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Different cross protection scopes of two avian influenza H5N1 vaccines against infection of layer chickens with a heterologous highly pathogenic virus

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ABSTRACT

Avian influenza (AI) virus strains vary in antigenicity, and antigenic differences between circulating field virus and vaccine virus will affect the effectiveness of vaccination of poultry. Antigenic relatedness can be assessed by measuring serological cross-reactivity using haemagglutination inhibition (HI) tests. Our study aims to determine the relation between antigenic relatedness expressed by the Archetti-Horsfall ratio, and reduction of virus transmission of highly pathogenic H5N1 AI strains among vaccinated layers.

Two vaccines were examined, derived from H5N1 AI virus strains A/Ck/WJava/Sukabumi/006/2008 and A/Ck/CJava/Karanganyar/051/2009. Transmission experiments were carried out in four vaccine and two control groups, with six sets of 16 specified pathogen free (SPF) layer chickens. Birds were vaccinated at 4 weeks of age with one strain and challenge-infected with the homologous or heterologous strain at 8 weeks of age. No transmission or virus shedding occurred in groups challenged with the homologous strain. In the group vaccinated with the Karanganyar strain, high cross-HI responses were observed, and no transmission of the Sukabumi strain occurred. However, in the group vaccinated with the Sukabumi strain, cross-HI titres were low, virus shedding was not reduced, and multiple transmissions to contact birds were observed.

This study showed large differences in cross-protection of two vaccines based on two different highly pathogenic H5N1 virus strains. This implies that extrapolation of *in vitro* data to clinical protection and reduction of virus transmission might not be straightforward.

1. Introduction

Since 1997, highly pathogenic avian influenza (HPAI) H5N1 strains have circulated in many countries (Sims et al., 2003; Eagles et al., 2009; Lupiani and Reddy, 2009). Common control measures such as stamping out of infected flocks, depopulation of contiguous flocks, and movement restrictions were often sufficiently effective with regard to elimination (Yee et al., 2009; Swayne et al., 2011), but in some Asian countries the disease has become endemic (Peyre et al., 2009; Kim et al., 2010; Swayne et al., 2011). In Indonesia, Vietnam, and China vaccination is applied as an additional measure to control the disease (Ellis et al., 2004; Siregar et al., 2007; 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). Since the first outbreaks of H5N1 in Indonesia in 2003, outbreaks have occurred despite wide spread vaccination, sometimes resulting in high mortality rates (Sims, 2007; Bouma et al., 2008). Poor biosecurity during vaccine administration, inappropriate vaccination procedures, antigenic diversity of avian influenza viruses and poor antigenic matching between vaccine virus and field challenge virus are believed to contribute to the limited efficacy of vaccination programmes (Siregar et al., 2007; Sims, 2007).

Since 2003, strains of H5N1 avian influenza (AI) virus, have been isolated from vaccinated and unvaccinated back yard flocks (Tiensin

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et al., 2005; Smith et al., 2006; Eagles et al., 2009; Nidom et al., 2012). These strains have been classified and clustered using a variety of methods, using phylogenetic trees (Smith et al., 2006; Nidom et al., 2012), serological data to determine antigenic relatedness between pairs of viruses (Archetti and Horsfall, 1950; Ndifon et al., 2009; Beato et al., 2010; Cai et al., 2012), and by antigenic cartography to obtain population level measures of relatedness (Smith et al., 2006; Mumford, 2007; Fouchier and Smith, 2010). Due to the emergence of new variants, it has been suggested that vaccines need to be updated regularly (Beato et al., 2010; Fouchier and Smith, 2010). For vaccine development it is therefore necessary to determine whether new strains should be used in vaccines and whether these vaccine strains are able to induce an immune response able to protect birds against (the consequences of) infection (Rauw et al., 2012; Abdelwehab et al., 2011; Swayne and Kapczynski, 2008).

Currently, all registered AI vaccines in Indonesia are classical inactivated vaccines produced by local vaccine manufacturers. The extent of protection conferred by novel inactivated vaccines is assessed by vaccination–challenge experiments, and by serological tests in which the haemagglutinin inhibition (HI) antibody titres are determined (Indonesian Pharmacopoiea, 2013). To reduce costs, number of experimental animals, and animal welfare problems, it would be convenient if an appropriate *in vitro* test was available that could replace the *in vivo* vaccine trials. The method developed by Archetti and Horsfall (1950), the so-called Archetti-Horsfall ratio (r value) (Archetti and Horsfall, 1950; Lee et al., 2004; Beato et al., 2010), might offer a simple solution. The method is based on comparing titres of sera in the HI test using homologous and heterologous viral antigens. There is, however, limited information whether this method adequately reflects the efficacy of a vaccine with respect to the induction of protection against clinical signs when infection of vaccinated birds occurs (Ndifon et al., 2009; Beato et al., 2010; Ducatez et al., 2011; Abbas et al., 2011). Moreover, vaccination against AI in poultry should preferably not only induce protection against disease, mortality, or production losses, but also should prevent virus spread. Only then can vaccination contribute to virus eradication, preventing potential massive of economic losses and significant human health risks.

As no information is available about a possible association between *in vitro* variables for protection and *in vivo* protection against transmission, we aimed to determine the relation between HI titres *in vitro*, expressed by the r value based on cross-HI data (Archetti and Horsfall, 1950), between two AI vaccines, and the level of transmission *in vivo* after challenge with a homologous or heterologous AI virus strains. This study contributes to elucidating the relation between the antigenic relation determined *in vitro*, and clinical protection and transmission after challenge with H5N1 AI virus field strains in poultry.

2. Materials and methods

The experiments were performed in accordance with the regulations for Research in Animal Health of the Indonesian Law on Livestock and Animal Health (UU/18/2009, article 80). All animal experiments in this study were handled under supervision of a veterinarian and performed in the high containment unit under Biosafety Level (BSL)-3 conditions at the manufacturing facilities of PT Vaksindo.[1]

2.1. Chickens and housing

The study consisted of six experiments (labeled A–F) with 16 SPF layer chickens in each, obtained from the SPF chicken farm of PT. Vaksindo. Birds were first housed at the BSL-2 animal facility. At the age of 4 weeks, the birds in groups (A–D) were vaccinated. At time of challenge, 4 weeks post vaccination (p.v.), all birds were moved to the BSL-3 facilities. The birds were housed in one experimental unit for the duration of the experiment, but each group was housed in a separate cage. Birds were fed with a commercial feed, and had tap water *ad libitum*.

2.2. Vaccines and challenge virus strain

Two avian influenza H5N1 viruses isolated from field cases at the laboratory of PT Vaksindo in Indonesia were used. The viruses were kindly provided by staff of PT Vaksindo: A/Ck/Wjava/Sukabumi/006/2008 (here referred to as Sukabumi strain), and A/Ck/Cjava/Karanganyar/051/2009 (here referred to as Karanganyar strain). The flock from which the Sukabumi strain was isolated had been vaccinated with a commercial inactivated vaccine containing an H5N1 strain; the flock from which the Karanganyar strain was isolated had been vaccinated with a commercial inactivated vaccine containing an H5N2 strain, more information was not provided by the farmers and practitioners (pers. comm. Dr. Bharoto, PT Vaksindo).

The vaccines were prepared according to standard operating procedures (SOPs) of the manufacturer, used for the production of their commercial vaccines. General information about these SOPs was provided by Dr. Bharoto of PT. Vaksindo, but details were considered confidential. The two vaccines were prepared as follows, using the two isolates mentioned above. The two strains were grown on 10-days-embryonated SPF chicken eggs. The allantoic fluid was harvested and the yield in terms of number of haemagglutination units (HAU) per ml fluid was determined. Subsequently, the virus in the fluid was inactivated using 0.2% v/v formaldehyde. The vaccines were then formulated by adding Montanide ™ ISA 70 VG (SEPPIC) adjuvant according to the manufacturer's instruction. The vaccine was administered once intramuscularly in the breast muscle using 0.5 ml vaccine containing 512 haemagglutination units (HAU) per dose per bird. This was the information provided by PT Vaksindo, not more information about the production process was made available.

The challenge virus strains were the same strains as used for vaccine production. Each group was challenged with either the homologous or the heterologous virus strain. Inoculation was done with a dose containing approximately 10^6 median egg infectious dose (EID_{50}) per ml. Two doses of inoculum of 0.1 ml each were administered, one intranasally and the other intratracheally.

2.3. Experimental design

Each experiment was carried out with 16 birds. Birds in groups A and B were vaccinated with Sukabumi vaccine; birds in groups C and D were vaccinated with Karanganyar vaccine strain; and birds in groups E and F consisted of unvaccinated birds (control groups).

The actual transmission experiment started four weeks p.v. with the challenge-inoculation. Half of each group was inoculated with either the Sukabumi or the Karanganyar strain. Eight birds in the vaccine groups A and C, and also in control group E were challenged with Sukabumi strain; eight birds in the vaccine groups B and D, and the control group F were challenged with the Karanganyar strain. Just before inoculation the eight birds per group (n = 16) that were to be inoculated were put in an empty cage (one cage per eight birds) in the same unit where the transmission experiment was planned (see Section 2.1 'Chickens and housing'), and kept in this cage during 8 h after inoculation. This was done to prevent spread of inoculum to pen mates. After these 8 h, the birds were moved back to their original cage with their eight pen mates.

In the transmission experiments, half of the group consisted of inoculated birds and the other half was contact-exposed to the inoculated pen mates. Inoculated birds are referred to as I birds; contact-exposed birds as S birds. An overview of the experimental design is given in Table 1.

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Table 1
Overview of the experimental design, serological responses and HI titre ratios.
Virus strains are A/Ck/WJava/Sukabumi/006/2008 (H5N1) and A/Ck/CJava/Karanganyar/051/2009 (H5N1) (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.

Group	Virus strain		Mean HI titre (^{2}log)[a]				HI titre ratio (95% CI)	
			At challenge (D0)[b]		End of the experiment (D28)[c]		Titre ratio (r)[e]	
	Vaccination	Inoculation	SMI[d]	KRA[d]	SMI	KRA	rs	rk
A	SMI[f]	SMI	3.94 ± 0.36^{A,B}	1.88 ± 0.35^{A}	4.38 ± 0.37^{A}	2.12 ± 0.39^{A}	0.25 (0.18–0.33)	N.A.
B	SMI	KRA	3.56 ± 0.26^{B}	0.88 ± 0.15^{B}	6.75 ± 0.84^{B}	5.12 ± 1.25^{A,B}		
C	KRA	SMI	4.6 ± 0.15^{A}	6.06 ± 0.17^{C}	5.31 ± 0.25^{A,B}	6.06 ± 0.17^{B}	N.A.	0.63 (0.45–0.81)
D	KRA	KRA	4.62 ± 0.2^{A}	5.25 ± 0.23^{D}	5.19 ± 0.26^{A,B}	5.44 ± 0.24^{B}		
E	None	SMI	0 ± 0^{C}	0 ± 0^{E}	1.5 ± 0.5 (n = 2)	0 ± 0	N.A.	N.A.
F	None	KRA	0 ± 0^{C}	0 ± 0^{E}	N.A.	N.A.	N.A.	N.A.

Values with different superscript (A, B, C, D, E) within column indicate a statistical difference significant ($p < 0.05$).
[a] The data are mean HI titres (^{2}log) ± standard error; N.A. not applicable.
[b] 4 week post vaccination.
[c] 4 week post challenge.
[d] The strain used in the HI test as antigen.
[e] Titre ratio rs and rk: heterologous titre divided by homologous titre; The ratio was calculated using mean HI titre at time of challenge.
[f] SMI, Sukabumi strain; KRA Karanganyar strain.

2.4. Sampling procedures and laboratory tests

After inoculation, all birds were monitored daily during the experimental period. Clinical signs were recorded during 4 weeks post challenge (p.c.). From all birds, swab samples were collected daily from the cloaca and the oropharynx until 14 days p.c. The swab samples were prepared according to standard procedures described by the World Organisation for Animal Health (O.I.E., 2012). The swabs were incubated for 1 h in medium, which was subsequently stored in duplo at − 70 °C until further testing. Blood serum samples were collected from the wing vein two days before challenge and at the end of the experiment. Sera were stored at − 20 °C until further testing (van der Goot et al., 2005; Spekreijse et al., 2011; Poetri et al., 2011).

Virus isolation was conducted to determine the presence of AI virus in swab samples using standard procedures (O.I.E., 2012). Briefly, swab samples were propagated in three 9-day old embryonated SPF chicken eggs using 0.2 ml swab medium. After 72 h (h), or when the embryo died before that time, the allantoic fluid was harvested (O.I.E., 2012). Alantoic fluid was tested using haemagglutination assay (HA) following standard procedure (O.I.E., 2012). The swab samples were considered to be virus positive when allantoic fluid of at least one of the eggs contained HA activity (Bouma et al., 2009; Poetri et al., 2009).

Serum samples were tested in a HI test to evaluate antibody titre to both strains. The testing was done following standard procedures (O.I.E., 2012). Briefly, HI tests were carried out in duplo in one run with 4 HAU of the Sukabumi and Karanganyar strains, using a sequence of twofold dilutions. Antibody titres were expressed as the reciprocal of the last serum dilution that caused complete inhibition of agglutination.

2.5. Genetic and phylogenetic characteristics

The HA sequence of the Sukabumi and Karanganyar strains were provided by PT Vaksindo. Both sequences were complemented with other sequences from viruses isolated in Indonesia and available in GenBank (A/Ch/Legok/03 strain: GU052416) and GISAID EpifFlu (http://gisaid.com) (Karanganyar strain: EPI824584; Sukabumi strain: EPI824583) databases. Sequences were aligned using Clustal W in Mega5 (Tamura et al., 2011) with the sequence of A/Goose/Guandong/96 H5N1 as root. Full length sequences were adjusted to include 48 Indonesian sequences of 1669 nucleotides in the analyses.

The phylogenetic tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree was 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 and Nei, 1993), and are represented by the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution with unit shape parameter. The analysis involved 48 nucleotide sequences. All positions containing gaps and missing data were discarded. The final dataset contained 1669 positions. Evolutionary analyses were conducted in MEGA 5 (Tamura et al., 2011). Trees were rooted to HA of A/Goose/Guandong/96.

2.6. Antigenic relatedness

The antigenic relatedness between the Sukabumi and Karanganyar strains was determined by conducting cross HI tests of 32 pairs of Sukabumi and Karanganyar antisera. The HI titre was determined in serum blood samples, collected four weeks after vaccination with the Karanganyar or Sukabumi strain. Pairs of HI titres were made by randomly selecting an HI titre from a bird vaccinated with Sukabumi, and from a bird vaccinated with Karanganyar strain.

The relatedness between the two virus strains was subsequently evaluated using the formula described by Archetti and Horsfall (1950), and the HI data. The titre of sera of birds vaccinated with the Sukabumi strain was normalized by dividing the heterologous HI titre obtained with Karanganyar virus antigen in the test by the homologous titre obtained with Sukabumi virus antigen. Likewise, the ratio of sera vaccinated with Karanganyar strain was calculated by dividing the heterologous HI titre obtained with the Sukabumi test antigen by homologous titre using Karanganyar test antigen. The titre ratio of Sukabumi (rs) or Karanganyar (rk) was calculated for each bird, and the geometric mean of the ratio per pair (R) was calculated as the square root of the product of rs and rk. (17). Here, the value of R provides an aggregate measure of the antigenic relatedness at the level of a pair of chickens. The consensus is that serologically related viruses have an Archetti Horsfall ratio larger than 0.5 (Archetti and Horsfall, 1950; Lee et al., 2004; Beato et al., 2010).

2.7. Data analysis

The serological responses were analysed using Mann-Whitney test. Differences were considered statistically significant at p-values smaller than 0.05. Mann-Whitney test was carried out to test the difference of HI titre between experimental groups with HI titre as dependent variable and group as independent variable. Mann-Whitney test was

performed using the software package SPSS 16.0. Inc.

Transmission rates in the control group were analysed using a Generalized Linear Model (GLM). 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 (Pawitan, 2001).

Maximum likelihood estimates of the infectious period in experiments with unvaccinated birds were obtained 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 are 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: $\hat{R}_0 = \hat{\beta}\hat{T}$ (Diekmann and Heesterbeek, 2000). 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).

3. Results

3.1. Genetic and phylogenetic characteristics

The amino acid motif at the HA cleavage site of the Sukabumi and Karanganyar strains comprises multiple basis amino acids: PQRESRRRKKR.GLF (Fig. 2), indicating both viruses are most likely highly pathogenic. Analysis of the nucleotide sequences of both strains in addition to 48 sequences of Indonesian viruses showed that they cluster in clade 2.1.3.2. For comparison, the well-known H5N1 virus A/Ch/Legok/2003 clusters in clade 2.1.1 (Fig. 1). Sequence analysis of both virus strains showed that the HA gene of the Sukabumi strain showed 23 amino acid differences, while the Karanganyar strain showed 14 amino acid differences in HA1 compared to H5N1 virus Ck/Legok/2003. A total of 29 amino acid differences and a deletion in HA (HA1) were observed between the Sukabumi and Karanganyar strains (Fig. 2). Of note, the Karanganyar strain had 7 potential N-linked glycosylation sites in HA1, while the Sukabumi strain had 5 potential N-linked glycosylation sites (Fig. 2).

3.2. Antigenic relatedness

The results of the analyses of antigenic relatedness between the Sukabumi and Karanganyar strains are presented in Table 1. The mean ratio of Sukabumi (rs) was 0.25 (95% CI: 0.18–0.33), and the mean ratio of Karanganyar (rk) was 0.63 (95% CI: 0.45–0.81). The overall estimated average antigenic relatedness (Archetti-Horsfall ratio) was R = 0.37 (95% CI: 0.29–0.45), indicating that the Sukabumi and Karanganyar strains had a relatively low level of antigenic relatedness. This was mainly due to the low ability of Sukabumi sera to inhibit agglutination by Karanganyar virus.

3.3. Serological response

After vaccination, all birds in the vaccine groups (A, B, C, and D) developed a positive HI antibody titre at 26 days post vaccination. On the other hand, none of the birds in the unvaccinated control groups (E and F) had developed antibody titres at time of challenge. An overview of the serological response data is showed in Tables 1-4.

3.3.1. Group A (vaccine Sukabumi/inoculation Sukabumi)

Birds had mean HI titre of $2^{3.9}$ against the homologous antigen and $2^{1.88}$ against the heterologous antigen at time of challenge. None of the birds in group A seroconverted after challenge virus with Sukabumi strain; at the end of the experiment, the mean HI titre against the challenge virus was $2^{4.38}$.

3.3.2. Group B (vaccine Sukabumi/inoculation Karanganyar)

Birds had a mean HI titre of $2^{3.56}$ against the homologous antigen and $2^{0.88}$ against the heterologous antigen at time of challenge. Three inoculated birds and one contact-exposed bird showed a four-fold increase in HI antibody titre against the Karanganyar challenge virus; the mean HI titre against challenge virus was $2^{5.12}$ at the end of the experiment.

3.3.3. Group C (vaccine Karanganyar/inoculation Sukabumi)

The mean HI titre against the homologous antigen was $2^{6.06}$ and $2^{4.6}$ against the heterologous antigen at time of challenge. Two inoculated birds showed a fourfold increase in HI antibodies to the Sukabumi challenge strain.

3.3.4. Group D (vaccine Karanganyar/inoculation Karanganyar)

Birds in this group had a mean HI titre of $2^{5.25}$ against the homologous antigen and $2^{4.6}$ against the heterologous antigen at time of challenge. None of the birds showed a fourfold titre increase to the Karanganyar challenge virus.

3.3.5. Groups E and F (control)

None of the control birds had antibody titres against the virus strains at time of challenge. The two surviving contact birds in group E had a HI titre below the cut off level at the end of the trial ($< 2^2$).

3.4. Clinical protection and virus shedding

An overview of HI titres at challenge, numbers of birds shedding virus, and mortality and seroconversion rates are shown in Table 5.

3.4.1. Group A (vaccine Sukabumi/inoculation Sukabumi)

There was no virus shedding and no transmission in this group. None of inoculated or contact exposed birds shed virus, all birds survived, and none of them showed any AI-like clinical signs.

3.4.2. Group B (vaccine Sukabumi/inoculation Karanganyar)

Six inoculated and four contact birds were positive in the virus isolation test. Four inoculated virus positive died within 4–7 days p.c., and three virus positive contact birds died after 10 days p.c.; all of these had AI-like clinical signs.

3.4.3. Group C (vaccine Karanganyar/inoculation Sukabumi) and Group D (vaccine Karanganyar/inoculation Karanganyar)

None of the inoculated birds or contact birds tested virus positive. All birds in both groups survived until the end of the experiment, and no AI-like clinical signs were observed.

3.4.4. Group E (unvaccinated/inoculation Sukabumi)

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, although all showed AI-like signs.

3.4.5. Group F (unvaccinated/inoculation Karanganyar)

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., after having developed AI-like signs post challenge.

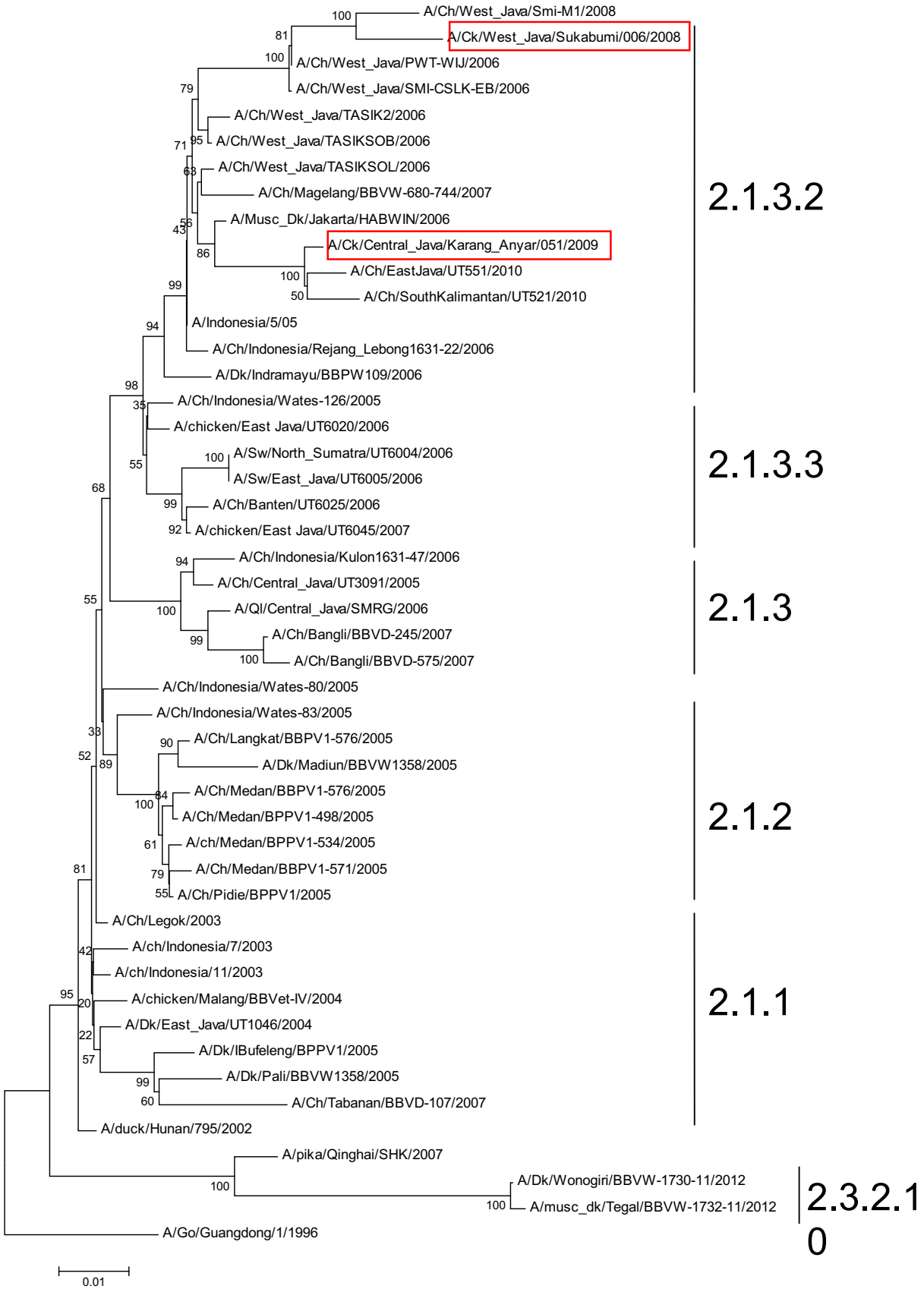

Fig. 1. Phylogenetic tree of Indonesian AI poultry isolates. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.39771100 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. 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 and 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).

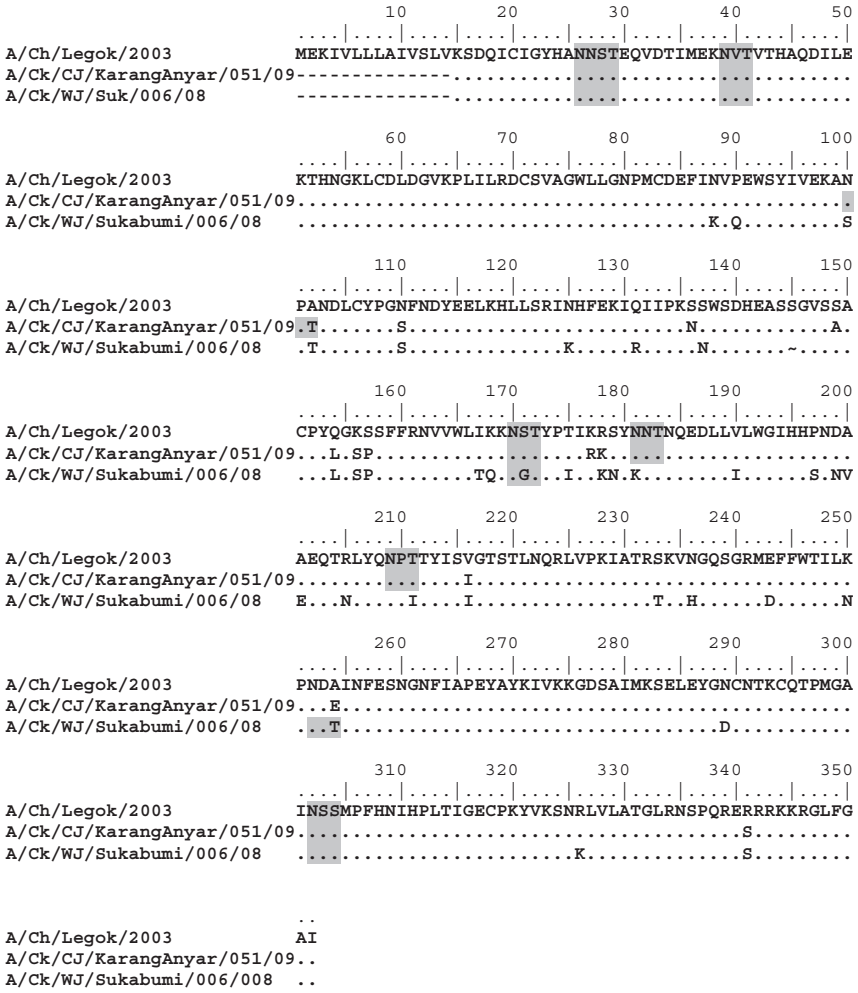

Fig. 2. Amino acid sequences of AI H5N1 virus A/Ch/Legok/2003, A/Ck/C.Java/Karanganyar/051/2009 and A/Ck/W.Java//006/2008. Currently part of HA ORF from position 1 to 352 is shown. Estimation of glycosylation sites were marked with grey.

3.5. Estimation of transmission efficiency

No virus transmission occurred in groups that were vaccinated, and in which the homologous strain was introduced. Also in the group vaccinated with the Karanganyar strain, and that was subsequently challenged with the Sukabumi strain (Group C) no virus transmission was observed. In the two control groups and group B (vaccination with Sukabumi/inoculation with Karanganyar), transmission was observed. For the control group challenged with the Sukabumi strain, we assumed a latent period of 2 days. The transmission rate parameter β was estimated at 2.4/day, the infectious period at 0.7 days (95% CI: 0.1–1.3), and the basic reproduction number at 1.7. For the control group challenged with the Karanganyar strain, we based the analysis on a latent period of 1 day. The transmission rate parameter β for the unvaccinated control groups was estimated at 2.5/day, the infectious period at 1.6 days (95%CI: 1.0–2.2), and the basic reproduction number at 4.1.

4. Discussion

The aim of this study was to assess whether the Archetti-Horsefall ratio, reflecting the antigenic relatedness between influenza field and vaccine strains, could be used to predict the efficacy against certain challenge virus with respect to vaccine potency and reduction in virus transmission among birds R value determined using the Archetti-Horsfall ratio had been used in previous study to select foot and mouth disease (FMD) virus vaccines (Ferris and Donaldson, 1992). Results in that study estimated the R value between 0.2 and 0.39, and this was said to be indicative of close antigenic relatedness The Archetti-Horsfall ratio has been used in a previous study to select. However, their interpretation of R value differs from those used for influenza viruses by Lee et al. (2004) and Beato et al. (2010) who suggested that viruses are related serologically if R value are larger than 0.5.

In the current study, the *in vitro* antigenic relatedness of the two virus/vaccine combinations based on the Archetti-Horsfall ratio was estimated at 0.37. Based on this estimate, we expected a poor efficacy of both vaccines against the heterologous challenge ($R < 0.5$). This was indeed so for the vaccine based on the Sukabumi strain, as it did not protect against transmission with the Karanganyar strain. The vaccine based on the Karanganyar strain, however, did provide protection against transmission of the Sukabumi strain. These findings indicate that protection induced by a vaccine based on a virus A to infection, disease, and transmission by a virus B may not be indicative of protection of a vaccine based on virus B to infection, disease and transmission by virus A. Of course, we only examined two vaccine strains and two challenge strains, implying that general conclusions cannot (yet) be drawn.

In our current studies we used an inactivated AI vaccine in water in oil emulsion representing all of the registered AI vaccines in Indonesia. For a more general statement more vaccine types and challenge strains combinations have to be tested which was beyond the scope of the current project. Previous studies of Rauw et al. (2012) and Kapczynski et al. (2015) showed limitations of classical inactivated AI vaccines to give protection against challenge. Both studies showed that the use of

Table 2
Serological response of chickens vaccinated with the Sukabumi strain.

Group[a]	Bird number	Treatment[b]	Mean HI titre (^{2}log)[c]			
			At challenge (D0)[d]		End of the experiment (D28)[e]	
			SMI[f]	KRA[f]	SMI[f]	KRA[f]
A	1	I	3	1	4	1
	2	I	2	2	2	2
	3	I	2	1	2	1
	4	I	5	4	5	4
	5	I	5	1	5	1
	6	I	2	1	3	2
	7	I	6	3	7	4
	8	I	3	0	4	0
	9	S	6	5	6	5
	10	S	4	1	5	2
	11	S	3	1	4	1
	12	S	3	2	3	2
	13	S	4	1	4	1
	14	S	4	1	4	1
	15	S	6	4	7	5
	16	S	5	2	5	2
B	1	I	4	1	8	7
	2	I	4	0	N.A.	N.A.
	3	I	3	0	N.A.	N.A.
	4	I	2	1	7	7
	5	I	4	2	7	7
	6	I	2	1	10	9
	7	I	4	1	N.A.	N.A.
	8	I	3	0	N.A.	N.A.
	9	S	5	1	9	8
	10	S	3	1	N.A.	N.A.
	11	S	4	1	N.A.	N.A.
	12	S	3	1	N.A.	N.A.
	13	S	4	1	4	1
	14	S	3	0	N.A.	N.A.
	15	S	3	1	6	2
	16	S	6	2	3	0

[a] A = challenge with Sukabumi strain (homologous challenge); B = challenge with Karanganyar strain (heterologous challenge).
[b] I = inoculated chickens; S = contact-exposed chickens.
[c] The data are mean HI titres (^{2}log) ± standard error; N.A. = not applicable since the bird died at end of experiment.
[d] 4 week post vaccination.
[e] 4 week post challenge.
[f] The strain used in the HI test as antigen (SMI = Sukabumi strain; KRA = Karanganyar strain).

Table 3
Serological response of chickens vaccinated with the Karanganyar strain.

Group[a]	Bird number	Treatment[b]	Mean HI titre (^{2}log)[c]			
			At challenge (D0)[d]		End of the experiment (D28)[e]	
			SMI[f]	KRA[f]	SMI[f]	KRA[f]
C	1	I	3	5	7	5
	2	I	4	6	5	6
	3	I	5	6	5	6
	4	I	4	6	7	7
	5	I	4	7	5	6
	6	I	5	6	5	6
	7	I	4	5	4	5
	8	I	6	7	6	7
	9	S	5	6	5	6
	10	S	3	5	4	5
	11	S	5	6	5	6
	12	S	6	7	7	7
	13	S	6	6	6	6
	14	S	5	7	5	7
	15	S	5	6	5	6
	16	S	4	6	4	6
D	1	I	4	5	5	6
	2	I	5	5	5	5
	3	I	3	6	6	6
	4	I	4	6	6	6
	5	I	6	5	5	5
	6	I	5	6	6	6
	7	I	4	4	4	4
	8	I	5	5	5	6
	9	S	4	5	5	5
	10	S	4	4	3	5
	11	S	6	7	7	7
	12	S	5	7	7	7
	13	S	5	6	5	6
	14	S	5	4	5	4
	15	S	4	5	4	5
	16	S	5	4	5	4

[a] C = challenge with Sukabumi strain (heterologous challenge); D = challenge with Karanganyar strain (homologous challenge).
[b] I = inoculated chickens; S = contact-exposed chickens.
[c] The data are mean HI titres (^{2}log) ± standard error.
[d] 4 week post vaccination.
[e] 4 week post challenge.
[f] The strain used in the HI test as antigen (SMI = Sukabumi strain; KRA = Karanganyar strain).

recombinant vector AI vaccine (rHVT-H5 vaccine) alone or in combination with classical inactivated AI vaccine provide better protection against challenge. Nevertheless, our results were in accordance with a previous study on avian influenza vaccine (Swayne et al., 2015) which showed that a serological response has predictive value for vaccine protection when vaccine and challenge virus were genetically and antigenically closely related, but when they had more distant relation, a serological response did not give consistent prediction on vaccine protection. A proper vaccination monitoring programme should be used by testing vaccinated birds in the HI test using vaccine virus and antigens prepared from recent field isolates. In addition this requires surveillance programmes to be conducted in the vaccinated populations to detect any new variant arising. Still, our study might contribute on improving vaccination strategies in order to decide if and when vaccines need to be updated.

According to the vaccine manufacturer, the antigenic load in both experimental vaccines was 512 HA units per dose. Nevertheless, the Sukabumi strain induced lower HI antibody titres in chickens than the Karanganyar strain. An explanation for this difference could be that the HA protein of Sukabumi strain was less immunogenic than HA of Karanganyar strain, as has been shown for influenza virus in the past (Hütter et al., 2013). Additionally, it is noteworthy that the Karanganyar strain has more N-glycosylation sites than Sukabumi strain which could result in increased antigenicity of the Karanganyar strain. This phenomenon was earlier reported for H1N1 influenza viruses which showed that addition of glycosylation sites may change the antigenicity of influenza viruses (Zhang et al., 2013). Another explanation is that the Karanganyar strain has a lower HA activity per μg HA than the Sukabumi strain and, as a consequence, vaccines based on the Karanganyar strain would contain more HA protein. Routinely HA content of poultry vaccines is measured by measuring the biological activity and not the HA concentration. However the difference in HA content would lower the response, but is not expected to have an effect on the cross-reactivity and thus should not affect the ratio of heterologous to homologous titre. The Sukabumi strain showed a lower r value (rs at 0.25) than the Karanganyar strain (rk at 0.63), indicating that the Sukabumi strain induced antibodies that had a lower capacity to cross-react than the antibodies induced by the Karanganyar strain.

In our challenge study, two surviving contact birds in group E (unvaccinated/inoculation Sukabumi) escaped from death. These birds had no detectable antibodies at the time of challenge and low HI titres at the end of the experiment. An explanation is that these surviving contact birds had become infected with an unusually low virus dose. These two birds showed AI-like clinical signs, and low antibody titres (below the cut-off) against challenge virus only (Table 5), indicating

Table 4
Serological response of unvaccinated chickens.

Group[a]	Bird number	Treatment[b]	Mean HI titre (^{2}log)[c]			
			At challenge (D0)[d]		End of the experiment (D28)[e]	
			SMI[f]	KRA[f]	SMI[f]	KRA[f]
E	1	I	0	0	N.A.	N.A.
	2	I	0	0	N.A.	N.A.
	3	I	0	0	N.A.	N.A.
	4	I	0	0	N.A.	N.A.
	5	I	0	0	N.A.	N.A.
	6	I	0	0	N.A.	N.A.
	7	I	0	0	N.A.	N.A.
	8	I	0	0	N.A.	N.A.
	9	S	0	0	2	0
	10	S	0	0	N.A.	N.A.
	11	S	0	0	N.A.	N.A.
	12	S	0	0	N.A.	N.A.
	13	S	0	0	N.A.	N.A.
	14	S	0	0	N.A.	N.A.
	15	S	0	0	N.A.	N.A.
	16	S	0	0	1	0
F	1	I	0	0	N.A.	N.A.
	2	I	0	0	N.A.	N.A.
	3	I	0	0	N.A.	N.A.
	4	I	0	0	N.A.	N.A.
	5	I	0	0	N.A.	N.A.
	6	I	0	0	N.A.	N.A.
	7	I	0	0	N.A.	N.A.
	8	I	0	0	N.A.	N.A.
	9	S	0	0	N.A.	N.A.
	10	S	0	0	N.A.	N.A.
	11	S	0	0	N.A.	N.A.
	12	S	0	0	N.A.	N.A.
	13	S	0	0	N.A.	N.A.
	14	S	0	0	N.A.	N.A.
	15	S	0	0	N.A.	N.A.
	16	S	0	0	N.A.	N.A.

[a] E = challenge with Sukabumi strain; F = challenge with Karanganyar strain.
[b] I = inoculated chickens; S = contact-exposed chickens.
[c] The data are mean HI titres (^{2}log) ± standard error; N.A. = not applicable since the bird died at end of experiment.
[d] 4 week post vaccination.
[e] 4 week post challenge.
[f] The strain used in the HI test as antigen (SMI = Sukabumi strain; KRA = Karanganyar strain).

that these birds did become infected.

The *in vitro* results in our current study corresponded with *in vivo* results, as we observed a difference between the two vaccine strains in HI antibody titres at moment of challenge. The Sukabumi vaccine induced a lower HI titre in chickens compared to the Karanganyar vaccine strain. Pre-challenge antibody titres to the challenge virus above 2^5 (Poetri et al., 2009) has been shown to be of sufficient level to induce protection against infection, as shown by Maas et al. (2009) and Abbas et al. (2011). In the group vaccinated with the Sukabumi strain (Group A), 6 of 16 birds had an HI titre of 2^5 or higher against the homologous strain, but none of the birds had such an HI titre against the heterologous strain. This might explain the lower level of *in vivo* protection. In contrast to the groups vaccinated with Sukabumi (group B), all birds in the group vaccinated with Karanganyar had an HI titre above the protective level against the homologous challenge virus, and 9 of 16 birds even had a protective HI titre against the heterologous strain. All birds in group C (vaccination Karanganyar/inoculation Sukabumi) had significantly higher titres against the heterologous strain than birds in group B (vaccination Sukabumi/inoculation Karanganyar) group. Virus shedding was only seen in the Sukabumi vaccine group in which 7 of 16 birds shed virus. Although we cannot give an explanation for the low HI titres against the heterologous strain in the Sukabumi vaccine groups, this finding might explain the observed different protection scope as well. We could not determine the association between HI titre and protection against shedding or infection, as this can only be done in pair-wise transmission experiments (Bouma et al., 2009; Poetri et al., 2011). For practical and logistical reasons this was not possible for the current study. However, a previous study (Kumar et al., 2007) has shown that HI titre ≥ 40 correlates with the absence of shedding of challenge virus by vaccinated birds, whereas in the current study, none of the birds in Sukabumi vaccine group (group B) developed HI titre ≥ 32 against challenge virus antigen. The emergence of antigenic variants in Indonesia has been documented since 2007 (Domenech et al., 2009; Dharmayanti et al., 2011). This antigenic drift may have occurred in the field due to immunological pressure of vaccination (Lekcharoensuk, 2008; Dharmayanti et al., 2011; Cattoli et al., 2011). The two strains we used did not differ significantly in transmission rate characteristics in unvaccinated groups, although the infectious period for the Karanganyar strain seemed to be slightly higher than for the Sukabumi strain. The latent period in the Sukabumi vaccine group challenged with the Karanganyar strain (Group B) was longer than in the unvaccinated group F challenged with Karanganyar, suggesting that the Karanganyar strain needed adaptation before replicating to such a level in vaccinated birds that virus shedding could be detected and transmission occurred. However, even if so, this did not seem to have altered the efficiency of transmission, as the rate remained comparable to the rate as measured for this strain in the unvaccinated group F.

Extrapolation of our results obtained in an idealized experimental setting to the situation in the field is not straightforward, and requires further validation in semi-experimental and field studies. For instance, one of the major issues that still needs to be addressed is the fact that chickens in our study had no maternally-derived antibodies (MDA), as

Table 5
Overview of HI titres at challenge, viral shedding, mortality rates, and seroconversion.

Group[a]	Treatment		Number of birds							
	Vaccination	Challenge	HI titre ≥ 32 at time of challenge		Shedding virus		Died		Seroconvert to challenge virus[d]	
			I[b]	S[c]	I	S	I	S	I	S
A	SMI	SMI	3	3	0	0	0	0	0	0
B	SMI	KRA	0	0	6	4	4	3	4	2
C	KRA	SMI	3	6	0	0	0	0	2	0
D	KRA	KRA	7	4	0	0	0	0	0	0
E	None	SMI	0	0	5	4	8	6	0	0
F	None	KRA	0	0	8	8	8	8	0	0

[a] Total numbers in each group were 8 inoculated chickens (I) and 8 contact-exposed chickens (S).
[b] I = inoculated chickens.
[c] S = contact-exposed chickens.
[d] Number of birds showing larger than 4 fold increase in HI titre.

our study had been performed with SPF chickens. As AI viruses are endemic to Indonesia, the existence of maternally-derived antibody (MDA) might interfere with vaccination efficacy in the field, as has been shown by De Vriese et al. (2010). Thus, it remains at present an open question to which extent vaccine efficacy will be molded by immunity mediated by maternally-derived antibodies.

Vaccination in Indonesia may have been suboptimal, allowing virus to spread unnoticed and thereby allowing new strains to emerge. A continuous surveillance is needed to understand genetic and antigenic variations of avian influenza virus in Indonesia. The Sukabumi and Karanganyar strains were isolated from clinical outbreaks in vaccinated flocks. The vaccine used in these flocks contained an H5N1 strain in the flock from which the Sukabumi strain originated, and a H5N2 strain in the flock from which the Karanganyar strain was isolated. Whether and how this has affected the outcome of the experiment, or affected the emergence of antigenic diversity between the two strains remains unclear.

Conflict of interest

The authors have declared no conflict of interest.

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