstract

Vaccination of poultry against highly pathogenic avian influenza (HPAI) is one of the control measures in endemically infected countries. The main goal of vaccination is to not only to protect flocks against production losses and mortality but also against infection. SPF layers, vaccinated under experimental conditions, are indeed protected against contact-infection. Improper use of vaccines in the field, however, may cause vaccination failure which may subsequently lead to silent virus spread. We carried out a transmission experiment with 66 commercial layers vaccinated in the field to determine whether a single vaccination with a vaccine containing HPAI virus strain H5N1 A/chicken/Legok/2003 would be sufficient to protect against infection. Birds were housed pair-wise, based on the titer of haemagglutination inhibition (HI) antibodies against H5N1. Each pair had similar HI titers. A group with unvaccinated SPF chickens was included as control group. Four weeks after vaccination, one bird per pair was inoculated intratracheally with H5N1 A/chicken/Legok/2003. Tracheal and cloacal swabs were collected daily for ten days, and serum samples were gathered at inoculation and at the end of the experiment, four weeks after inoculation. The majority of the birds had HI titers below protective levels. No clinical signs were observed in the vaccinated pairs and virus shedding was limited, and only observed in pairs (13 out of 27) with pre-existing HI vaccination titers smaller than 2log 2. However, a high proportion of vaccinated birds, inoculated ones as well as contact exposed (32 out of 33 pairs) demonstrated a four-fold increase in HI titer, which would be an indication of infection. Then transmission would have occurred among nearly all pairs. In conclusion, our study showed that a single vaccination applied under field condition induced clinical protection, but had been insufficient to induce protection against virus transmission, suggesting that silent spread of virus in vaccinated commercial flocks may occur.

Introduction

Highly pathogenic avian influenza (HPAI) H5N1 has been present in South-East Asia since 1996 (Xu *et al* 1999). It is considered to be a major threat for both the poultry industry and public health. Since the first outbreak, the virus has spread to many countries in Asia, Europe and Africa (Yee *et al* 2009, Peyre *et al* 2009, Li *et al* 2011, OIE 2013). In several countries, outbreaks of HPAI have been successfully controlled by culling infected flocks, pre-emptive culling of contiguous flocks, biosecurity measures, and movement restrictions (Capua and Marangon 2006, Domenech *et al* 2009, Hsu *et al* 2010). In some countries in Asia, however, these measures could not be sufficiently applied, and the disease has become endemic (Domenech *et al* 2009, Peyre *et al* 2009).

In endemically affected areas - Indonesia, Vietnam, Egypt and China - vaccination of commercial poultry was applied as additional tool to control HPAI (Suarez 2005, Swayne 2006, Sims 2007). The main goal of this strategy was to induce protection against clinical signs, production losses, and mortality. Although vaccination seems to have been rather successful in reducing production losses and mortality (Capua & Marangon 2007, Peyre *et al* 2009), HPAI outbreaks in vaccinated flocks still occur, for example in Indonesia (Bouma *et al* 2008, Loth *et al* 2011, El Zhogby *et al* 2012, Swayne 2012). Several explanations for these outbreaks have been suggested such as an insufficient vaccine coverage in flocks, inappropriate biosecurity during vaccine administration, poor vaccine quality, an insufficient immune response, or antigenic drift of virus strains (Siregar *et al* 2007, Sims 2007, Bolz *et al* 2009, Fouchier & Smith 2010, Swayne 2012), but up to now it remains unclear why vaccination programmes may fail.

The currently registered AI vaccines are usually evaluated experimentally by measuring vaccine-induced titers of haemagglutination inhibition (HI) antibodies, the capacity to provide protection against clinical signs, virus shedding, and mortality after a challenge infection (Alexander 2007, Capua 2007, Swayne 2006, Swayne 2009). This HI titer is used in the field to determine whether or not a flock is sufficiently protected against infection or should be revaccinated.

In general, birds vaccinated under experimental conditions are well protected against a challenge infection (Van der Goot *et al* 2005, Bouma *et al* 2009, Sasaki *et al* 2009, Rauw *et al* 2012). The efficacy of vaccination under field conditions is, however, often lower as suggested by outbreaks of AI in vaccinated flocks even with a presumed sufficient coverage, i.e. the percentage of birds in a flock with the presumed protective HI titer. It is possible that,

for a certain period, virus may spread unnoticed, if for example the HI titer levels protect against the occurrence of clinical signs or production losses, but not against transmission. Therefore, we conducted a transmission experiment using layers with different levels of vaccine-induced HI titer. We measured whether infection occurred, and whether inoculated birds could transmit virus to cage mates. This study might help to develop or improve a vaccination strategy in the field.

Materials and methods

The experiment was performed in the high containment unit under Biosafety level 3 (BSL3) conditions at PT. Medion, Bandung, Indonesia. The experiments complied with the article 80 on 'Research in Animal Health' of the Indonesian 'Law on Livestock and Animal Health UU/18/2009'.

Animals and housing

Two groups were included in the experiment. Group 1 consisted of 11 pairs of unvaccinated specified pathogen free (SPF) layers, and the other group consisted of 33 pairs of vaccinated layers. Birds for group 1 were obtained from the SPF layer flock of PT. Medion. This flock was regularly checked for the absence of various poultry diseases, including AI. At time the embryonated eggs for the trial were obtained for hatching at the experimental facilities, the parent flock was free from HI antibodies against AI. The layers for the vaccine group were purchased from a commercial layer farm in West Java. At the age of 4 weeks these birds were vaccinated once against H5N1. When they were 7 weeks old they were moved to the PT. Medion BSL3 facilities. The vaccinated birds were first housed in one group to acclimatise, and moved to cages shortly before the start of the actual experiment.

For the actual experiment, birds were housed pair-wise according to their HI titer induced by vaccination, based on similar HI titer. Two separate units were available for the experiment, each with two rows with cages on three floors. Birds were housed in cages of the upper and lower floors. Birds from the lowest HI titer were housed in one unit; birds with the higher titers in the other unit. The cage between each pair remained empty. Unvaccinated SPF layers from the SPF unit of PT. Medion were placed in empty cages in the middle floor below each pair in the upper row. These birds served as sentinels to demonstrate independence of the observations in each cage and to determine whether between-cage virus

transmission was indeed absent. The sentinels were approximately of the same age as the experimental birds.

Birds were fed with a commercial ration, and had tap water ad libitum. Each cage had a separate feeding and drinking system. The floor and wall of each cage were covered with plastic to prevent spread of manure or other material between cages.

Vaccine and challenge virus

The vaccine used at the commercial farm was an inactivated oil-emulsion vaccine, containing HPAI virus strain H5N1 A/Chicken/Legok/2003 (Medivac®, PT. Medion, Bandung, Indonesia). The vaccine was administrated intramuscularly in one leg using 0.5 ml containing 256 haemagglutination units (HAU) per dose per bird. The birds in the vaccine group (2) were vaccinated once when they were 4 weeks old. Birds in the control group (1) and the sentinel birds remained unvaccinated.

The homologous HPAI virus strain H5N1 A/Chicken/Legok/2003 was used for challenge. The strain was provided by PT. Medion Bandung Indonesia. The virus has been used in other transmission experiments, and was able to induce symptoms, virus shedding and transmission to contact birds (Bouma *et al* 2009, Poetri *et al* 2011).

At day of challenge, one bird per pair was inoculated intranasally and intratracheally with 0.2 ml inoculum containing 10^6/ml median egg infectious dose (EID_{50}) (Bouma *et al* 2009, Poetri *et al* 2011). Before inoculation each bird that had to be inoculated was put in the empty cage near its pen mate. Eight hours after inoculation, they were placed back in their original cage.

Infection experiments

Before allocation to the pairs and cages, the HI titer of each bird was determined in the HI test (see section ***Test***). Serum samples were collected when the birds were 7 weeks old, 3 weeks post vaccination. In the vaccine group (2), homogenous pairs were formed, based on the HI antibody titer. Titers varied between 0-32 (see Table 2 for details).

Inoculation was done when birds were 8 weeks old. Per pair one bird was inoculated, while the other bird was contact-exposed to the inoculated bird. We refer to each pair as one trial; in each trial birds had similar HI titer; in total the vaccine group consisted of 33 pairs: 33 inoculated and 33 contact-exposed birds.

Shedding patterns and spread of virus was monitored by taking daily swab samples from the cloaca and the oropharynx from all birds for 10 days post inoculation (p.i.). All birds that died during the experiment were swabbed thoroughly. The samples were taken and stored in duplo at -70ºC until further testing. Serum blood samples were taken two days before inoculation and at the end of the experiment. Sera were stored at -20ºC until further testing. Clinical signs were recorded during 4 weeks after inoculation. Sentinels were used to monitor between-cage virus transmission to determine whether observations in each trial could be considered to be independent. The experiments lasted 4 weeks.

Tests

The presence of AI virus in swabs was determined by virus isolation according to standard procedure (OIE 2012). Briefly, three SPF embryonated chicken eggs, incubated for 9 days, were inoculated with 0.2 ml swab medium per egg. After 72 hours (h), or when the embryo had died before that time, the allantoic fluid was harvested. A haemagglutination assay (HA) was performed following standard procedure. When at least one of the eggs was positive in the HA, the swab was considered to be positive. The test results were recorded as positive for AI virus or negative (Bouma *et al* 2009, Poetri *et al* 2011).

Serum samples were tested in a HI test according to standard procedures (OIE 2012). Tests were carried out in duplo using 4 HA units (HAU) of the HPAI virus strain H5N1 A/chicken/Legok/2003. Two-fold dilutions of the serum samples were made, and titers are expressed as the serum dilution that caused complete inhibition of agglutination (OIE 2012).

A bird was considered infected if at least one sample (either tracheal or cloacal) tested positive in the virus isolation assay, or if the HI titer determined at the end of the experiment was increased four-fold compared to the HI titer determined shortly before inoculation or exposure (here referred to as seroconversion).

Statistical analysis

To investigate the relation between pre-existing antibody titer and virus shedding (a measure for infectiousness) we analysed the data using a logistic regression with the log2 HI titer as independent variable. To enable the analyses, we assigned a value of -1 to samples that tested negative in the original twofold dilution. Other assumptions yielded similar outcomes (not shown). Further, birds were classified as infectious if they shed virus on at least one day; otherwise they were classified as negative (i.e. not infectious).

In the transmission analyses we considered two definitions to classify birds as infected. In the first, birds were considered infected if they shed virus or died. In the second, the classification was based on a four-fold increase in HI titer between the moment of challenge and the end of the experiment only. The transmission efficiency, as measured by the reproduction number, was estimated by analysis of the final size, i.e. the number of contact-exposed birds that were infected over the course of the experiment (Van der Goot *et al* 2005, Velthuis *et al* 2007, Bouma *et al* 2009). Here we present results under the assumption of a fixed infectious period. Alternative analyses, assuming exponentially distributed infectious periods, led to comparable results (not shown). Logistic regression was performed using the geometric linear model (GLM) function of R 3.0.1. The final size analyses were performed using Mathematica 9.0.1.

Results

Clinical sign, viral shedding and mortality

All inoculated and 4 contact-exposed birds in the control group tested positive in the virus isolation test on swab samples, and all virus shedders except 1 contact-exposed bird died within 3-4 days after challenge (p.c.). In the vaccine group, 13 inoculated birds and 2 contact-exposed birds shed virus; these virus positive birds had vaccine-induced HI titer < 2log 2. Six inoculated shedders died within 10 days p.c. No contact-exposed birds in the vaccine group died of AI. More detailed information is shown in Tables 1 and 2.

Serological response

The HI titers are shown in Tables 1 and 2. None of the surviving contact-exposed birds in the control group showed a seroconversion p.c. (Table 1). In the vaccine group, 32 inoculated birds and 27 contact-exposed birds showed a four-fold seroconversion (Table 2). One inoculated bird which did not seroconvert had a HI titer of 2log 5 before inoculation; the other birds that seroconverted had HI titer varying between 2log 0 and 2log 4.

Table 1. Overview of HI titers in the control group

Trial number	Type of bird[1]	HI titer[2] at moment of challenge (HAU)	HI titer 4 weeks post challenge (HAU)	Four fold increase of HI titer yes/no	Number of days virus positive	Bird died yes/no	Bird infected[3] yes/no
1	I	neg	na	na	2	yes	yes
	C	neg	na	na	2	no	yes
2	I	neg	na	na	2	yes	yes
	C	neg	na	na	2	yes	yes
3	I	neg	na	na	1	yes	yes
	C	neg	neg	no	neg	no	no
4	I	neg	na	na	1	yes	yes
	C	neg	neg	no	neg	no	no
5	I	neg	na	na	2	yes	yes
	C	neg	neg	no	neg	no	no
6	I	neg	na	na	2	yes	yes
	C	neg	neg	no	neg	no	no
7	I	neg	na	na	2	yes	yes
	C	neg	0	no	neg	no	no
8	I	neg	na	na	2	yes	yes
	C	neg	0	no	neg	no	no
9	I	neg	na	na	2	yes	yes
	C	neg	0	no	neg	no	no
10	I	neg	na	na	2	yes	yes
	C	neg	na	na	1	yes	yes
11	I	neg	na	na	1	yes	yes
	C	neg	na	na	2	yes	yes

[1] I: inoculated bird; C: contact-exposed bird

[2] HI titer data in log2; neg : negative; na : not applicable

[3] based on VI result

Table 2. Overview of HI titers in the vaccine group

Pair Number	Type of bird	HI titer[1] at moment of challenge (HAU)[1]	HI titer 4 weeks post challenge (HAU)	Four fold increase of HI titer yes/no	Number of days virus positive	Bird died yes/no	Bird infected[2] yes/no
1	I	neg	3	yes	1	no	yes
	C	neg	6	yes	0	no	no
2	I	neg	9	yes	2	no	yes
	C	neg	7	yes	0	no	no
3	I	neg	5	yes	0	no	no
	C	neg	6	yes	0	no	no
4	I	neg	10	yes	0	no	no
	C	neg	6	yes	0	no	no
5	I	neg	na	na	2	yes	yes
	C	neg	3	yes	1	no	yes
6	I	neg	10	yes	3	no	yes
	C	neg	6	yes	0	no	no
7	I	neg	4	yes	0	no	no
	C	neg	5	yes	0	no	no
8	I	0	na	na	5	yes	yes
	C	0	3	yes	0	no	no
9	I	0	na	na	8	yes	yes
	C	0	3	yes	0	no	no
10	I	0	2	yes	0	no	no
	C	0	4	yes	0	no	no
11	I	0	na	na	6	yes	yes
	C	0	3	yes	0	no	no
12	I	0	3	yes	0	no	no
	C	0	4	yes	0	no	no
13	I	0	4	yes	0	no	no
	C	0	4	yes	0	no	no
14	I	0	9	yes	4	no	yes
	C	0	5	yes	0	no	no

Pair Number	Type of bird	HI titer[1] at moment of challenge (HAU)[1]	HI titer 4 weeks post challenge (HAU)	Four fold increase of HI titer yes/no	Number of days virus positive	Bird died yes/no	Bird infected[2] yes/no
15	I	1	na	na	6	yes	yes
	C	1	4	yes	0	no	no
16	I	1	na	na	3	yes	yes
	C	1	7	yes	0	no	no
17	I	1	10	yes	7	no	yes
	C	1	7	yes	0	no	no
18	I	1	4	yes	0	no	no
	C	1	6	yes	0	no	no
19	I	1	5	yes	0	no	no
	C	1	na	na	0	no	no
20	I	1	5	yes	0	no	no
	C	1	4	yes	0	no	no
21	I	1	7	yes	1	no	yes
	C	1	4	yes	1	no	yes
22	I	1	4	yes	1	no	yes
	C	1	3	yes	0	no	no
23	I	2	5	yes	0	no	no
	C	2	neg	no	0	no	no
24	I	2	6	yes	0	no	no
	C	2	6	yes	0	no	no
25	I	2	5	yes	0	no	no
	C	2	8	yes	0	no	no
26	I	2	8	yes	0	no	no
	C	2	4	yes	0	no	no
27	I	2	6	yes	0	no	no
	C	2	3	no	0	no	no
28	I	3	8	yes	0	no	no
	C	3	3	no	0	no	No

Pair Number	Type of bird	HI titer[1] at moment of challenge (HAU)[1]	HI titer 4 weeks post challenge (HAU)	Four fold increase of HI titer yes/no	Number of days virus positive	Bird died yes/no	Bird infected[2] yes/no
29	I	3	6	yes	0	no	no
	C	3	8	yes	0	no	no
30	I	3	8	yes	0	no	no
	C	3	6	yes	0	no	no
31	I	3	6	yes	0	no	no
	C	3	3	no	0	no	no
32	I	4	6	yes	0	no	no
	C	4	10	yes	0	no	no
33	I	5	6		0	no	no
	C	5	6		0	no	no

[1]HI titer data in log2; neg: in starting dilution negative, no detectable titer; na : not applicable
[2] based on VI result

Virus shedding and transmission efficiency

In the trials with unvaccinated birds, inoculation led to a productive infection in all 11 birds (Table 1). Transmission to contact-exposed birds was less efficient, leading to infection in 4 out of 11 contact-exposed birds. The reproduction number, i.e. the number of birds that would be infected in a large uninfected population, was estimated at 0.90 (95%CI: 0.23-2.4).

In trials with vaccinated birds, logistic regression showed that there was a significant and strong negative relation between pre-existing antibody titer and virus shedding in the inoculated birds ($p<0.001$). Inoculation resulted in detectable virus shedding in 13 inoculated birds in 22 trials with birds with a HI titer < 2log 2, and in 0 out of 11 inoculated birds with a pre-existing antibody titer ≥ 2log 2. In the trials with chickens with antibody titers < 2log 2, 2 out of 22 contact-exposed birds showed signs of infection, based on shedding, leading to estimates of the reproduction number in this group of 0.19 (95%CI: 0.02-0.69). In vaccinated birds with a HI titer ≥ 2log 2 none of the inoculation attempts resulted in a productive infection (i.e. virus shedding) and none of the contact-exposed birds shed virus or showed signs of AI..

The reproduction number was also estimated using the fourfold increase of HI titer as the criterium for infection (n=26). As 32 out of 33 inoculated ones seroconverted, this resulted in an estimate of $\hat{R} = 3.1$ (95%CI: 1.9-4.8), which was significantly higher than 1 (p=0.0000045).

Discussion

The efficacy of vaccination against AI is generally determined experimentally using SPF birds. From these experiments it turns out that a single vaccination is often sufficient to reduce transmission of virus (Van der Goot *et al* 2005, Van der Goot *et al* 2007, Bouma *et al* 2008, Bos *et al* 2008, Sasaki *et al* 2009, Spekreijse *et al* 2011, Van der Goot *et al* 2008). In the field, however, outbreaks still occur, and new variants of AI strains arise, suggesting vaccine failure (Dharmayanti *et al* 2011, Cattoli *et al* 2011, Moneim *et al* 2011, FAO 2012). The aim of this study was to determine whether a single vaccination of commercial layers, if carried out under field conditions, were sufficient to protect a chicken against infection with a HPAI H5N1 strain and against subsequent transmission to a pen mate. Unvaccinated SPF birds became infected, but virus transmission in this group was not efficient, as the majority of the contact-exposed birds escaped infection. After a single vaccination, the majority had titers that varied between 2log 0-1, much lower than the presumed protective level of 2log 5 (Lee *et al* 2004, Ellis *et al* 2004, Phillipa *et al* 2007). These low titers did not fully protect chickens against virus replication in the trachea, as several birds tested positive in the VI test. However, the reproduction number for HPAI amongst vaccinated birds with a low vaccine-induced HI titer < 2log 2 was estimated to be 0.19, suggesting that spread of virus in a population with vaccinated birds that only had low HI titers did not occur to an extensive level. Nevertheless, the reproduction number, based on the assumption that birds that showed a four-fold increase in HI titer were infected, was estimated to be significantly above one. As clinical signs were not observed, the latter finding suggests that silent virus spread in vaccinated flocks with low HI titer (<2log 2) may occur. Silent spread can facilitate the emergence of new variants, which may pose a risk for poultry flocks but also for human health (Savill *et al* 2006).

Viral shedding and transmission were observed among unvaccinated birds, but was not very efficient. Inefficient transmission in pairs of unvaccinated chickens has been observed more often, like for example in studies carried out by Spekreijse *et al* (2011) and

Bouma *et al* (2009). Using larger groups improves the probability of transmission, but our aim was to link HI titer with transmission, which can only be done properly in a pair-wise design (Velthuis *et al* 2007). An explanation for the lack of efficient transmission is that inoculated unvaccinated birds died rapidly after inoculation, and did not have enough time to shed a large amount of virus to contact-exposed birds to become infected. Also the rather clean circumstances, for example due to the absence of bedding material, may have reduced the probability of transmission.

Vaccination did not induce high HI titers in the majority of the layers vaccinated in a commercial farm. Nearly all birds had titers < 2log 5, which is in contrast to findings in SPF birds which usually develop high HI titer (Bouma *et al* 2008, Sasaki *et al* 2009, Spekreijse *et al* 2013, Pfeiffer *et al* 2010). Nevertheless, despite these low HI titer, the birds were protected against clinical signs and mortality, and also virus shedding was limited, as has also been seen by e.g. Spekreijse *et al* (2013). This seems consistent with a previous study on the efficacy of vaccination in ducks against HPAI H5N1 A/Duck/Laos/25/06, in which it was shown that low or undetectable HI titer (<2log 3,3) provided clinical protection against a challenge infection (Kim *et al* 2008). The efficacy of vaccination of SPF layers and commercial layers, with respect to protection against disease, seemed to be comparable, and even low HI titer were able to protect inoculated birds against clinical signs and mortality. With respect to prevention of virus transmission, the efficacy differs substantially, as SPF birds are generally fully protected against transmission (Bouma *et al* 2008, Poetri *et al* 2009), whereas the commercial birds in our experiment seemed to be unprotected, assuming that the interpretation of HI titer 4 weeks p.v. is correct with respect to predicting whether a flock is protected against infection or not. Of course it should be realised that SPF and commercial layers were not compared in one experiment simultaneously. Nevertheless, our study showed that repeated vaccination of commercial layers should probably be carried out to reach sufficiently high HI titer.

In transmission studies it is more common now to determine transmission using data on infectiousness, indicated by shedding of the pathogen of interest (Van der Goot *et al* 2005, Velthuis *et al* 2007). Using the virus shedding data for estimating the level of transmission, the conclusion would be that transmission in vaccinated groups was limited. Using the variable 'seroconversion' as indication for infection, which has been done more often (Buckley *et al* 2006, Leschnik *et al* 2007, Van Boven *et al* 2008), would, however, change the conclusion drastically, as then the reproduction number would be estimated to be larger than one, suggesting that major outbreaks could occur if contact birds were indeed infected,

the question remains whether they would be able to transmit the virus to other contacts, as no virus was detected in samples from these birds. An extended transmission experiment could contribute to determine whether non-shedders are indeed infectious to others (Velthuis *et al* 2007). Moreover, this approach assumes that the titer increase induced by vaccination had come to an end before infection, as we did not include a vaccine control group that was not challenged with H5N1. The reason was that we expected virus spread in the control group and that virus shedding could be used as parameter for spread. In our experiment, however, virus shedding by unvaccinated inoculated birds was not sufficient to transmit virus to contact pen mates, and shedding by vaccinated birds was not consistent. We therefore did not use this measure to quantify the spread. Instead we used the four-fold increase in HI titer before and after inoculation, a measure that is also often used to demonstrate infection due to exposure to a virus (Leschink *et al* 2007, Van Boven *et al* 2008).

Vaccination without good application of a monitoring system, and biosecurity could lead to vaccination failure, where the disease has become endemic (Capua & Alexander 2004). The effectiveness of field vaccination in Indonesia has been doubted due to low vaccine coverage, improper vaccination programme and insufficient quality of some vaccines (Bouma *et al* 2008, FAO 2011, Swayne *et al* 2011). In our experiment, layers were not even protected against transmission with a homologous strain. If silent spread of even homologous strains can occur, improper use of vaccines may promote mutation on circulating virus in the field, and if this occurs then the vaccine protective efficacy would be reduced even more (Lee *et al* 2004, Capua & Alexander 2004, Lekcharoensuk 2008, Peyre *et al* 2009, Fouchier & Smith 2010, Cattoli *et al* 2011).

In summary, our results show that a single vaccination applied under field conditions might not be sufficient to induce protection against virus reproduction and subsequent transmission, implying that silent spread of virus may occur and that multiple vaccination of layers is necessary.

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CHAPTER 5

A lack of antibody formation against inactivated influenza virus after aerosol vaccination in presence or absence of adjuvantia

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Abstract

In the poultry industry, infections with avian influenza virus (AIV) can result in significant economic losses. The risk and the size of an outbreak might be restricted by vaccination of poultry. A vaccine that would be used for rapid intervention during an outbreak should be safe to use, highly effective after a single administration and be suitable for mass application. A vaccine that could be applied by spray or aerosol would be suitable for mass application, but respiratory applied inactivated influenza is poorly immunogenic and needs to be adjuvanted. We chose aluminum OH, chitosan, cholera toxin B subunit (CT-B), and Stimune as adjuvant for an aerosolized vaccine with inactivated H9N2. Each adjuvant was tested in two doses. None of the adjuvanted vaccines induced AIV-specific antibodies after single vaccination, measured 1 and 3 weeks after vaccination by aerosol, in contrast to the intramuscularly applied vaccine. The aerosolized vaccine did enter the chickens' respiratory tract as CT-B-specific serum antibodies were detected after 1 week in chickens vaccinated with the CT-B-adjuvanted vaccine. Chickens showed no adverse effects after the aerosol vaccination based on weight gain and clinical signs. The failure to detect AIV-specific antibodies might be due to the concentration of the inactivated virus.

Introduction

In the poultry industry, infections with avian influenza virus (AIV) can result in significant economic losses. The risk and the size of an outbreak of AIV could be restricted by vaccination of poultry. Possible vaccination strategies are rapid intervention (emergency vaccination of poultry in the area around an outbreak), preventive vaccination of specific categories of poultry that are more at risk for a new introduction of AIV (e.g. free range layers in areas with many wild ducks and geese), and general preventive vaccinations of poultry in areas in which AIV is endemic. A vaccine used for rapid intervention during an outbreak should be safe, highly effective after a single administration and be suitable for mass application. A vaccine that could be applied by spray or aerosol would be suitable for mass application, which is regularly performed for e.g. infectious bronchitis virus (De Witt *et al* 2010), Newcastle disease virus (NDV), avian metapneumovirus and most *Mycoplasma galliseptum* vaccines (Ley 2003). In the case of AIV, aerosol vaccination using live virus is not desirable because of its zoonotic potential and because of the risk for virus reassortment. Mucosal vaccination via the respiratory route has several advantages: it induces both local and systemic immune responses (Atmar *et al* 2007, Tseng *et al* 2009, Worrall *et al* 2009), it could halt infection already at portal of entry (Yoshikawa *et al* 2004) and it is suitable for mass application.

The respiratory tract (RT) mucosa constantly comes into contact with inhaled Ag and in normal circumstances these Ag do not provoke strong immune responses, but induce a state of tolerance (Akbari *et al* 2001) leading to a tolerogenic environment in the RT. Intranasally (i.n.) applied whole inactivated AIV (WIV) is poorly immunogenic (Hagenaars *et al* 2008), as was also described in chicken for i.n. applied WIV (Worrall *et al* 2009) and i.n. applied inactivated NDV (Tseng *et al* 2009). To enhance the immunogenicity of WIV it needs to be adjuvanted. We chose aluminum (alum) OH, chitosan, cholera toxin B subunit (CT-B), and Stimune as adjuvant for in an aerosolized vaccine with inactivated H9N2. Alum adjuvants are the most widely used adjuvants for human vaccines (Lambrecht *et al* 2009) and it is an effective adjuvant in influenza vaccines in mice (Chang *et al* 2010) and chicken (Reemers *et al* 2010). However, in human influenza trials results are less consistent, with an aluminum phosphate adjuvanted H9N2 WIV i.m. vaccine being well tolerated and immunogenic (Nicholson *et al* 2009), while other groups found that H1N1 split virus (detergent-disrupted virion) vaccine formulations containing alum were less immunogenic than formulations without adjuvant when given i.m. (Zhu *et al* 2009). Chitosan has shown

promising results in i.n. split virus vaccines in mice (Bacon *et al* 2000), split virus and protein vaccines in human (Read *et al* 2005, Sui *et al* 2010a, Sui *et al* 2010b) and a split virus vaccine in poultry (Worrall *et al* 2009, Rauw *et al* 2010). It was previously shown that CT is an effective mucosal adjuvant in chicken (Vervelde *et al* 1998), but CT cannot be used in the field because of its toxicity. CT-B containing trace amounts of CT is an effective adjuvant in i.n. delivered split virus vaccines in mice (Matsuo *et al* 2000), but because of the toxicity of CT and the mass application as intended in the field, we decided to use pure CT-B. CT-B already showed protective effects in chickens with an i.n. applied inactivated NDV vaccine (Takada & Kida 1996). Stimune, also known as Specol, has been used successfully in an i.m. vaccine using soluble trimeric H5 protein in chicken (Cornelissen *et al* 2010).

In this study, different adjuvants were tested in an aerosolized vaccine using WIV for use in a rapid intervention strategy. None of the adjuvanted vaccines induced AIV-specific Ab after a single vaccination, measured 1 and 3 weeks post vaccination (wpv) by aerosol, in contrast to the i.m. applied vaccine. The aerosolized vaccine did enter the chickens' RT as CT-B-specific Ab were detected in serum from 1 wpv onwards. Chickens showed no adverse effects on weight gain and no clinical signs after aerosol vaccination.

Materials and methods

Chickens

One-day old specific pathogen free broiler chickens (a crossbred of Hybro and Cobb) of both sexes (Animal Health Service, Deventer, the Netherlands) were housed at the Utrecht University animal facilities. Animals were housed in groups on the floor with sawdust bedding and received food and water ad libitum. Aerosol vaccination was performed at Animal Health Service Deventer. In compliance with Dutch law, all experiments were approved by the Animal Experimental Committee of the Faculty of Veterinary Medicine of Utrecht University, the Netherlands, in accordance with the Dutch regulation on experimental animals.

Virus

H9N2 A/Chicken/Saudi Arabia/SP02525/3AAV/2000 (Animal Health Service, Deventer, the Netherlands) was used for vaccination. The virus titer was 1.2 x 10^8 EID50/ml or 405 haemagglutinating units (HAU)/ml. Before vaccination, the virus was inactivated using beta-propiolactone (BPL; Acros organics). Briefly, a 10% BPL solution was prepared in a 125

mM sodiumcitrate and 150 mM sodium chloride buffer and 10 µl/ml was added to the virus. Virus-BPL solution was then incubated for 24h at 4^0C under continuous stirring. Inactivation was confirmed by inoculation in embryonated chicken eggs performed by Animal Health Service Deventer.

Vaccines

Each adjuvant was used in 2 doses: a low dose and a high dose. Imject alum (Pierce) was used 1:3 and 1:1 mixed with WIV. Stimune (Prionics) was mixed 1:2 and 1:1 for i.m. vaccination, Stimune was mixed with WIV according to manufacturer's instructions (5 parts Stimune and 4 parts water phase). CT-B (Sigma) was used at a concentration of 10 µg/chicken and 50 µg/chicken. Chitosan (Protasan UP CL 213; Novamatrix) was used as a 1.5% (w/v) and a 3% (w/v) solution in sterile saline.

Chickens were vaccinated with approximately 75 HAU WIV. To calculate the amount of virus needed, the breathing volume was estimated at 44 liter/kg body weight per hour (Fedde *et al* 1998) and the volume of the isolator was 1.38 m^3. The i.m. control group was vaccinated with 700 µl containing 75 HAU WIV adjuvanted with Stimune.

Experimental setup

Ninety-five three-week-old SPF broilers were randomized into 9 groups of 10 chickens and one control group of 5 chickens. Group 1 was aerosol-vaccinated with WIV only; group 2 and 3 were aerosolized with alum-adjuvanted vaccine in a low and high dose respectively; group 4 and 5 were treated with chitosan-adjuvanted vaccine in a low and high dose; group 6 and 7 were treated with the CT-B-adjuvanted vaccine in low and high dose; group 8 and 9 were aerosolized with the Stimune-adjuvanted vaccine and group 10 was i.m. vaccinated with Stimune-adjuvanted vaccine.

At the age of 3 weeks chickens were vaccinated by aerosol. Animals were vaccinated in groups in isolators (Beyer & Eggelaar) with a volume of 1.38 m^3 (1.94 m long, 0.75 m wide and 0.95 m high). The vaccine was aerosolized using a Walther Pilot I spray-head with 0.5 mm nozzle (Walther Spritz- und Lackiersysteme), as described previously (Corbanie *et al* 2008). After aerosolizing the vaccine, chickens were left for 1 hour in the isolator to inhale the vaccine. To check whether any of the aerosolized vaccines had adverse effects, chickens were weighed weekly and were checked for adverse effects on eyes and RT right after vaccination and in the week following vaccination. Blood was collected before and 1 and 3 wpv and tracheal swabs were taken 3 wpv. Chickens were killed 3 wpv.

Tracheal swabs

Swabs were put in 350 µl PBS and mixed for 1h at room temperature to elute tracheal swab content. Swabs fluid was used for ELISA.

Avian Influenza Virus ELISA

IDEXX FlockChek Avian Influenza MultiS-Screen Ab Test Kit (IDEXX) was used according to manufacturer's instructions. This ELISA is based on a competitive blocking approach, the specific sample antibodies block the enzyme-labeled, specific antibody in the conjugate. The addition of an enzyme substrate-chromogen reagent causes color to develop. This color is inversely proportional to the amount of bound sample antibody. The more antibodies present in the sample, the less color development in the test wells. Sample/negative (S/N) ratio was calculated as follows: S/N ratio = sample absorbance / negative control absorbance. AIV-specific Ab were determined in serum and in tracheal swab samples. Serum samples were diluted according to manufacturer's instructions. Tracheal swab elutes were used without diluting.

CT-B antibody ELISA

To detect CT-B-specific Ab, a ganglioside M (GM)-1 ELISA was performed as described previously by Stok *et al* (1994, 521-526). Briefly, high bind microplates (Corning) were coated with 2 µg/ml GM-1 (Sigma) at 4 °C overnight. Then 0.2 µg/well CT-B (Sigma) was added and plates were incubated for 1h at room temperature. Serum was added starting at a dilution of 1:50 and 1:1 serially diluted to 1:6,400 and incubated for 1h at 37 °C. Horseradish peroxidase-labeled goat-anti chicken IgG (H+L; Southern Biotech) was added and plates were incubated for 1h at room temperature. Plates were developed using one-step ultra TMB (Pierce). Colour development was stopped using 1M H_2SO_4 and extinction was measured at 450 nm.

Results and discussion

In this study, inactivated AIV antigen with different adjuvants was tested in an aerosolized vaccine for use in a rapid intervention strategy. A vaccine used for rapid intervention vaccination strategies is aimed at stopping an ongoing outbreak. It should therefore induce a rapid protection, ideally after a single vaccination by a mass application method to be effective in the field situation. Aerosol vaccinations were performed in an isolator, using a

Walther Pilot I spray-head. This set-up was used in a previous experiment to aerosolize NDV (Corbanie *et al* 2008) and the spray-head was used previously to aerosolize fluorescent microspheres (Corbanie *et al* 2006). It was found that using this set-up the concentration of NDV in the isolator did not decrease when measured up until 20 minutes after nebulization (Corbanie *et al* 2008) and fluorescent microspheres were present throughout the respiratory tract, including air sacs, at 20 minutes after administration (Corbanie *et al* 2006). Furthermore, in a previous experiment we performed aerosol inoculation with live H9N2 AIV (Reemers *et al* 2009). In another study chickens were inoculated with virus via the intratracheal route and found similar immune responses and similar kinetics of the responses (Rebel *et al* 2011).

Chickens were aerosol-vaccinated at 3 weeks of age with H9N2 WIV adjuvanted with either alum, chitosan, CT-B or Stimune. To determine if the adjuvants had any adverse effects on weight gain of the chickens, birds were weighed weekly. To adjust for growth differences between the sexes, we set the pre-vaccination bodyweight (day 19) at 100% for each individual chicken and used this to calculate relative bodyweights. We found no significant differences in relative body weight between any of the treatment groups (data not shown). Furthermore, we did not observe any respiratory or eye problems right after vaccination and in the weeks following vaccination. The vaccines were therefore safe to use.

From previous experiments using an i.m. applied vaccine, it was determined that 42-128 HAU were needed for an efficient protection against a high-pathogenic H7N7 infection (Maas *et al* 2009). In our experiments, chickens were vaccinated with approximately 75 HAU by aerosol. Chickens were bled before and 1 and 3 wpv to detect AIV-specific serum Ab after the vaccination.

The presence of AIV-specific Ab in serum at 1 and 3 wpv was determined using a well validated ELISA. In this commercial ELISA, the samples containing AIV-specific antibodies have a sample/negative (S/N) ratio <0.5. Pre-vaccination, no AIV-specific Ab were present in serum. From 1 wpv, AIV-specific serum Ab were detected in the i.m. vaccinated group, but not in any of the aerosol-vaccinated animals (Figure 1).

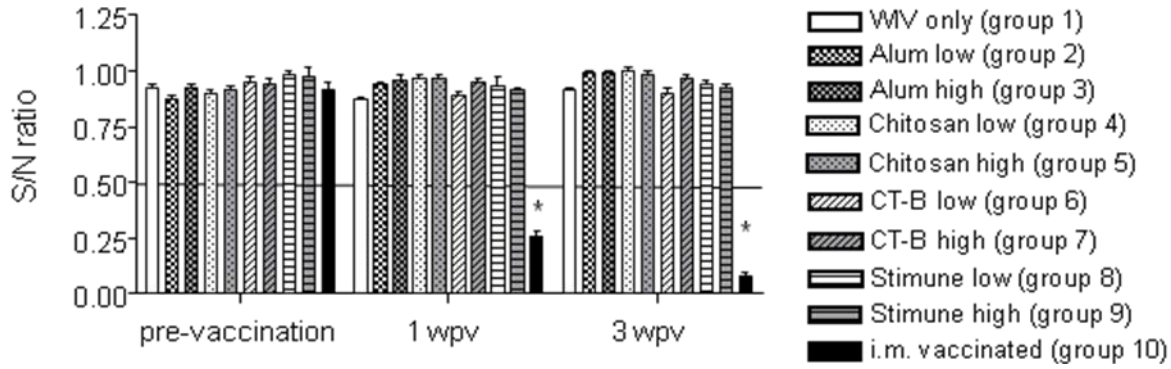

Figure 1. AIV-specific Ab in serum of aerosol-vaccinated chickens. Presence of AIV-specific Ab in serum was determined pre-vaccination, 1 wpv and 3 wpv using the IDEXX multispecies AIV antibody ELISA. Data are shown as mean S/N ratio + SEM. AIV-specific serum antibodies are detected in samples with S/N ratios <0.5.

Primary i.n. vaccination in chicken with 100 HAU split H5N1 virus + sialidase and 0.5% w/v chitosan already induced mucosal AIV-specific IgA responses at 1 wpv as determined in tracheal swab elutes (Worrall *et al* 2009). When the antigen was given with chitosan alone, AIV-specific IgA responses were lower and IgA was not detected at all time points (Worrall *et al* 2009). When chickens were vaccinated at 1 day of age with replication-competent adenovirus-free AIV H7 vaccine via coarse spray, no HI titers were detected in serum, however AIV-specific IgA was detected in tears at day 10 post vaccination (Toro *et al* 2010). To test whether in our experiment Ab were locally induced following aerosol vaccination, AIV-specific Ab were determined in tracheal swab elutes. None of the samples contained detectable levels of influenza-specific Ab (data not shown).

To test if the aerosolized vaccine had entered the RT and had induced immune responses, a CT-B antibody ELISA was performed using sera of chickens vaccinated with CT-B-adjuvanted vaccines. Pre-vaccination no CT-B-specific Ab were detected. When the low dose of CT-B (10 μg/chicken) was given, CT-B-specific Ab were detected at 3 wpv. However, the high dose CT-B (50 μg/chicken) already induced CT-B-specific Ab at 1 wpv and Ab titers were increased at 3 wpv. Vaccination with the high dose CT-B significantly increased the titer of CT-B specific Ab as compared to the low dose of CT-B (Figure 2).

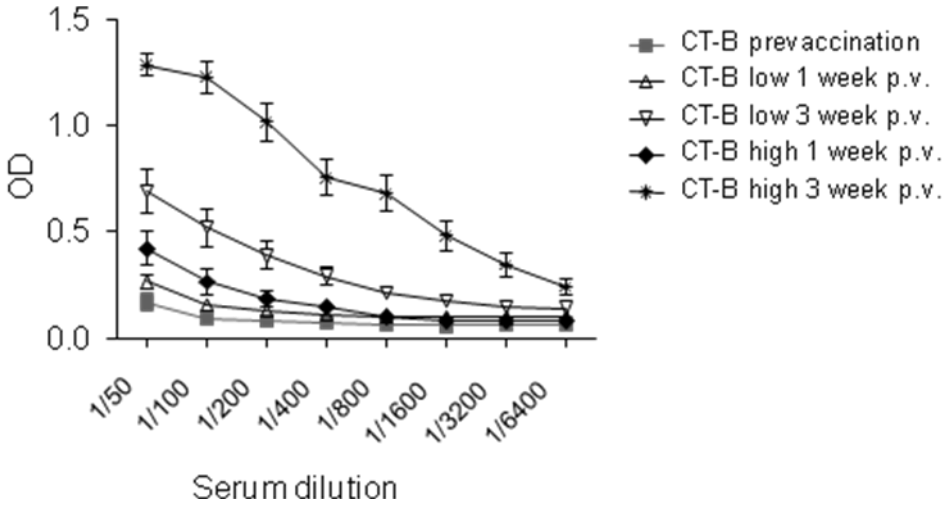

Figure 2. CT-B-specific Ab in serum of chickens aerosol-vaccinated with CT-B adjuvanted vaccine. The presence of CT-B-specific Ab in serum was determined pre-vaccination, 1 wpv and 3 wpv. Data are shown as mean OD +/- SEM.

In summary, although the aerosolized vaccines did enter the chicken RTs, either the dose of influenza virus used was insufficient or a booster vaccination would be needed in order to induce detectable levels of Ab. It has been reported in different mouse studies that i.n. vaccination with adjuvanted WIV (Jo *et al* 2010) or split viruses (Ichinohe *et al* 2005, Ichinohe *et al* 2006, Saluja *et al* 2010) requires a boost vaccination for effective induction of Ab. Furthermore, in chicken LPS-containing liposomal inactivated NDV vaccine also required a booster vaccination for effective induction of serum IgG titers (Tseng *et al* 2009). As we already observed CT-B-specific antibodies in serum at 1 wpv, the aerosol vaccination technique is in principle feasible to use in a rapid intervention strategy. The number of HAU needed for protection was determined from i.m. applied vaccine and therefore in a future experiment we will increase the dose in order to determine the number of HAU needed in a respiratory applied vaccine.

Conflict of interest

The authors declare that they have no conflict of interest.

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CHAPTER 6

Asymmetrical protection of two avian influenza H5N1 vaccines against infection of layer chickens with a heterologous highly pathogenic AI strain

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Background Antigenic differences between Avian Influenza virus strains and vaccines affect the effectiveness of vaccination of poultry.

Objective To determine the relation between the degree of the antigenic relatedness *in vitro,* using cross-HI test results, and protection against virus transmission *in vivo* between AI vaccine and challenge virus strains.

Methods We conducted an experiment to quantify protection of two vaccines, derived from H5N1 AI strain A/Ck/WJava/Sukabumi/006/2008 and A/Ck/CJava/Karanganyar/051/2009 against challenge with the homologous or heterologous strain. We used six groups of 16 layers each. All birds in groups A and B were vaccinated with the Sukabumi strain, in groups C and D with the Karanganyar strain. Birds in groups E and F remained unvaccinated. Four weeks later, half of groups A, C and E were challenged with Sukabumi strain; half of groups B, D and F with Karanganyar strain. Oropharyngeal and cloacal samples were taken daily for ten days after inoculation, and analysed using egg culture. Serum samples were collected at challenge and the end of the trial.

Results No transmission or virus shedding occurred in groups A and D. Group C was also protected against challenge with the Sukabumi strain; group B was only partly protected against challenge with and transmission of the Karanganyar strain, as virus shedding was not reduced and transmission to contact birds occurred.

Conclusion This study showed asymmetrical cross-protection between two H5N1 virus strains, which implies that extrapolation of *in vitro* data to clinical protection and reduction of virus transmission might not be straightforward.

Introduction

Since 1997, highly pathogenic avian influenza (HPAI) H5N1 strains have circulated in several Asian countries (Sims *et al* 2003, Eagles *et al* 2009, Lupiani & Reddy 2009). Common control measures such as stamping out of infected flocks, depopulation of contiguous flocks, and movement restriction were effective with regard eradication of virus in several countries (Yee *et al* 2009, Swayne *et al* 2011) but in countries like Indonesia, Vietnam, Egypt, and China the disease has become endemic (Peyre *et al* 2009, Kim *et al* 2010, Swayne *et al* 2011).

One of the additional measures to control the disease in these countries is the application of vaccination (Ellis *et al* 2004, Siregar *et al* 2007, Swayne & Kapczynski 2008, Swayne *et al* 2011) mainly aiming at the prevention or reduction of clinical signs and production losses in case of virus incursion in a flock (Suarez 2005, Swayne 2006). Despite wide-scale vaccination, however, large outbreaks of HPAI in flocks still occur, sometimes resulting in high mortality rates (Sims 2007, Bouma *et al* 2008). Poor biosecurity during vaccine administration, inappropriate vaccine application, limited vaccination coverage, and antigenic drift of virus strains are some factors considered for the limited efficacy of vaccine programmes (Siregar *et al* 2007, Sims 2007).

Since the first outbreaks of H5N1 in Indonesia in 2003, antigenic drift of virus strains has been demonstrated, possibly facilitated by suboptimal vaccination practices (Domenech *et al* 2009, Dharmayanti *et al* 2011, Cattoli *et al* 2011, Grund *et al* 2011). Avian influenza A isolates have been classified based on their cross-reaction in a haemagglutinin inhibition (HI) test using different antisera. Based on these results, an antigenic relationship can be defined by antigenic relatedness using the Archetti-Horsfall ratio (Archetti & Horsfall 1950, Lee *et al* 2004, Ndifon *et al* 2009, Beato *et al* 2010), or, in case of high dimensional data, by antigenic cartography (Smith *et al* 2004, Mumford 2007, Fouchier & Smith 2010).

Some of the currently circulating virus strains isolated in Indonesia differ antigenically and genetically from the strains used in the locally produced vaccine (Indriani *et al* 2011). These strains were isolated from clinically affected flocks, suggesting suboptimal protection by vaccination. Because of recent clinical outbreaks and the isolation of new variants, it has been suggested that avian influenza (AI) vaccine seed strains need to be updated when there is at least a fourfold difference in HI titers between the vaccine strain and

the reference strain from a recent field virus isolate (Fouchier & Smith 2010, Pfeiffer *et al* 2010).

The antigenic relationship, however, have mostly been determined *in vitro*, and there is limited information whether this method adequately reflects clinical protection and protection against infection *in vivo* (Ndifon *et al* 2009, Beato *et al* 2010, Ducatez *et al* 2011, Abbas *et al* 2011). Moreover, the major goal of vaccination against AI in poultry preferably is to prevent spread of the virus rather than inducing protection against clinical signs, as this could aid eradication efforts and thereby contribute strongly to reduction of economic losses and reduction of health risks by human exposure.

The aim of our study was therefore to determine the relation between the degree of the antigenic relatedness *in vitro*, expressed by the *r* value, based on cross-HI data,19 and protection against shedding and transmission *in vivo* between AI vaccine and challenge virus strains. By doing so this study contributes to elucidating the relation between the antigenic relation determined *in vitro* and protection against challenge and transmission of H5N1 AI virus field strains in poultry.

Methods

The experiment complied with article 80 on "Research in Animal Health" of the Indonesian "Law on Livestock and Animal Health UU/18/2009".

Chickens and housing

Specified pathogen free (SPF) layer chickens were obtained from PT. Vaksindo Satwa Nusantara, Bogor, Indonesia. Birds were housed at PT Vaksindo Biosafety Level-3 (BSL-2) facility in one experimental unit. Birds were fed with a commercial ration, and had tap water ad libitum.

Vaccines and challenge virus strain

Two HPAI H5N1 viruses isolated in Indonesia were used: A/Ck/Wjava/Sukabumi/006/2008 (referred to as Sukabumi strain), and A/Ck/Cjava/Karanganyar/051/2009 (referred to as Karanganyar strain), provided by PT. Vaksindo. These strains were isolated after a clinical outbreak of AI in vaccinated flocks. The flock from which the Sukabumi strain was isolated had been vaccinated with an H5N1 strain; the flock from which the Karanganyar strain was

isolated with an H5N2 strain (pers. comm. Bharoto, Vaksindo PT). The relationship between the strains is shown in Figure 1.

For vaccine preparation, both strains were grown on 10-days-embryonated SPF chicken eggs. The allantoic fluid was harvested and inactivated using 0.2 % v/v formaldehyde. The vaccines were subsequently formulated by adding Montanide TM ISA 70 VG (SEPPIC) adjuvant (SEPPIC 2013). The vaccine was administered once intramuscularly in the breast muscle using 0.5 ml vaccine containing 512 haemagglutination units (HAU) per bird.

The challenge strains were the same strains as used for vaccination. Inoculation was done with 0.2 ml containing approximately 106 median egg infectious dose (EID50) per ml; 0.1 ml was administered intranasally and the other intra-tracheally. During eight hours after inoculation, inoculated birds were put in an empty cage.

Experimental design

The experiment consisted of six groups (A-F) of 16 chickens. Each group was housed in a separate cage, each consisting of two levels (2x98cmx70cmx50cm). Birds in groups A and B were vaccinated with Sukabumi vaccine; birds in groups C and D were vaccinated with Karanganyar vaccine strain; birds in groups E and F consisted of unvaccinated birds. Four weeks after vaccination (p.v.), eight birds in the vaccine groups A and C, and also in control group E were challenged with Sukabumi; eight birds in the vaccine groups B and D, and the control group F were challenged with Karanganyar. The other half of each group was contact-exposed. Inoculated birds are referred to as *I* birds; contact-exposed birds as *S* birds (Table 1). Five sentinel SPF layer birds were housed between the cages to determine whether events in each cage could be considered as independent observations.

After inoculation, clinical signs were recorded. Swab samples were collected daily from the cloaca and the oropharynx for 14 days, and stored in duplo at -70ºC until further testing. Blood serum samples were collected from the wing vein two days before challenge and at the end of the experiment, and stored at -20ºC until further testing.

The presence of AI virus in swabs was determined by virus isolation according to standard procedure (OIE 2009). Briefly, three embryonated SPF chicken eggs were inoculated with 0.2 ml swab medium per egg. After 72 h the allantoic fluid was harvested. A haemagglutination assay (HA) was performed.30 When at least one of the eggs was positive in the HA, the swab was considered to be virus positive (Bouma *et al* 2009, Poetri *et al* 2009).

Serum samples were tested in a HI test according to standard procedures.30 The tests were carried out in duplo in one run using 4 HAU of the Sukabumi or Karanganyar strain as antigen. The HI titer was expressed as the serum dilution that caused complete inhibition of agglutination.

Antigenic relatedness

The antigenic relatedness between the virus strains was evaluated using the formula described by Archetti and Horsfall (1950), based on HI data. The degree of the antigenic relatedness, indicated by the *r* value, was determined for both strains. The HI titer was determined in samples collected 2 days before inoculation. Pairs of HI titers were made by randomly selecting an HI titer from a bird vaccinated with Sukabumi, and from a bird vaccinated with Karanganyar. The titer ratio for the Sukabumi strain was calculated by dividing the normalized heterologous HI titer obtained with Karanganyar strain as antigen in the test by the normalized homologous titer obtained with Sukabumi strain as antigen. Likewise, the ratio for Karanganyar was calculated by dividing the normalized heterologous HI titer in serum obtained with Sukabumi by the normalized homologous titer obtained from chickens vaccinated with Karanganyar. The titer ratio of Sukabumi (*rs')* or Karanganyar (*rk')* was calculated for each bird, and the geometric mean of the ratio per bird pair was calculated as: $r' = \sqrt{rs'.rk'}$ (Archetti & Horsfall 1950).

The general consensus is that viruses related serologically have *r* values larger than 50% (Archetti & Horsfall 1950, Lee *et al* 2004) and influences vaccine efficacy in terms of clinical protection and virus shedding (Shortridge *et al* 1998).

Data analysis

One way ANOVA was carried out to test the difference of HI titer between experimental groups with HI titer as dependent variable and antigen as independent variable. ANOVA was performed using SPSS 17.0 (SPSS Inc.,Chicago, Illinois).

Transmission rates in the control group were analysed using a Generalized Linear Model (Van Der Goot *et al* 2005). Briefly, we assumed that daily number of cases are binomially distributed with binomial totals given by the available number of susceptible birds on the previous day, and with the infection probability determined by the transmission rate parameter β (unit: *per day*) and prevalence of infectious birds (on the previous day). We analysed scenarios with a latent period of 1 or 2 days, and report results from the best fitting

model. Confidence bounds (95%, equal-tailed) of the parameter estimates were based on chi-squared approximations of the profile likelihood.

Maximum likelihood estimates of the infectious period in experiments with unvaccinated birds were obtained by assuming that the infectious periods follow a normal distribution, taking into account interval censoring of the observations. Other two-parameter continuous distributions (log normal, gamma) yielded similar results (data not shown).

Estimates of the overall transmissibility of the virus were given by the basic reproduction number, here defined as the product of the infectious period and the transmission rate parameter: $R_0 = \beta T$. Hence an estimate of the basic reproduction number is given by the product of the estimates of the transmission rate parameter and infectious period: $\hat{R}_0 = \hat{\beta}\hat{T}$. Notice that this formulation makes the implicit assumption that each bird makes a fixed expected number of contacts with other birds per unit of time regardless of population composition (Van Boven *et al* 2007).

Results

Serological response and virus shedding

Group A (vaccine Sukabumi/ inoculation Sukabumi). The mean HI titer was $2^{3.9}$ against the homologous antigen and $2^{1.9}$ against the heterologous antigen at time of challenge. Four weeks p.c., the mean HI titer against the challenge virus was $2^{4.4}$. The mean HI titer to both antigens differed significantly at time of challenge and at the end of the experiment ($p < 0.05$). None of inoculated or contact exposed birds shed virus and all survived.

Group B (vaccine Sukabumi/ inoculation Karanganyar). The mean HI titer was $2^{3.6}$ against the homologous and $2^{0.9}$ against the heterologous antigen at time of challenge. Three inoculated and one contact-exposed bird showed a four-fold increase against Karanganyar strain: the mean HI titer was $2^{5.1}$ at the end. There was no association between HI titer against Sukabumi or Karanganyar at time of inoculation and probability of infection. The mean HI titer to both strains differed significantly at time of challenge ($p < 0.05$), but did not differ significantly post challenge ($p > 0.05$). Six inoculated and four contact birds shed virus. Four inoculated birds died within 4-7 days p.c.; three contacts died after 10 days p.c.

Table 1. Overview of the experimental design, serological responses and HI ratio. Virus strains are A/Ck/WJava/Sukabumi/006/2008 (H5N1) and A/Ck/CJava/Karanganyar/051/2009 (H5N1) (in Table 1 referred to as SMI and KRA, respectively). For the statistical analyses, the HI data at D0, from groups A and B, and from C and D were combined, as birds in these groups received the same treatment.

			Mean HI titer (^{2}log)					
	Virus strain		At challenge (D0)[b]		End of the experiment (D28)[c]		HI titer ratio (95% CI)[e]	
Group	Vaccination	Inoculation	SMI[d]	KRA[d]	SMI	KRA	SMI	KRA
A (Vs/Is)	SMI	SMI	3.75±1.2^A	1.4±1.2^B	4.4±1.5 ^{A}	2.1±1.5 ^{B}	0.25 (0.18-0.33)	0.63 (0.45-0.81)
B (Vs/Ik)	SMI	KRA			6.8±2.4 ^{A}	5.1±3.5^A		
C (Vk/Is)	KRA	SMI	4.6±0.9^A	5.7±0.9^B	5.3±1.0 ^{A}	6.1±0.7 ^{B}		
D (Vk/Ik)	KRA	KRA			5.2±1.1^A	5.4±1.0 ^{A}		
E (UV/Is)	Unvaccinated	SMI	0	0	1.5±0.3	0		
F (UV/Ik)	Unvaccinated	KRA	0	0	nd	nd		

[a] The data are mean HI titers (^{2}log) ± standard deviation; nd=not determined
[b] 4 week post vaccination ; [c]4 week post challenge
[d] The strain used in the HI test as antigen
[e] Ratio: heterologous titer divided by homologous titer; The ratio was calculated using mean HI titer at time of challenge
[A,B] Values with different superscript within row indicate a statistical difference significant ($p < 0.05$).

Group C (vaccine Karanganyar/ inoculation Sukabumi). The mean HI titer against the homologous antigen was $2^{6.1}$ and $2^{4.6}$ against the heterologous antigen at time of challenge. Two inoculated birds showed a fourfold increase in HI antibodies to Sukabumi strain. The mean HI titer to Karanganyar strain was significantly higher than the titer to the Sukabumi strain, both at time and after challenge ($p < 0.05$). Virus transmission was completely halted, and none of the birds shed virus.

Group D (vaccine Karanganyar/ inoculation Karanganyar). The mean HI titer was $2^{5.3}$ against the homologous and $2^{4.6}$ against the heterologous antigen at time of challenge. None

of the birds showed a fourfold increase to Karanganyar challenge virus. The mean HI titer to both strains did not differ significantly, neither before nor after challenge ($p > 0.05$). Virus transmission was completely halted, and none of the birds shed virus.

Groups E and F (control). None of the control birds had antibody titers against the virus strains at time of challenge. In group E, five inoculated birds and four contact birds shed virus for 1-2 days p.c. Eight inoculated and six contact birds died within 2-7 days p.c. Of these birds, three inoculated and two contact birds never tested positive in the virus isolation test. In group F all inoculated and contact-exposed birds shed virus for 1-3 days p.c. and all virus-positive birds died within 3-7 days p.c.

Virus transmission and antigenic relatedness

No virus transmission occurred in groups that were vaccinated and subsequently infected with the homologous strain. Also in the group vaccinated with Karanganyar and challenged with Sukabumi no virus transmission occurred. In the control groups and group C (vaccination Sukabumi/inoculation Karanganyar), transmission was observed. For the control group challenged with Sukabumi, we assumed a latent period of 2 day. The transmission rate parameter β was estimated to be 2.4 / day. The infectious period T was estimated to be 0.7 (day (sd 0.3) and the reproductive rate 1.7. For the control group challenged with Karanganyar, we assumed a latent period of 1 day; β was estimated to be 2.5/d. The infectious period T was estimated to be 1.6 (day (sd 0.3) and the reproductive rate 4.1. The ratio of Sukabumi (*rs*) was 0.25 (95% CI: 0.182-0.326), and of Karanganyar (*rk*) 0.63 (95% CI: 0.45-0.81); the overall average antigenic relatedness was $r\hat{} = 0.37$ (95% CI: 0.287 0.448), indicating a low level of antigenic relatedness between the strains, mainly due to the low ability of Sukabumi sera to inhibit agglutination by Karanganyar virus (Table 1).

Discussion

The aim of this study was to determine whether *in vitro* antigenic relatedness between two H5N1 AI vaccine strains was associated with the efficacy of these vaccine strains *in vivo*. *In vivo* antigenic relatedness between the two strains was estimated at 37% suggesting a poor efficacy of both vaccines against the heterologous challenge. This was indeed so for the vaccine based on the Sukabumi strain, as virus shedding and transmission occurred. The vaccine based on the Karanganyar strain, however, provides protection against infection with

Sukabumi, as no transmission was observed. These findings suggest that it might be difficult to decide if and when vaccines need to be updated, and to determine in advance, based on *in vitro* results, whether updated vaccines are indeed able to provide more protection than 'outdated' vaccine strains.

Cross-reactivity between AI virus strains in general could be explained by the presence of common antibody epitopes in the HA protein (Sui *et al* 2009, Ekiert *et al* 2009, Ekiert *et al* 2012) but does not explain the asymmetrical response. Asymmetrical crossprotection was described by Lin *et al* (2013) for a swine H1N1 strain, which was explained by the presence of adjuvant in the vaccine. Boon & Webby (2009) mentioned that strains from one clade induced a broad cross-reactive response than strains from another clade suggesting that a single vaccine antigen is unlikely to produce the desired coverage of circulating strains. An explanation for the asymmetrical response in our experiment could be that the HA protein of Sukabumi strain was less immunogenic than HA of Karanganyar strain. Another possibility is that the Karanganyar isolate contained a broader suite of minor genetic variants than Sukabumi resulting in a wider spectrum of protective antibodies in chickens. It is also possible that Karanganyar strain evolved from Sukabumi-like strains and was possible to evade immunity elicited vaccination by Sukabumi.

The *in vitro* results corresponded with *in vivo* results, which was also found in a study by Ndifon *et al* (2009). The Sukabumi vaccine induced a lower HI titer in chickens compared to the Karanganyar vaccine strain. Antibody titers above 2^5 has been shown to be sufficient to induce protection against infection (Abbas *et al* 2011, Philippa *et al* 2007). In the group vaccinated with Sukabumi, 6 birds had an HI titer > 2^5 against the homologous strain, but none against Karanganyar, which could explain the lower level of *in vivo* protection. All birds in the Karanganyar vaccine groups had an HI titer > 2^5 against the homologous challenge virus and 9 birds against Sukabumi strain. All birds in group C (vaccination Karanganyar/inoculation Sukabumi) had significantly higher titers against the heterologous strain than birds in group B (vaccination Sukabumi /inoculation Karanganyar) group. Virus shedding was only seen in Sukabumi vaccine group. Although we cannot give an explanation for the low HI titers against the heterologous strain in the Sukabumi vaccine groups, this finding might explain the observed asymmetrical protection as well.

The emergence of antigenic variants in Indonesia has been documented since 2007 (Domenech *et al* 2009, Dharmayanti *et al* 2011) which may have occurred due to immunological pressure of vaccination (Lekcharoensuk 2008, Dharmayanti *et al* 2011, Cattoli *et al* 2011). The two strains did not differ significantly in transmission rate

characteristics in the control groups. The latent period in the Sukabumi vaccine group challenged with Karanganyar was longer than in the unvaccinated group challenged with Karanganyar, suggesting that the Karanganyar strain needed adaptation before replicating, although this did not seemed to have altered the efficiency of transmission. The Sukabumi and Karanganyar strains were isolated from clinical outbreaks in flocks vaccinated with an H5N1 and H5N2 strain respectively. Whether and how this has affected the outcome of the experiment, or affected the emergence of antigenic diversity between the two strains remains unclear.

Vaccination can only be successful if the antigenicity of the vaccine strain matches with circulating virus (Beato *et al* 2010, Smith *et al* 2004, Fouchier & Smith 2010). An alternative way to determine whether influenza vaccines need to be updated is based on antigenic cartography. We did not have sufficiently detailed data to put our isolates on an AI map exactly, making it rather difficult to use this map. In addition, for AI it is easy to make use of sera from vaccinated chickens to determine cross-reaction against various isolates, providing r-values. This method may be easier to provide relevant information on vaccine efficacy than the cartography map. Our current study, however, also demonstrated that extrapolation from *in vitro* results to the field might not always be straightforward, and yet animal experiments are still needed to determine vaccine efficacy.

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Figure 1. Phylogenetic tree of various AI isolates

The evolutionary history was inferred using the Neighbor-Joining method.43 The optimal tree with the sum of branch length = 0.39771100 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Tamura-Nei method (Tamura & Nei 1993) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 48 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1669 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al* 2011).

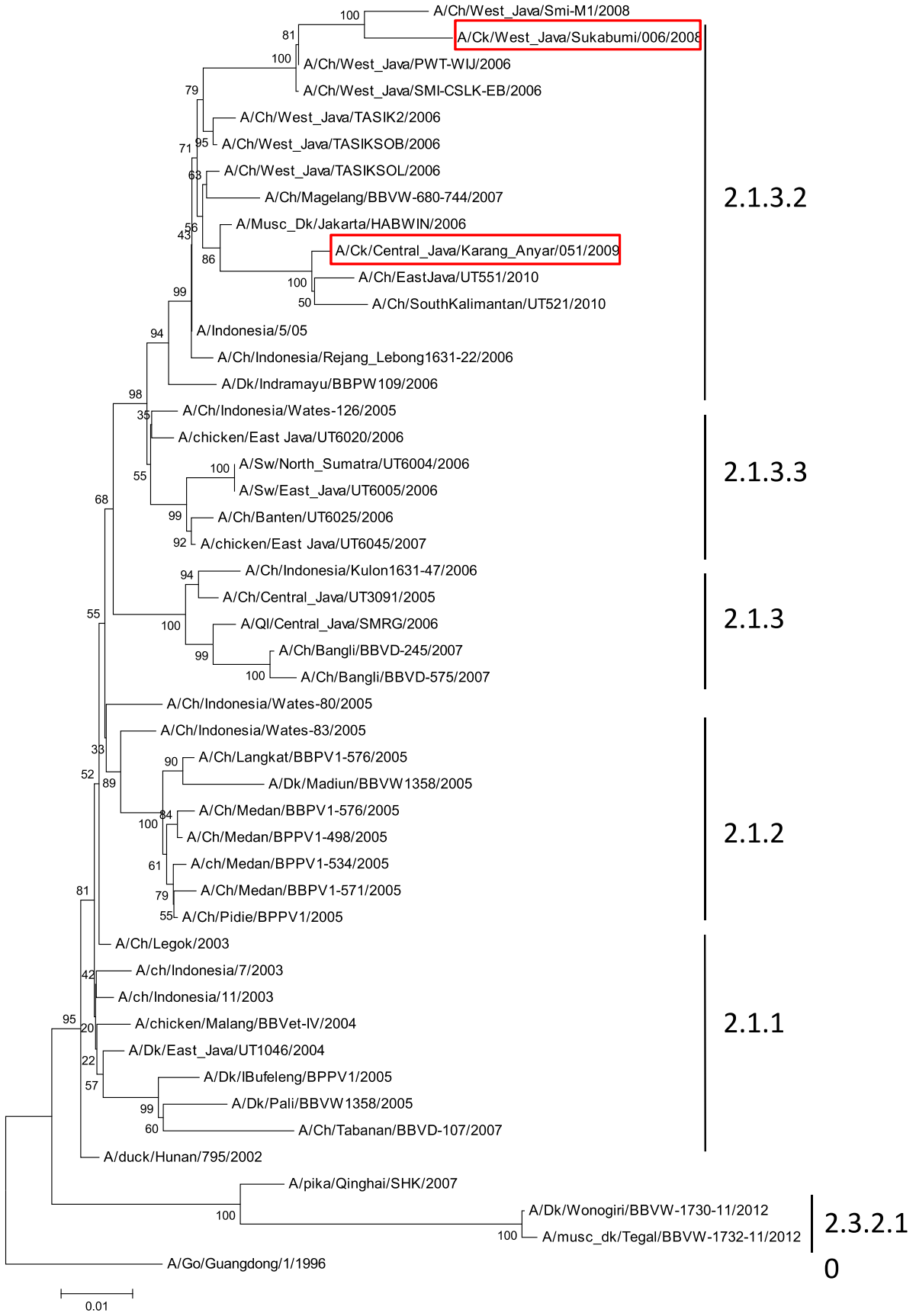

Figure 1 Phylogenetic tree of various AI isolates

CHAPTER 7

General Discussion

Introduction

The first outbreak of highly pathogenic avian influenza (HPAI) virus subtype H5N1 was reported in Hongkong in 1997, but the first isolate was obtained in Guangdong, China (Xu *et al* 1999). Since then, virus spread to various other Asian countries such as Vietnam, Thailand and Indonesia (Sims *et al* 2003, Eagles *et al* 2009, Yee *et al* 2009), and some European and African countries. Eradication of the virus in Hongkong, Thailand, and European countries was achieved by depopulation of infected and contiguous flocks, movement restrictions and stringent biosecurity measures. The disease is still known to be present in some South-east Asian countries (Sims 2012, Swayne 2012, OIE 2013).

For several reasons, related to cultural, financial and logistical factors, large-scale culling was not applied in Indonesia, and the disease became endemic in most of the provinces (FAO 2011, Sims 2012, DGLS 2013a). Because of the epidemiological situation, vaccination was introduced as one of the control measures, and implemented in all poultry sectors including back yard flocks. The main goal of vaccination was, if virus incursion occurred, to protect flocks against clinical signs, in order to reduce production losses, and to reduce the human health risk by reducing virus shedding. However, an even more important requirement for a successful vaccination campaign against such a severe poultry disease, also being a zoönosis, should be to reduce virus transmission within a region to such a level that the virus may be eradicated (Sims 2007, Capua & Marangon 2007).

Most of the vaccines used in Indonesia are locally produced. Since the production of the first vaccines in 2003, the quality has improved, and now seems to be effective in inducing protection against clinical signs, as for example demonstrated in vaccination-challenge experiments and in transmission experiments, mainly with specified pathogen free (SPF) layer birds (Bouma *et al* 2008, Bouma *et al* 2009). Nevertheless, the virus is still present, despite large-scale vaccination programmes, and outbreaks in vaccinated flocks still occur (Wibawan 2012, DGLS 2012), although infection seems not to result in high mortality rates. Various explanations for the presumed vaccine failure in commercial flocks have been suggested (Domenech *et al* 2009, Kim *et al* 2010, Swayne *et al* 2011, Swayne 2012), but no clear causes have been identified yet. In addition to this information, various overviews of requirements that need to be fulfilled to launch an effective vaccination campaign have been published (Hinrichs *et al* 2010, Swayne *et al* 2011, Swayne 2012, Sims 2012).

This thesis provides more knowledge of the effectiveness of vaccination of chickens against HPAI H5N1 in Indonesia, especially with respect to transmission by conducting

vaccine efficacy studies. The goal is to provide more insight in vaccine effectiveness and causes of vaccine failure, knowledge that might be used to improve the current vaccination programmes in Indonesia. In this chapter, the results of various studies, described in Chapters 2-6, are discussed following the guidelines described by Hinrichs *et al* (2010). Factors that will be described are related to vaccine quality, vaccination strategy, surveillance programmes and incentives of HPAI H5N1 vaccination in Indonesia. This might provide information that will be needed to develop future strategies to control HPAI H5N1 in Indonesia and other Asian countries.

Characteristics of commercial HPAI H5N1 vaccines in Indonesia

Route of vaccine administration

All current vaccines contain inactivated virus, and therefore need to be formulated with adjuvant and administered by injection, which is time consuming and costly. In Indonesia, back yard flocks are vaccinated by local official veterinarians from the Indonesian Government. Vaccination coverage in this sector is generally low and reasons for this low coverage might be the large area in which these veterinarians have to operate meaning that only a few households can be visited per day, in combination with the necessity to catch free range chickens. In contrast, poultry farmers in other sectors have their own personnel to do this job which may reduce costs. Intramuscular administration is however rather laborious and also requires skilled operators. Inappropriate injection may harm birds and leads to vaccine failure.

It would be more convenient if vaccines could be delivered by other routes of administration, and this may be cheaper and less harmful for birds. Spray vaccination is a vaccination method which is usually carried out for other poultry diseases like Newcastle disease (ND) (Gough & Alan 1976, Yadin 1981) and Infectious Bronchitis (IB) (De Wit *et al* 2000). This route of vaccination requires, however, live virus, resulting in infection and virus replication in the respiratory tract to evoke a proper induction of immunity. Up to now, this is not feasible for AI vaccines, mainly because live influenza virus vaccines are considered not to be safe neither for poultry nor for humans, as low pathogenic influenza viruses may mutate to highly pathogenic strains, and because AI of H5 and H7 subtype have a zoonotic potential.

Several concepts have been tried to overcome the risks associated with the use of live vaccines. Administration of inactivated vaccines by spray with the low pathogenic avian influenza (LPAI) H9N2 strain was not successful, as it did not induce detectable levels of

antibodies or protection against challenge with H9N2 strain (De Geus *et al* 2011, Chapter 5). Alternatively, influenza virus genes are inserted into the genome of another virus, the so called vector virus. The vector virus is infectious and can be applied via natural routes. Examples of AI vector vaccines are Trovac AI H5, in which the H5 gene is inserted in the fowl pox virus as vector (Bublot *et al* 2006, Meeusen *et al* 2007), or Vectormune HVT-AI vaccine consisting of turkey herpesvirus (HVT) as vector with the HA gene of AI H5N1 virus as insert (Rauw *et al* 2012, Soejoedono *et al* 2012). Trovac induces a solid response even in the presence of maternally derived antibodies (MDA) to influenza but still needs to be injected (Fuchs *et al* 2009, Steensels *et al* 2009). HVT-AI injected at day old has been shown to protect birds in the presence of MDA against AI challenge under experimental conditions, but so far it has not been widely used in the field (Rauw *et al* 2012). Interference may occur because of (maternal) immunity against the vector. Newcastle disease virus (NDV) vectored vaccines for chickens which expressed the HA and NA from A/goose/Guandong/1996 (Ge *et al* 2007, Qiao *et al* 2009, Qiao *et al* 2006) were produced and widely used in China. Thus live ND vector vaccine can be mass applied by spray but the use of this vaccine has been abandoned among others because of interference by existing immunity against ND. Vector vaccines allow for using a DIVA system as antibodies against influenza proteins other than HA are an indication of an influenza virus infection (Spackman & Swayne 2013). Vector vaccines have not been used in Indonesia so far (TROBOS 2011, DGLS 2011).

Alternative routes for inactivated vaccines have been investigated as well. One study has been published in which intranasal administration of an inactivated AI vaccine was examined (Worral *et al* 2009). This study demonstrated that the inactivated AI vaccine, which consisted of three homologous HPAI H5N1 strains in combination with bacterial sialidase of *Clostridium perfringens* type A 107, administered intranasally, was able to induce production of IgA and IgG, and reduced the incidence of clinical signs in intensively reared poultry. Unfortunately, intranasal application of such a vaccine is still laborious.

Vaccine efficacy

There are several factors that may affect vaccine efficacy in poultry flocks such as chicken breed and type of poultry production, levels of MDA or the presence of other diseases at time of vaccination. The ability of the host to respond to AI infection or vaccination varies greatly between species as demonstrated for chickens, turkeys, ducks, pheasents, teal and other species (e.g. Suarez & Cherry 2000, Alexander 2000, Marangon & Busani 2006, Van der

Goot *et al* 2007, Bos *et al* 2008). Most of the studies carried out to show species differences focused on protection of individual birds by measuring clinical protection, virus shedding and HI antibody titers.

However, the most important aim of vaccination against HPAI is to eradicate the virus, because of the devastating effects on poultry production systems and to reduce risk of infection of humans. From traditional vaccination-challenge experiments, this cannot be determined properly. Transmission experiments may provide this essential information (De Jong and Kimman, 1994, Velthuis *et al* 2007). In few studies also the effect of vaccination on transmission of HPAI virus has been studied (Van der Goot *et al* 2005, Bos *et al* 2008). These transmission studies were carried out with H7N7 or H7N1, and only few have been carried out with H5N1 (Spekreijse *et al* 2011, Bouma *et al* 2009). In addition to these studies, transmission studies described in this thesis were carried out with various chicken types: native chickens (Chapter 2), broilers (Chapter 3), and layers (Chapter 4).

Native chickens

In general, native chickens are not vaccinated against any disease, and thus are considered to be highly at risk for AI virus infections. Sources for virus incursion in native chicken flocks are assumed to be spill over from infected neighbouring commercial flocks (Sedyaningsih *et al* 2006, Tiensin *et al* 2005), and, probably to a lower degree, migratory birds (Sumiarto & Arifin 2008, Beato & Capua 2011).

It was suggested that native chickens respond inadequately to vaccination (FAO 2008a). In addition, it was hypothesised that these breeds are not or less susceptible to infection (GRAIN 2006). To test these hypotheses, a transmission experiment was carried out (Chapter 2). The results showed that native chickens were not resistant to infection, and that the course of the infection in native chickens was comparable to that in layer chickens. Virus transmission among unvaccinated native chickens was efficient as the reproduction number was estimated to be 12 (4.7-28.7), and all unvaccinated native chickens (inoculated and contact birds) died 2-6 days post challenge. Moreover, native chickens also responded to AI vaccination, applied under experimental conditions. Native chickens vaccinated twice with an inactivated H5N2 vaccine developed a rather high mean HI titer of $2^{7.9}$ (range 2^3-2^{10}) against H5N1 antigen. After challenge with a HPAI H5N1 strain these birds were protected against virus shedding, clinical signs and did not produce contact-infection. Reduction of transmission occurred to such a level that eradication should be possible as transmission between vaccinated native chickens was completely halted. These results were similar to

those performed in SPF layer chickens, as carried out by others (Bouma *et al* 2009, Spekreijse *et al* 2011). Consequently, the study in Chapter 2 also showed that native chickens are responsive both to infection and vaccination.

Broilers

In contrast to commercial layer and breeder chickens, most commercial broilers are not vaccinated routinely against AI, but routine vaccination programmes against other diseases e.g. ND and IB are operational. Due to the short production cycle of broilers (4-5 weeks) vaccination is assumed not to be effective, but this was not demonstrated in challenge experiments or field studies.

In chapter 3, the results of a transmission experiment with commercial broilers, obtained from a flock vaccinated against AI, are presented, showing that a single vaccination with an inactivated H5N1 vaccine at day of hatch or ten days of age was not effective. Only very low HI titers (mean HI titer: $2^{0.8}$; range 2^0-2^3) were induced, and virus transmission occurred extensively. Day-old chicks were partly protected against infection, probably due to the presence of MDA. This phenomenon has been seen more often, not only for AI (Maas *et al* 2011), but also for IB (De Wit 1998, De Wit 2000), and ND (Van Eck *et al* 1991). The advantage of the presence of MDA for protection of young animals against infection is a disadvantage for poultry producers who wish to vaccinated birds early in the production period. Vaccine manufacturers need to invent a vaccine that may circumvent MDA interference, as the mechanism that prevents effective viral antigen replication will interfere with induction of antibodies. Repetition of vaccination, to evade the interference of MDA eventually resulting in an adequate immune response, is an option, as for example seen for ND (Huang *et al* 2004) and IB (Davelaar *et al* 1977, De Wit 2000), but at the time immunity is fully developed broilers are ready to be slaughtered. Thus as vaccination practice this is not feasible in commercial broiler production systems.

Considering the short production period for broilers, and the small number of contacts between this type of flock and other types of poultry flocks, it is not unreasonable to assume that the probability of a broiler flock to become infected will most likely be lower than for layer or breeder farms. For LPAI this has been demonstrated by Gonzales *et al* (2012) and for HPAI H7N7 Thomas *et al* (2005) demonstrated a higher risk for layers than for broilers. Nevertheless, outbreaks have occurred as demonstrated in a field study on poultry collector houses (IDP 2012). Poultry farmers should therefore implement measures to reduce the probability of incursion of AI virus.

Layers

In Chapter 4, it was shown that a single vaccination with an inactivated H5N1 vaccine of commercially kept layer chickens induced low to medium HI titers to H5N1 antigen (range 2^0-2^5). Despite the low HI titer, birds were protected against clinical signs and mortality, but most of the vaccinated birds showed a four-fold seroconversion after challenge and after exposure to inoculated pen mates. A four-fold seroconversion (measured with paired sera) is generally considered as indication of infection after virus exposure (Buckley *et al* 2006, Leschnik *et al* 2007, Van Boven *et al* 2008), and therefore "seroconversion" was used as parameter for infection. Consequently, the results in Chapter 4 indicated that a single vaccination of commercial layers under field conditions induced clinical protection, but was not sufficient to induce high levels of HI antibodies, and did not prevent transmission, implying that "silent spread" of virus might occur among vaccinated birds. The study also showed that challenge experiments with SPF birds, usually done to demonstrate the efficacy of vaccination before a new vaccine or seed strain is released, likely does not accurately demonstrate vaccine effectiveness in the field, as for example shown in a study by Bouma *et al* (2008) in which it was shown that a single vaccination of SPF layers with a H5N1 vaccine induced a sufficient level of HI antibody titer (2^5) (Philippa *et al* 2007).

Comparison between poultry types

Extrapolation of the results shown in Chapters 2-4 suggests that vaccination gives better results in native and layer chickens compared to broiler chickens, as vaccination provides clinical protection and prevention of mortality in native chickens and layers. It also reduced transmission of AI virus in native chickens, which did not occur in vaccinated broilers. Of course, a comparison between results obtained in different experiments carried out at different times and different locations should be done with caution. An explanation for the difference between broilers and native chickens is the presence of MDA at time of vaccination. The native chickens in chapter 2 were vaccinated at the age of 4 weeks, and at that time MDA were assumed to have disappeared. The broilers were vaccinated at hatch or at day 10 of life. MDA in general persist in birds for approximately 10-21 days after hatch (King *et al* 2010), but for broilers this period might be shorter, mainly caused by the rapid growth (Van den Berg & Meulemans 1981), resulting in undetectable levels of MDA at 10 days of age (Gharabeih & Mahmoud 2013). For AI it has been demonstrated that MDA titers are already low at 7 days after hatching (Ka Oud *et al* 2008). It has been demonstrated that MDA interfere with vaccine efficacy, for example for AI H5N1 (Maas *et al* 2011, Faulkner *et*

al 2013), but also for other diseases (Klipper *et al* 2004, Ganapathy *et al* 2006), like for example for ND (Rauw et *al* 2009) and IB (Terregino *et al* 2008).

Another likely explanation for the ineffective AI vaccination in broilers is the presumed weaker immune system of broiler. Broilers have been bred for growth characteristics and this seems to have reduced and modified the capability to respond with a proper humoral and cellular immune response after vaccination or infection (Koenen *et al* 2002). Another study by Parmentier *et al* (2010) showed, however, that there was no difference between broilers and layers during first 6 weeks of age, kept under similar conditions, with respect to the (primary) innate and specific immunocompetence after challenge with human serum albumin (HuSA) and lipopolysaccharide (LPS) at the respiratory mucosal level. Differences in immune responses of different types of chickens, seen in the field, may be explained by the fact that broilers and layers have different levels of metabolism and housing condition (Parmentier *et al* 2010), which may have an impact on the stress level that may influence immune responses.

Onset and duration of immunity

Not much information is available on onset and duration of immunity after vaccination. Onset of immunity is an important characteristic of vaccines applied for the control of emerging diseases in previously free countries, but less relevant if a disease is endemic in a region. Studies on onset of immunity have been performed for example for Aujeszky's disease, foot and mouth disease (FMD) and classical swine fever (CSF) (Van Oirschot *et al* 2003, Kaden *et al* 2001, Golde *et al* 2005, Bouma *et al* 2006, Li *et al* 2008, Rodriguez & Marvin 2009, Shao *et al* 2011). For AI not much information is available, because emergency vaccination has not often been considered. Therefore, the relevance for manufactures to focus on this feature is not high and most experiments are carried out to determine vaccine efficacy three weeks after vaccination (Pharmacopeia 2007). Considering the fact that AI is endemic in Indonesia, early onset of immunity is not of primary importance for the country.

Determination of the duration of immunity is common practice for manufacturers of vaccines, as the specific product characteristics (SPC) requires this type of information. For AI vaccines, duration of immunity is usually guaranteed until 20 weeks after vaccination (Swayne 2006). Whether or not the duration of immunity induced by vaccination should be extended depends on, amongst others the costs of revaccination.

A few studies carried out in Indonesia have been described. A laboratory study by Indriani *et al* (2005) with layer chickens vaccinated with one dose containing a prototype

inactivated AI H5N1 vaccine (A/Chicken/West Java/67-2/2003) at 3-4 weeks (25 days) of age showed an average peak of HI antibody titer of $2^{4.1}$ at 8 weeks post vaccination (p.v.). Afterwards the titer decreased to $2^{2.4}$ at week 12 p.v. When vaccination was done at 1 and 2 weeks of age, the peak of antibody titer was only $2^{2.2}$ at 3 weeks p.v. and the titer decreased as well. Another vaccine study was done by Suardana *et al* (2009) with a commercial, inactivated AI H5N1 vaccine (Vaksiflu®, PT Vaksindo) in ducks with a half, one, and two vaccine doses. A double vaccination with these doses demonstrated a significant increase in antibody titers. In all groups, the antibody response started to rise at 2 weeks post first vaccination and reached a peak at 2 weeks after the second vaccination.

Other AI vaccine studies done by Tian *et al* (2005) showed longer duration of immunity than the Indonesian studies mentioned above. They showed that single vaccination of 3-week-old SPF chickens with inactived oil emulsion AI H5N1 vaccine showed an average peak of HI antibody titer of 2^{10} at 6 weeks p.v., then slowly decline to 2^4 at week 43, and this vaccine provide protective immunity until approximately 10 months p.v.. However, these studies included different vaccines or adjuvants than the Indonesian studies.

Cold chain

Vaccines usually have to be stored within a temperature range of 2°-8° C to maintain vaccine quality. Inappropriate vaccine storage, the absence of standard operating procedures for cold chain and a lack of information for animal health workers about cold chain, logistic and management have been observed in Bali province (Vogel *et al* 2011), and it is believed that such poor conditions will also be present in other provinces in Indonesia. More focus on the need for cold chain and proper vaccine handling should be addressed in order to improve the success of vaccination programmes in Indonesia.

Immunosuppressive agent

The presence of immunosuppressive agents may interfere with vaccination effectiveness (Marangon & Busani 2006, Abdelwhab & Hafez 2012). The poultry population in Indonesia not only faces AI, but also other diseases such as chicken anaemia virus, infectious bursal diseases (IBD/Gumboro) (Sumiarto & Arifin 2008, Wibawan 2012, Xiao *et al* 2012). These diseases could lead to immunosuppression in chickens resulting in a poor immune response upon vaccination. Other immunosuppressive agents, e.g. mycotoxin, are also a problem in Indonesia (TROBOS 2013). No proper information about the prevalence and incidence of these diseases or the number of mycotoxin related feed problems is available, nor about the

relevance of these factors with respect to AI. Nevertheless, it seems reasonable to assume that biosafety measures that are applied to control AI would also contribute to the improvement of poultry health in general, as the incidence of immunosuppressive diseases may also be reduced by increased hygienic measures (Van de Giessen *et al* 1998, Vandeplas *et al* 2010). Moreover, the report from TROBOS (2013) suggests that also non-infectious immunosuppressive agents should be controlled.

Antigenic variants

Antigenic drift is a gradual evolution of viral strains due to frequent mutation and selection, and drift is hypothesised to allow a virus to persist in a population for many years (Carrat & Flahault 2007, Tizard 2009). Because of antigenic drift the virus is able to escape neutralisation by antibodies, described for example for human influenza A virus (Thompson *et al* 2006), and as a result, human influenza vaccines have to be updated regularly. Selection of appropriate vaccine seed strains that match with circulating field virus is important to conserve vaccine effectiveness (WHO 2013). To be able to select suitable seed viruses, the World Health Organization (WHO) coordinates a global influenza surveillance network for the purpose of vaccine seed strain selection (Ndifon *et al* 2009, Fouchier & Smith 2010).

Antigenic drift is also observed for HPAI H5N1 (Domenech *et al* 2009, Fouchier *et al* 2010, Cattoli *et al* 2011, Moneim *et al* 2011) and is considered as one of the causes of AI vaccination failures in poultry flocks. The latter may be correct, but it may also be the other way around: vaccine failure (suboptimal vaccination) may be the cause of the emergence of new variants. Proper surveillance programmes should be implemented to rapidly detect changes in virus that may be relevant for vaccination programmes. A systematic surveillance programme is, however, not carried out in Indonesia and other countries. Using DIVA would simplify such programmes but is not very well accepted in most Asian countries. As a result isolates are obtained sparsely and not systematically. The rate at which new variants emerge is therefore unknown, but it seems reasonable to assume that new variants may cause losses in the poultry industry and increase the risk for a human pandemic (Cattoli *et al* 2011).

It is highly recommended that influenza vaccine seed strains antigenically match with circulating field strains to provide proper protection (Smith *et al* 2004, Beato *et al* 2010, Fouchier & Smith 2010). Selection of appropriate seed strains could be based on information of antigenic differences between circulating influenza virus strain (Ndifon *et al* 2009). Antigenic differences can be determined by the antigenic relatedness (Archetti & Horsfall 1950) or by antigenic cartography (Fouchier & Smith 2010). Both methods are based on the

HI assay (Archetti & Horsfall 1950, Fouchier & Smith 2010). It has been shown that antigenic differences correlate with vaccine efficacy (Gupta *et al* 2006), and could predict vaccine efficacy (Ndifon *et al* 2009). This relationship is, however, based on *in vitro* data (HI assay data), and the validity of this type of data for the efficacy of vaccines in blocking transmission needs to be demonstrated.

The study in Chapter 6 aimed to determine the level of cross-protection between two HPAI H5N1 strains (Sukabumi strain and Karanganyar strain). Both strains were isolated in different years and regions, but both strain were isolated from an AI outbreak in a vaccinated flock (pers. comm. Bharoto, Vaksindo PT). The *in* vitro results showed that the two strains had a rather low antigenic relatedness of only 37%, suggesting a poor cross protection between these strains. This was indeed so for the vaccine based on the Sukabumi strain, as virus shedding and transmission of challenge virus Karanganyar strain occured. However, the vaccine based on the Karanganyar strain, provides protection against infection with Sukabumi strain as no viral shedding and transmission occured. The results in Chapter 6 imply that extrapolation of *in vitro* data to clinical protection, and reduction of virus transmission might not be straightforward.

HPAI H5N1 vaccination strategies and vaccination coverage in Indonesia

Before discussing the vaccination strategies, first the poultry production system in Indonesia is described, as different strategies are applied to different sectors, and the control measures and reporting systems may vary as well. Moreover, the Indonesian government does not have access to farms of the large commercial poultry producers, neither do they have information about their disease management.

Poultry production systems

There are four main poultry farm types in Indonesia: 1) breeders, 2) layers, 3) broilers and 4) backyard/native chicken. Breeder flocks only have grandparent stock and parent stock of layers and broilers, whereas pedigree pure lines and great-grandparents are not raised in Indonesia.

Breeders are reared in sectors 1 or 2 in Indonesia and have a life span between 65-67 weeks. Commercial layers are generally reared in a semi-intensive way in sector 3. Layers are kept for 60-72 weeks depending on the level of egg production. Broilers are also often reared in sector 3. Backyard poultry which consist of native chickens are reared in sector 4 with two

rearing systems: 1) extensive traditional, meaning birds scavenge during daytime and kept inside at night; 2) semi-intensive, meaning birds are kept in cages in sheds and provided with feed, water, feed supplements and medication if required. The growth period for native chickens in the intensive system takes about 4.5 months (FAO 2008b). In general, native chickens that are raised traditionally for complementing the income of the owners are not vaccinated for any disease, and thus are assumed to be highly at risk of AI virus infection.

Indonesia has a high density of poultry, especially the province Java, where poultry farms are located closely together. There is often a mix of different farm types in one region, with commercial farms in close proximity to backyard flock. Live poultry markets and poultry collecting facilities receive birds from all over the island. Most birds are sold the same day, and brought to homes when still alive. This system with intensive mixing of poultry from different sources and lack of hygienic measures could be one of the reasons of H5N1 persistence in Indonesia (Indriani *et al* 2011, Loth *et al* 2011, IDP 2012).

Changing the structure of the poultry sector may reduce the risk of infection with HPAI H5N1, but this is not easy to achieve as many factors are economically or culturally determined. Therefore, structural changes of the poultry sector will probably require years to become reality, and even than it will be difficult to eliminate all risky practices, such as free grazing ducks or using poultry collector houses (FAO 2011). Even so, Indonesia is trying to establish changes in the marketing chain: the targets were live bird markets and collector yards in Jakarta province, since both have been shown to be sources for HPAI H5N1 virus (FAO 2011, IDP 2012). An official order (PERDA no. 4/2007) has been released by the Jakarta provincial government to eliminate the sale of live poultry in markets by 2010 (FAO 2011). Moreover, by 2012, the Indonesian government included ‘restructruring poultry sector’ as part of the strategies for controlling HPAI in Indonesia (Iwantoro 2013).

However, elimination of the sale of live poultry by 2010 has not been achived and ratherthan changing the poultry system, improving the management with respect to professional visitors, hygiene measures, all in all out systems etc., and commitment of stakeholders e.g. government, farmers (sector1-4) and veterinarians to report AI outbreaks and destroy infected flocks might be more successful to eradicate HPAI from Indonesia. The commitment has improved through the use of participatory diseases surveillance and response (PDSR) method that aims to gain trust of community village level producers and through the engagement of market traders (FAO 2011), since PDSR facilitates links between government, farmers (most are sector 3-4) and veterinary services (Azhar *et al* 2010). However, PDSR only covered information from sectors 3 and 4, consequently, an improving

engagement between government veterinary services and farmers from sectors 1 and 2 did not occur and this is required for sharing information about the incidence of diseases and control method applied in these sectors. This will probably not occur within the near future.

HPAI vaccination strategies

There are three vaccination strategies described by OIE (2007) : (1) emergency vaccination; (2) preventive (prophylactic) vaccination; (3) routine vaccination. Emergency vaccination is a strategy that is applied in the face of an epidemic or if an infectious disease is introduced in a previously unaffected area/country and for which the epidemiological situation indicates that there could be a massive and rapid spread of infection (OIE 2007, Marangon & Busani 2006). Preventive vaccination is a measure that may be applied if there is a high risk of introduction and further spread of a contagious disease. The advantages of vaccination in the absence of any outbreak of disease, together with the application of good biosecurity measures, could maximise protection whenever a risk of exposure exists. Routine vaccination is an option in an area/country where an infectious disease is endemic, and if used properly, this strategy may effectively reduce mortality and production losses (Marangon & Busani 2006).

Besides these three vaccination strategies, there are other ways to describe vaccination strategies (OIE (2007) : (i) mass vaccination : vaccination that is applied to all susceptible birds; (ii) targeted vaccination : vaccination is applied to defined categories of birds; (iii) ring vaccination : vaccination that is applied in a defined area around an outbreak.

Mass compulsory vaccination was introduced as one of the first responses to outbreaks of HPAI H5N1 in Indonesia, initially targeting all poultry in Indonesia (Sumiarto & Arifin 2008). However, the Indonesian government provided vaccine and vaccination services free of charge for back yard poultry owners (sector 4) and to owners of sector 3 flocks up to a maximum of 5000 birds. The other farms had to carry out their own vaccination programme and did not get compensation from the government. Morover, duck populations were not vaccinated in a 'sponsored' programme (Siregar *et al* 2007, Domenech *et al* 2009).

Due to limited resources , in 2006, mass vaccination programmes were discontinued, and targeted vaccination was applied. Vaccination was restricted to high risk areas only (e.g. Java, Lampung, North Sumatera, West Sumatera, Bali, and South Sulawesi). The criteria for prioritisation were: occurrence of human cases, high poultry density, and high number of

HPAI reports in poultry (Sumiarto & Arifin 2008). Ring vaccination of back yard poultry was also applied as a response to an HPAI H5N1 outbreak in an industrial flock.

Initially, the government programme used vaccines containing an Indonesian virus seed strain A/Chicken/Legok/2003 (Siregar *et al* 2007). In 2006, however, the policy was changed and only vaccines containing low pathogenic AI (LPAI) virus strains of the H5N2 subtype were used, either produced locally or imported. In 2008, imported vaccines containing LPAI H5N9 were also used (Siregar *et al* 2007, Sumiarto & Arifin 2008, Indriani *et al* 2011). In 2011, the policy was changed again, and the government only gave permission to use vaccines produced in Indonesia containing a local isolate of H5N1.

The vaccination of back yard poultry was not successful, probably due to the problem of limited vaccine availability, equipment and facilities, cold chain, personnel and operational budget (Siregar *et al* 2007, Sumiarto & Arifin 2008, Domenech *et al* 2009). Targeted vaccination for backyard poultry under government programme was stopped in 2007, and from then the government no longer provided AI vaccines for free (Siregar 2008).

Vaccination may contribute to reduction of losses, but outbreaks are still reported, both in commercial farms, as well as in poultry facility houses and back yard flocks (Wibawan 2012, DGLS 2013a). AI vaccination continues to be used on breeder and layer farms, and some broiler farmers (FAO 2011). At the end of 2012, outbreaks of HPAI H5N1 clade 2.3.3 were reported in commercial duck farms (Wibawa *et al* 2012), and in 2013 the government considered to apply a vaccination programme in the commercial duck population (MoA 2012, DGLS 2013b). The government asked local vaccine companies to produce new inactivated AI vaccine using H5N1 clade 2.3.2 as seed strain. Theses vaccines currently are in the process of registration (MoA 2012, DGLS 2013b). The routine vaccination in commercial poultry farms is still applied by integrations with more or less success.

Surveillance and disease reporting

A participatory disease surveillance and response (PDSR) programme which applies rural appraisal methods to disease surveillance (Azhar *et al* 2010) was implemented in 2006. The initial programme was only applied in 12 districts in Java, but in 2007 all Java, parts of Sumatra and Bali were under PDSR surveillance for HPAI (Azhar *et al* 2010, Bett *et al* 2013). PDSR programme was succesful in identifying and reporting outbreaks in back yard and commercial sector 3 farms (FAO 2011, Bett *et al* 2013). However, PDSR programmes only focused on sector 3 and 4 farms. Owners of large farms do not necessarily report outbreaks consistently. Therefore, this system does not seem sufficient for gathering all

information of the HPAI incidence. Recommendations made by FAO (2011) for improving surveillance are :

1. Supporting healthier poultry by establishing a certification programme for poultry.
2. Strengthening veterinary services by supporting the development of a National Veterinary Services (NVS) and building capacity of animal services to effectively address other animal and zoonotic diseases of concern.
3. Facilitating the establishment of a functional and dynamic public private partnership between poultry industry and government.

The PDSR system may create more awareness by smallholders, and may improve participation of farmers to contribute to the control of AI in Indonesia. However, because the PDSR is limited to part of the poultry population only, it cannot control the disease. The poultry production system is divers, and the collaboration between the farmers and producers of various sectors is limited. The control of AI is therefore hampered.

Commercial farmers seem to monitor HI titers of their flocks, to determine whether the flock should be revaccinated or not, but as mentioned before, information is lacking. Moreover, they do not seem to report outbreaks. Isolates are obtained, though not systematically, both by the government, the vaccine producers and poultry farmers, but neither information nor the strains are shared (Wibawan 2013). Even although phylogenetic trees are constructed (Lam *et al* 2008, Takano *et al* 2009, Mulyono & Asmara 2012), the question remains whether this is a correct representation of the situation in Indonesia as the number of isolates is limited and also because not all data are publically available. Moreover, new strains attract a lot of attention because of the fear for a human pandemic, but the relative prevalence of these strains also remains unclear. Thus the surveillance system in Indonesia is not systematic, but focused on incidents, and therefore does not provide a good overview of the real situation.

Vaccination coverage in the various poultry production sectors

The minimal vaccination coverage considered to be necessary to reduce transmission can be derived from estimates of the within-flock reproduction ratio (R_0) (Tiensin *et al* 2007, Bouma *et al* 2009). Information of within flock transmission in Indonesia is hardly available, but Walker *et al* (2013) estimated the transmissibility of HPAI H5N1 in sector 3 and back yard flocks in Java, and the estimated within flock *R* was 2.5 (95% CI : 2.35-2.74). Based on the upper limit of *R* (Walker *et al* 2013), vaccination coverage needs to be at least 63.5% to achieve eradication of the virus.

The vaccination coverage assessed by the Ministry of Agriculture of Indonesia rarely exceeded 30% of poultry population based on the number of reported vaccine dosages used and number of poultry present (Siregar *et al* 2007). Swayne *et al* (2011) stated that the vaccination coverage rate of Indonesia between 2004 and 2010 was approximately 14 % of the whole poultry population based on the number of reported vaccine dosages used and number of poultry. Another study by Walker *et al* (2013) estimated the vaccination coverage among semicommercial and back yard flocks in Java to be 11 % based on post vaccination seromonitoring. The interpretation of these figures is difficult as vaccinated birds may be clustered, and it is not known which birds were sampled or whether the titers were due to vaccination or infection. It may make more sense to use the percentage of flocks and coverage of vaccination within a flock than an overall coverage for the whole country. Moreover, more detailed information should be provided about the local situation. Nevertheless, it seems clear that vaccination coverage in Indonesia is insufficient.

Vaccination of grandparent and parent layer flocks is carried out four times before the production starts, with one or two booster vaccinations during the production period (Siregar *et al* 2007). Vaccination of layers is applied two or three times before the onset of the egg production and sometimes complemented with one booster vaccination (Siregar *et al* 2007). In contrast to commercial layer and breeder chickens, most commercial broilers are not vaccinated against AI regularly. Only in identified high risk areas broilers sometimes receive a half dose at day 7 of life. The efficacy of this is however doubtful as shown in chapter 3. A study by CIVAS (2008) has demonstrated that a single vaccination of broilers with HPAI H5N1 at day 1, 4, 7, 10, or 14 days of age did not induce HI antibody titer to the presumed protective level (2^5). Moreover, there was also concern about the spread of virus by vaccination teams when used as an emergency measure if an outbreak had occurred. Whether this has actually occurred remains unclear (Siregar *et al* 2007, Hinrich *et al* 2010).

For breeders and commercial layers, it should be feasible to apply and maintain a vaccination coverage > 63.5%, since birds on these type of farms are routinely vaccinated against AI. The vaccination coverage of broilers is irrelevant, because vaccination is not effective. Back yard flocks are certainly not vaccinated to a sufficient coverage (Bouma *et al* 2008). However, the focus on back yard flocks in a vaccination programme should not be exaggerated as their role in virus spread is probably limited (Tiensin *et al* 2007; Bavinck *et al* 2009).

Costs and benefits for vaccination against HPAI in Indonesia

Cost components of mass vaccination programme consist of: planning, monitoring and communication, labour and equipment, purchase, storage and distribution of vaccines and equipment, post vaccination seromonitoring, and vaccine production. Not much information is available about the costs for vaccination in sectors 1 and 2. Hinrichs *et al* (2010) estimated the total costs of vaccination per chicken/dosage in Indonesia between US$ 0.03 in broiler flocks and US$ 0.12 in back yard flocks. This difference in vaccination costs is due to differences in accessibility and flock-size that strongly influence the numbers of vaccinations per vaccinator per day. Vaccination of extensive back yard flocks with scavenging chickens in rural areas demand more labour than birds in large commercial flocks. Moreover, also the storage and distribution costs for the vaccination are higher for back yard poultry than for large commercial flocks (Hinrichs *et al* 2010).

The benefits of vaccination for farmers are reduction of mortality and other production losses if virus infection of birds in a flock occurs (Marangon & Busani 2006, Hinrichs *et al* 2010). Considering the wide scale at which AI vaccines are applied in sectors 1 and 2, it seems reasonable to assume that integrations are aware of the benefits of vaccination. The challenge is to convince owners of flocks in sectors 3 and 4 to vaccinate. Factors such as immunosuppressive agents which are better controlled in sectors 1 and 2 however may at the end reduce the benefits for the small holders. If smallholders and personnel are not convinced of the need of vaccination then it is unlikely they will cooperate (Hinrichs *et al* 2010). The rumours that vaccination itself causes outbreaks of AI also hamper the efficacy of vaccination campaigns. The benefits for the government are a sustainable poultry production, and protection of human health. The government should also inform citizens how to handle live poultry and may advise stopping with slaughtering birds for own use.

Ilham & Iqbal (2011) reported type of poultry production system, farmers' experience with HPAI, poultry farm management, scale of poultry farm, cost of vaccination, mortality rates in poultry farms and the contribution of poultry farms to the household income as factors determining farmers decision to implement HPAI vaccination. Independent poultry farmers, breeder farmers, layer farmers and farmers with experiences with HPAI appear to be more likely to implement HPAI vaccination on their poultry farms. For breeder (sector 1 & 2) and layer (sector 2 & 3) production systems the cost-effectiveness seems higher in comparison to other production systems, and thus these farmers are more inclined to adopt

HPAI vaccination. Due to the short life span of broilers, producers have little benefits from applying HPAI vaccination since its only fully effective about 13 to 21 days after the first of two injections.

Other management measures

Vaccination is only an additional tool to control of HPAI H5N1 and should therefore be combined with other measures like improved biosafety, depopulation of infected flocks, surveillance, movement control of poultry and poultry products. The incentives for the implementation of management measures to prevent incursion of virus are not clear. Of course hygienic measures are not only effective for AI but also prevent the probability of incursion of other diseases. Therefore, the costs per disease are reduced. For small farms the incentives are unknown, and may depend on the risk of incursion as well, which does not seem to be very high. However, proper information on this topic is lacking.

Conclusions

The purpose of vaccination is to increase herd immunity, to prevent mortality, virus shedding to the environment and between flock virus transmission. Large poultry producers in Indonesia use routine vaccination and apply management measures to prevent outbreaks of AI, but the effectiveness is unknown. On broilers and back yard farms no good vaccination programme has been implemented. Vaccination is however only one tool among the measures aiming at the control of HPAI in Indonesia, and a successful strategy needs commitment from all parties engaged. Important issues that need to be addressed beside vaccination are the development of a good surveillance system and the willingness to report outbreaks of AI and to take adequate control measures should an outbreak occur, not only in back yard or sector 3 farms, but also in sectors 1 and 2.

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Summary

Highly pathogenic avian influenza (HPAI) H5N1 has been present in South-East Asia since 1996 and is considered to be a major threat for both the poultry industry and public health. Since the first outbreak, the virus has spread to many countries in Asia, Europe and Africa. In many countries e.g. Thailand, and European countries, eradication of the virus was achieved by depopulation of infected and contiguous flocks, stringent biosecurity measures etc. After the first outbreaks in Indonesia, in 2003, large-scale culling was not applied due to cultural, financial and logistical factors. The virus has since then spread in the country and the disease is now considered to be endemically present. To reduce the impact on poultry health, and production losses, vaccination was applied as control measure, mainly by the commercial poultry producers but also by the Indonesian government.

Vaccination has been applied since 2004, mainly in large commercial breeder and layer flocks, but outbreaks continued to occur. Various explanation can be given e.g. inappropriate vaccine administration, low quality of available vaccines, antigenic drift resulting in suboptimal protection induced by the vaccines against newly emerged field strains, and the presence of immunosuppressive diseases. However, no clear causes have been identified yet. Moreover, it is known from experimental infections with SPF layer birds that vaccines can induce protection against symptoms. It has, however, not been determined extensively whether vaccines are able to protect birds against infection and whether it can protect a flock against transmission. For AI this should be the ultimate goal of vaccination, as reduction of transmission might result in eradication of the virus which is both of benefit for poultry and human health.

The main goal of this thesis was to provide more insight in vaccine efficacy with respect to virus transmission and possible causes of vaccine failure. To this end, the knowledge that was gained from the studies might be used to improve the current vaccination programmes in Indonesia or other endemic countries.

Back yard flocks, small flocks of native chickens, have been suggested to be a source for outbreaks in the field. Vaccination against AI is not widely applied to back yard chickens, mainly for practical and financial reasons. It was suggested that these types of bird would not be able to respond adequately upon vaccination. In Chapter 2, it was demonstrated that native

chickens can be vaccinated effectively and that these birds responded similary to infection and vaccination as layer type chicken did, implying that protection against AI using vaccines would be feasible.

AI vaccination is also not applied extensively in commercial broiler flocks mainly due to their short life span, but also because of the presumed interference of maternally derived antibodies with the build-up of immunity. In Chapter 3, the efficacy of vaccination in broilers was determined. The results indicated that vaccination is not effective in commercial broilers, as early vaccination at an age of 1 and 10 days did not induce a good immune response probably due to maternally derived antibody. If vaccination is applied later, the birds may be protected only at the moment they are slaughtered. Vaccination of broilers therefore does not seem to be a cost-effective control measure.

Animal trials are often carried out to determine vaccine efficacy, by measuring HI antibody titers after vaccination and measuring protection against a challenge infection. A HI titer larger than 1:32 is considered to be sufficient to protect a chicken against disease. However, it is not known which HI antibody titer can be considered to prevent transmission. In Chapter 4, a transmission experiment was conducted using layers with two levels of vaccine-induced HI titers. It was measured whether infection occurred, and whether inoculated birds could transmit virus to cage mates. The study showed that a single vaccination applied under field condition induced clinical protection, but seemed to be insufficient to induce protection against virus transmission, indicating that silent spread of virus in commercial flocks may occur.

All available AI vaccines in Indonesia contain inactivated virus, and are formulated with adjuvant and administered by injection. Intramuscular administration is however rather laborious and also requires skilled operators. Inappropriate injection may harm birds and lead to vaccine failure. It would be more convenient if vaccines could be delivered by other routes which may less harmful for birds and can be carried out more easily on a large scale. Spray vaccination is a vaccination method which is usually carried out for large flocks, as for example for Newcastle disease and Infectious Bronchitis. This route of vaccination requires live virus that result in infection and virus replication in the respiratory tract which leads to a proper induction of immunity. Up to now, this is not feasible for AI vaccines, mainly because live influenza virus vaccines are considered not to be safe neither for poultry nor for humans, as influenza viruses may mutate to highly pathogenic strains, and because AI of H5 and H7 subtype have a zoonotic potential. Several concepts have been tried to overcome the risks associated with the use of live vaccines. In Chapter 5, the results of a challenge experiment

showed that spray vaccination with an inactivated vaccine with a low pathogenic AI (LPAI) H9N2 strain was not successful, as it did not induce detectable levels of antibodies or protection against challenge with H9N2 strain. This shows that more research is needed to determine whether other not concepts of vaccination might be suitable for mass vaccination programmes.

Update of vaccines for Influenza needs to be done regularly, mainly due to antigenic drift. Antigenic drift seems to occur in Indonesia since 2007, and it has been suggested that current vaccines are no longer protective against the newly emerged field strains. It would be convenient if it could be predicted whether certain vaccines are still adequate to be used. One of the methods to do so is determination of the antigenic relatedness. This can be assessed by measuring serological cross-reactivity using haemagglutination inhibition (HI) tests. However, it is not known how this relatedness, determined *in vitro*, reflects vaccine efficacy *in vivo* with respect to reduction of virus transmission upon challenge of vaccinated birds. In Chapter 6 a transmission experiment was conducted to quantify protection of two vaccines, derived from two strains of highly pathogenic H5N1 AI virus against challenge with the homologous or heterologous strain. The study showed asymmetrical cross-protection between two highly pathogenic H5N1 virus strains, but this response could be expected based on the *in* vitro response. Nevertheless, extrapolation of *in vitro* data to clinical protection and reduction of virus transmission in the field might not always be straightforward.

The experiments performed in this thesis may have provided more knowledge on vaccine efficacy and the epidemiology of HPAI, which may be useful for decision support systems for the control of HPAI H5N1 in Indonesia. Aiming at eradication of AI in Indonesia, vaccination of broilers or native chickens in small back yard flocks does not seem to be useful, although for different reasons. Silent spread in flocks with low herd immunity seems to be possible, posing a risk for the control programme and public health. An explanation for outbreaks in properly vaccinated flocks has not been found. More research on risk factors that contribute to the development of poor responses on vaccination or to the occurrence of outbreaks may be needed. This does, however, also require the cooperation of stakeholders to provide information about virus isolates, the management and contact structure between farms. More research is also needed on vaccine effectiveness in the field in different types of poultry, and on improving new vaccination techniques or development of new vaccines. This might help to develop better vaccination strategies in Indonesia. Furthermore, studies on the poultry production and market chain, and socio-economic effects

of various control measures might contribute to improving the AI control in Indonesia as well.

Samenvatting

Hoogpathogene aviair influenza (HPAI) H5N1 is in Zuidoost Azië sinds1996 en wordt beschouwd als een grote bedreiging zowel voor de pluimveehouderij als voor de volksgezondheid. Sinds de eerste uitbraak heeft het virus zich verspreid naar veel andere landen in Azië, Europa en Afrika. In veel landen, zoals Thailand en de getroffen Europese landen, is het virus uitgeroeid door middel van het ruimen van besmette en van contactbedrijven, strikte hygiënemaatregelen enz. Na de eerste uitbraak in Indonesië, in 2003, werd grootschalige ruiming niet toegepast om culturele, financiële en logistieke redenen. Het virus heeft zich daarna verspreid door het hele land en de ziekte wordt nu verondersteld endemisch te zijn. Om de impact op pluimveegezondheid, en productieverliezen te verminderen wordt vaccinatie toegepast als interventiemaatregel, vooral door de commerciële pluimveehouders, maar ook door de Indonesische overheid.

Ondanks vaccinatie vinden er nog steeds uitbraken plaats. Hiervoor kunnen verschillende verklaringen worden gegeven, zoals slechte vaccinatiedekking, slecht uitgevoerde vaccinatie, matige kwaliteit van de beschikbare vaccins, antigene drift van het virus waardoor vaccins geen of onvoldoende bescherming geven tegen infectie, en de aanwezigheid van immunosuppressieve ziektes in pluimvee. Duidelijke oorzaken voor vaccin falen zijn echter nog niet gevonden. Daarbij is ook niet bekend of vaccins, die meestal wel in staat zijn om bescherming te bieden tegen verschijnselen van vogelgriep, geschikt zijn om verspreiding van het virus tegen te gaan. Voor HPAI is dit laatste wel het ultieme doel, aangezien vermindering van verspreiding kan resulteren in uitroeiing van het virus.

Het belangrijkste doel van dit proefschrift was meer inzicht te krijgen in de werkzaamheid van vaccins met betrekking tot de overdracht van virus en in mogelijke oorzaken van vaccin falen. De kennis die is op gedaan in dit onderzoek kan worden gebruikt om de huidige vaccinatieprogramma’s in Indonesië of andere endemisch besmette gebieden te verbeteren.

Kleinschalig gehouden pluimveekoppels, veelal bestaande uit inheemse kippen, zijn wel verantwoordelijk gesteld voor het herhaaldelijk optreden van uitbraken, o.a. in de commerciële pluimveehouderij. Vaccinatie wordt niet grootschalig toegepast in deze koppels om praktische en financiële redenen. Ook is wel gesuggereerd dat dit type pluimvee niet goed

reageert op vaccinatie. De resultaten in Hoofdstuk 2 laten zien dat deze kippen echter effectief kunnen worden gevaccineerd en dat ze vergelijkbaar op vaccinatie en infectie reageren als leghennen die worden gebruikt in de commerciële sector. Dit betekent dat deze inheemse kippen ook effectief kunnen worden gevaccineerd.

Ook vleeskuikens worden in het algemeen niet gevaccineerd, vooral omdat de productieperiode erg kort is, maar ook omdat deze dieren meestal antistoffen hebben van de moeder, die kunnen interfereren met de respons na vaccinatie. De resultaten in Hoofdstuk 3 laten zien dat vaccinatie van commerciële vleeskuikens niet effectief is indien toegepast op een leeftijd van 1 of 10 dagen. Dit is waarschijnlijk te wijten aan de aanwezigheid van de maternale immuniteit. Later toedienen van vaccin, als deze antistoffen zijn verdwenen, zou wel kunnen, maar dan is een eventuele immuunrespons pas opgebouwd op het moment dat de dieren naar de slacht gaan. Vaccinatie van vleeskuikens lijkt daarom geen kosteneffectieve maatregel.

Dierproeven worden vaak uitgevoerd om de effectiviteit van vaccin te bepalen. Dit gebeurt dan door het meten van de HI antistof titer en vast te stellen of dieren beschermd zijn tegen een experimentele infectie. HI titers gelijk aan of groter dan 1:32 worden verondersteld bescherming te geven tegen verschijnselen. Het is echter niet bekend of bepaalde HI titers ook bescherming bieden tegen verspreiding van virus. In Hoofdstuk 4 is een experiment beschreven dat is uitgevoerd met legkippen die onder commerciële omstandigheden waren gevaccineerd. Deze dieren werden ingedeeld in twee groepen, met een hoge en een lage HI titer. Vervolgens was gekeken of er infectie optrad en of besmette dieren het virus konden verspreiden naar hokgenoten. Uit de proef bleek dat als een eenmalige vaccinatie was uitgevoerd onder veldomstandigheden er wel bescherming kon worden geïnduceerd tegen verschijnselen, maar dat deze onvoldoende leek om bescherming te bieden tegen virus verspreiding, wat zou kunnen betekenen dat subklinische verspreiding van virus in commerciële pluimveekoppels zou kunnen optreden.

Alle beschikbare AI-vaccins in Indonesië bevatten geïnactiveerd virus en worden geformuleerd met adjuvans en door injectie toegediend. Intramusculaire toediening is echter nogal omslachtig en vereist ook ervaren personeel. Een verkeerd toegediende injectie kan welzijnsproblemen geven voor de vogel en ook leiden tot vaccin falen. Het zou handiger zijn als vaccins op andere manieren kunnen worden toegediend. Sprayen is een vaccinatiemethode die gewoonlijk wordt uitgevoerd voor ziekten zoals pseudovogelpest en infectieuze bronchitis. Deze vaccinatieroute vereist echter levend virus voor het opwekken van een goede immuniteit. Tot nu toe is dit niet haalbaar gebleken voor AI vaccins, vooral

omdat levende influenzavaccins niet veilig wordt geacht, noch voor pluimvee noch voor mensen. Influenza virussen kunnen namelijk muteren van laagpathogeen in hooghpathogene stammen. Bovendien zijn de H5 en H7 stammen zoönotisch. De studie beschreven in hoofdstuk 5 toonde aan dat het toedienen van een geïnactiveerde laagpathogene aviair influenza H9N2 stam door sprayen niet succesvol was, zoals bleek uit het ontbreken van detecteerbare niveaus van antilichamen.

Antigene drift van virus treedt al enige jaren op en is sinds 2007 ook aangetoond in Indonesië. Er is verondersteld dat de huidige vaccins niet meer de gewenste bescherming geven tegen deze nieuwe veldvirusstammen, zoals bekend van humane griepvirussen, en een update van vaccins vereisen. Dit wordt mede gedaan aan de hand van het vaststellen van antigene verwant schap tussen vaccins en virusstammen. Dit kan worden gedaan door het meten van serologische kruisreactiviteit van HI antistoffen. Het is echter niet bekend of kruisbescherming, *in vitro* vastgesteld, *in vivo* resulteert in vermindering van virusoverdracht. In hoofdstuk 6 is een transmissie-experiment uitgevoerd om de kruisbescherming van twee vaccins te kwantificeren. Deze waren gebaseerd op twee recente hoogpathogene H5N1 AI-virus stammen. De studie toonde asymmetrische kruisbescherming aan, die ook al op basis van de *in* vitro proeven kon worden voorspeld. De *in vitro* methode lijkt daarom een goede manier om de geschiktheid van vaccins vast te stellen.

De experimenten uit gevoerd in dit proefschrift werden gedaan om meer inzicht te krijgen in vaccinatie-effectiviteit wat betreft vermindering van virus transmissie om zo bij te dragen aan een betere vaccinatiestrategie voor Indonesië. Meer onderzoek naar de oorzaak van uitbraken onder gevaccineerde pluimveekoppels moet echter nog worden gedaan. Ook is er meer onderzoek nodig naar vaccins die via massa-applicatie kunnen worden toegediend . Dit type onderzoek richt zich onder andere op nieuwe vaccinatietechnieken en genetische karakterisering van stammen. Daarvoor is wel inzet nodig van alle betrokken partijen om virus stammen te leveren voor vaccins, maar ook om informatie te delen over uitbraken die plaatsvinden op bedrijven en over de genetische karakterisering van nieuwe stammen. In combinatie met studies naar de kosten en baten van verschillende maatregelen en de contactstructuren tussen pluimveehouders kan dit wellicht leiden tot verbeterde controlestrategieën die passen bij de Indonesische situatie.

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Okti Nadia Poetri

Curriculum vitae

Okti Nadia Poetri was born on October 27th 1980 in Bogor, West Java, Indonesia. In 2004 she receieved the degree of Doctor of Veterinary Medicine (DVM) from Bogor Agricultural University, Indonesia. Then, in 2005, she started her career as a lecturer and researcher in Department of Infectious Diseases and Veterinary Public Health, Faculty of Veterinary Medicine, Bogor Agricultural University, Indonesia. She completed her master degree in Veterinary Science from Bogor Agricultural University, Indonesia, in 2007.

In the same year, 2007, she involved with the Indonesian Dutch Partnership Programme on Highly Pathogenic Avian Influenza Control (IDP-HPAI) for project on vaccination as a control measure for HPAI H5N1. In 2009, she left for Utrecht, the Netherlands to joined postgraduate programme Veterinary Epidemiology in Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht Utrecht and completed her master degree in 2011. Then she started her PhD in the same university under supervision Prof. Dr. Arjan Stegeman, Dr. Annemarie Bouma and Dr. Guus Koch. Her research focus on efficacy of vaccination on controlling HPAI H5N1 in Indonesia. Her studies in Utrecht University was supported by IDP-HPAI and Utrecht University.