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ORIGINAL ARTICLE

Characterization of the M2e antibody response following highly pathogenic H5N1 avian influenza virus infection and reliability of M2e ELISA for identifying infected among vaccinated chickens

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A surveillance method able to differentiate between vaccinated and infected poultry is required for those countries that practice vaccination against highly pathogenic avian influenza H5N1. The external domain of the M2 protein (M2e) of influenza virus is a potentially useful differentiating-infected from vaccinated animals (DIVA) antigen but little is known about the M2e antibody response and factors influencing its detection. In this study, the M2e antibody response was characterized in layer birds vaccinated and challenged with an Indonesian H5N1 virus isolate, using a single M2e peptide or four-branched multiple antigenic peptide form of M2e (MAP-M2e) as antigens in two separate ELISAs. Anti-M2e antibodies were absent in chicks with high level of maternal haemagglutination inhibition antibodies and also in all layers vaccinated once, twice or three times with an inactivated commercial H5N1 vaccine. In contrast, anti-M2e antibodies were detected in vaccinated layers challenged with H5N1 virus and their presence was associated with virus isolation and an increase in haemagglutination inhibition titres. The number of birds that developed M2e antibodies, as well as the strength and duration of the M2e antibody response were strongly influenced by the length of the interval between vaccination and challenge. Birds challenged at six weeks after vaccination all developed M2e antibodies by 14 days that lasted until at least 56 days after infection. In birds challenged at two weeks after vaccination, only a proportion of birds developed M2e antibodies by 14 days that lasted only until 28 days post-infection. Both single M2e peptide and MAP-M2e ELISAs had high diagnostic specificity but the diagnostic sensitivity of MAP-M2e ELISA was significantly higher and more effective in detecting M2e antibody in immune and infected birds. The results show that MAP-M2e ELISA would be useful for surveillance in countries using vaccination to control highly pathogenic avian influenza H5N1.

Introduction

Highly pathogenic avian influenza caused by the subtype H5N1 (HPAI H5N1) has been an important disease in Indonesia since 2003. Following incursion, the H5N1 spread rapidly causing severe outbreaks in many parts of the country (Forster, 2009; Loth *et al*., 2011) that resulted in significant economic losses to the poultry industry and a high number of human H5N1 cases with significant fatalities (Forster, 2009; Basuno *et al*., 2010; FAO, 2013). The widespread and high incidence of the disease compelled the application of vaccination as an important complement to other control measures. Since 2005, vaccination has been applied intensively to both breeder and layer birds. Due to technical constraints, no vaccination is generally carried out in backyard poultry (Bett *et al*., 2015), nor in commercial broilers (Spackman & Swayne, 2013).

One of the biggest challenges in applying vaccination for the control of avian influenza viruses (AIV) is the use of vaccines that match the circulating virus(es) (Capua & Alexander, 2008; van den Berg *et al*., 2008), which are often changing as a result of mutations or new introductions (Cattoli *et al*., 2011; Zhong *et al*., 2014a). Since it is not feasible to update frequently the H5N1 vaccine for poultry, protective immunity in vaccinated chickens may often be unable to withstand challenge by circulating H5N1 strains (El-Zoghby *et al*., 2012; Connie Leung *et al*., 2013) leading to infection and shedding of the virus into the environment (Rudolf *et al*., 2010; Abdelwhab *et al*., 2011). In Indonesia, it has been suspected that village chickens and ducks, which roam freely and are usually not vaccinated, may act as a source of HPAI H5N1 infection for nearby commercial poultry. The infected commercial poultry may then in turn unrecognizably act as virus reservoirs and be a source of new H5N1 strains (Yupiana *et al*., 2010).

Routine monitoring for the presence of circulating H5N1 virus based on virus isolation or antigen detection is not feasible because of the short duration of virus infection and

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antigen secretion, as well as the high cost of tests and their limited sensitivity (Loth *et al*., 2008). In contrast, serological tests are practical, cheap, sensitive and reliable due to their ability to detect specific antibodies for a long duration following exposure to a pathogen. However, currently available serological tests for AIV cannot detect whether vaccinated birds have been infected. This shortcoming was realized in the early stages of H5N1 vaccination in Indonesia, and in order to address this drawback, the Government of Indonesia had planned to use a DIVA (differentiating infected from vaccinated animals) strategy as previously applied in Italy (Capua *et al*., 2004). This strategy is based on the use of an inactivated vaccine containing the haemagglutinin (HA) of the same subtype as the challenge virus (i.e. H5), but with a different neuraminidase, i.e. N2 instead of N1 (Capua *et al*., 2003; Cattoli *et al*., 2006). Nevertheless, this strategy failed to be implemented in Indonesia partly because of the reluctance of the poultry farmers to use heterologous H5N2 vaccines, which were not fully protective against challenge with Indonesian H5N1 strains (Swayne, 2006; Swayne *et al*., 2006; Poetri *et al*., 2009).

Antibodies to the external domain of the M2 protein (M2e) have been reported to be present only in chickens infected with live AIV but not in experimentally vaccinated chickens, thereby enabling the use of M2e protein as DIVA antigen (Lambrecht *et al*., 2007; Kim *et al*., 2010; Hemmatzadeh *et al*., 2013; Hadifar *et al*., 2014). The M2 protein is packed into the virion in a low copy number in comparison to the other influenza virus surface proteins: HA and neuraminidase (Zebedee & Lamb, 1988). For this reason, antibodies to M2e are absent in animals immunized with a killed vaccine. However, the amount of M2 protein in infected cells is almost equal to that of HA and hence the infected hosts are capable of mounting specific antibodies to the M2e (Lamb *et al*., 1985).

Despite its potential as a candidate for a DIVA test, there have been only a few studies on the characterization of the M2e antibody response in vaccinated and infected chickens (Lambrecht *et al*., 2007; Kim *et al*., 2010; Marché & van den Berg, 2010; Hemmatzadeh *et al*., 2013). In studies conducted in pigs and humans, the M2e antibodies were either absent or induced only at low titres (Feng *et al*., 2006; Kitikoon *et al*., 2008; Zhong *et al*., 2014b). Currently, it is unknown whether the M2e antibodies are present in birds after multiple vaccinations, which is a common practice in breeders and layers in Indonesia. Also, the time course of M2e antibody development is largely unknown, including the strength and duration of M2e antibodies, all of which would be expected to have a major influence on the efficacy of M2e-based DIVA tests.

The objectives of the present study were to provide information on the status of M2e antibodies in repeatedly vaccinated birds and to characterize the M2e antibody response in vaccinated layers challenged with HPAI H5N1. In addition, the diagnostic specificity and sensitivity of the two M2e ELISAs, as possible DIVA tests, were evaluated.

Materials and Methods

Vaccination of layer chicks. Two hundred, day-old commercial layer chicks were purchased from a local layer breeding farm and reared on the floor of a closed henhouse. They were fed with a commercial ration and had access to chlorinated drinking water *ad libitum*. Blood samples were collected from 40 randomly selected birds at 1, 7, 14, 21, 28, 35 and 42 days of age. At 48 days of age, the birds were wing-tagged, bled and randomly divided into 4 groups (A, B, C and D) with 50 per group. They were then placed into four separate compartments, one group per compartment, in the same henhouse.

Birds in group A were vaccinated at 8, 12 and 16 weeks of age, those in group B at 12 and 16 weeks, and in group D at 16 weeks of age with the commercial inactivated vaccine Medivac-AI® (Pt. Medion, Bandung, Indonesia). The H5N1 strain contained in the vaccine is not specified but is likely to be A/Chicken/West Java/PWT-WIJ/2006 (H5N1) (GenBank accession no: EU124148.1). Birds in the group C were not vaccinated and served as controls. All the birds in groups A, B and D (n = 50/group) were bled prior to each vaccination.

Experimental infection of vaccinated layers with H5N1. Two separate experiments were carried out. In Experiment 1, vaccinated birds were challenged 2 weeks after their last vaccination, i.e. at 18 weeks of age. As described above, vaccinated birds from group A were vaccinated thrice (at 8, 12 and 16 weeks of age), those in group B were vaccinated twice (at 12 and 16 weeks of age), and those in group D only once (at 16 weeks of age). Eight birds from each of the vaccinated groups A, B and D and six birds from the control group C were randomly selected and moved into a biosecurity level 3 experimental facility for infection. The facility has two separate positive pressure isolators and 16 birds were housed in each isolator, each with birds from each group. Each bird was inoculated oropharyngeally with 10^5 EID_{50}, in 100 µL of H5N1 virus strain A/chicken/West Java/Sbg-29/2007 (GenBank accession no: KC831453.1). Just before inoculation, all birds were bled for collection of sera. Cloacal and oropharyngeal swabs were collected into 2 mL of virus isolation media (Medium-199, Bovine albumin, antibiotics and antimycotics) (Sigma Co. Singapore). Serum and swab samples were also collected at 3, 7, 14, 21 and 28 days post-infection (dpi). Feed and water were provided *ad libitum*. Birds were observed several times during the day, at regular intervals, for clinical signs and discomfort.

In Experiment 2, vaccinated birds were challenged 6 weeks after the last vaccination, i.e. at 22 weeks of age. This experiment was carried out after Experiment 1 had been completed and in the same biosecurity level 3 facilities. Twenty-four birds, six from each of the vaccinated groups (i.e. A, B and D) and six birds from the control group (C), were randomly selected and housed in two positive pressure isolators, with 12 birds per isolator, each with three birds from each group. Only birds from the unvaccinated control group (C) were inoculated as in Experiment 1 whereas birds from the vaccinated groups (A, B and D) were not inoculated but were expected to be infected via the in-contact route. Cloacal and oro-pharyngeal swabs and blood samples were collected at the time of infection and subsequently at 3, 7, 14, 21, 28, 35, 42, 48 and 56 dpi.

Animal ethics approval. All experiments were carried out in strict accordance with the article 80 on Research in Animal Health of the Indonesian Law on Livestock and Animal Health ("UU No 18 tahun 2009"). Protocols were approved by the Committee on the Ethics of Animal Experiments of the Indonesian Research Center for Veterinary Sciences (Registration number: BB/V/A/01/2013) and carried out in accordance with the standard procedures described in the Manual of Diagnostic Tests and Vaccines (OIE, 2009).

Haemagglutination and haemagglutination inhibition tests. Haemagglutination (HA) and haemagglutination inhibition (HI) tests were performed according to the OIE (2009) standard procedures using the HA antigen prepared from H5N1 strain A/chicken/West Java/Sbg-29/2007 (GenBank accession no: KC831453.1). For the HI test, the serum to be tested was serially diluted in 25 µL of phosphate-buffered saline (PBS) in V-bottom microtitre plates and an equal volume of HA antigen containing 4 HA units was added. After incubation at 25°C for 30 min, 25 µL of a 1% suspension of chicken red blood cells was added and incubated for 40 min at 25°C. The HI titre was expressed in $\log_2$ units of the highest dilution of sera that completely inhibited haemagglutination.

Virus re-isolation. Isolation of H5N1 challenge virus from swab samples was performed according to the OIE standard procedures (OIE, 2009). Briefly, the cloacal or oro-pharyngeal swabs, immersed in the virus isolation media, were vortexed, clarified by centrifugation at 1000 × g and the supernatant was collected. Three specific pathogen-free chicken eggs at

11 days of incubation were inoculated into the allantoic cavity with 100 µL of the clarified swab sample. After incubation at 37°C for 72 h, the allantoic fluid was collected and the HA test carried out. If the allantoic fluid did not have HA activity, it was passaged for another one or two rounds using the same procedure. If the fluid had HA activity and the activity could be neutralized by the H5N1-specific antiserum in the HI test, the swab sample used for inoculation was considered positive for H5N1 virus.

Single M2e peptide (sp-M2e) ELISA. A peptide with a sequence identical to the 23–amino acid sequence of the N-terminal part of the M2 protein of the Indonesian H5N1 isolate, A/Indonesia/CDC540/2006 (GenBank accession no: EU014132.1) was used as the antigen in the ELISA (Hemmatzadeh *et al*., 2013). The sp-M2e peptide, synthetized by VCPBIO Ltd. (Shenzhen City, China), to a purity of >95%, was diluted in carbonate buffer pH 9.6 at concentration of 5 µg/mL, and added to a 96-well microtitration plate (Maxisorp, Nunc, Roskilde, Denmark), with 100 µL/well. Six wells in every plate were left uncoated as controls. After incubation at 4°C overnight, the plate was blocked with 1% non-fat skim milk in PBS buffer pH 7.2. The serum sample was diluted 1:100 in ELISA buffer [0.7 M NaCl, 0.05 M EDTA, 3% (v/v) Triton X-100, 3% (v/v) Tween-20, non-fat skim milk (2% (w/v)), rabbit serum (5% (v/v), 0.1 M Tris, pH 7.4)] and then added in a final volume of 100 µL to the wells. In each negative plate, vaccinated and infected control sera were added, diluted 1:100. After incubation at room temperature (25°C), the plate was washed four times with washing buffer [640 µM NaCl, 3 µM KCl, 8 µM Na_2HPO_4, 1.5 µM KH_2PO_4 and 5% (v/v) Tween-20]. A solution of horseradish peroxidase–labelled rabbit anti-chicken IgG (Sigma Chemicals, St Louis, MO, USA) diluted in ELISA buffer was added and incubated at 25°C for 2 h. After washing four times, chromogenic substrate solution, 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (Sigma) in phosphate-citrate buffer [57 µM citric acid, 86 µM Na_2HPO_4, 0.6%(v/v) H_2O_2, pH ≈ 4.2] was added. The optical density (OD) at 420 nm was measured after about 5 min using a microplate reader (Multiskan Ex, Thermo Scientific, Waltham, MA USA).

The test was considered valid if (1) the OD value of negative and vaccinated control sera were ≤0.2; (2) the OD of positive control sera was ≥0.9; (3) the positive/negative OD ratio was ≥7 and (4) the OD of all non-coated wells, either with or without serum, were ≤0.2. The ODs of positive control sera diluted serially 1:100, 1:200, 1:400, 1:800 and 1:1600 were linear and the ODs at 1:6400 were equal to those of the negative control sera. The OD value of serum sample was standardized using the following formula: (OD_{420} sample−OD_{420} negative control)/(OD_{420} positive control −OD_{420} negative control). This standardization allowed comparison between assays performed at different times. The cut-off OD_{420} value for the standardized sp-M2e ELISA was ≤0.1.

Multiple antigenic form of M2e ELISA. The multiple antigenic peptide form of M2e (MAP-M2e) ELISA was similar to the sp-M2e ELISA except that the coating antigen was in the form of a four-symmetry branched MAP-M2e, wherein each M2e branch was identical in sequence and length to the sp-M2e peptide. This peptide was synthetized by VCPBIO Ltd. (Shenzhen City, China), but its purity was not known. The cut-off OD_{420} value for the standardized MAP-M2e ELISA was ≤0.03.

Negative, vaccinated and infected control sera. These sera were used in all the ELISAs as controls. The negative control sera were collected from 18-week-old layers not vaccinated against any disease and raised in isolated housing in our laboratory. HI tests conducted on the sera collected from these birds between 1 and 18 weeks of age indicated that they were H5N1 antibody negative throughout this period. The OD readings of these sera in the sp-M2e ELISA were comparable to those of specific pathogen-free sera. The vaccinated control sera were collected from the hatchmates but they were vaccinated at 8 and 12 weeks of age with the inactivated H5N1 vaccine Medivac-AI® (PT Medion, Bandung, Indonesia). Blood was collected from these birds two weeks after the second vaccination and the collected sera were pooled, distributed in small volume and kept at −20°C until use. As a large quantity of M2e positive sera was required for the development and validation of the test, IgY was purified from the eggs collected from vaccinated layers infected with A/chicken/West Java/Sbg-29/2007 (H5N1) virus (Jensenius & Koch, 1997). The purified IgY was suspended in PBS and the concentration adjusted so that its titre in the sp-M2e ELISA was comparable to the sera of layers vaccinated and infected with H5N1 and from which the eggs for IgY isolation were collected.

Data analyses. Differences in the HI titre between groups of birds were analysed by the Kruskal–Wallis non-parametric one-way analysis of variance (Petrie & Watson, 2006). Receiver operating characteristic (ROC) curve analysis was used to determine the comparative diagnostic accuracy of the sp-M2e and MAP-M2e ELISAs. For this analysis, as in the previous studies, all sera collected prior to the challenge (in total 450 sera from vaccinated groups A, B and D) were classified as negative ("non-infected"), whereas those collected after the challenge (142 sera from Experiment 1 and 120 sera from Experiment 2) were classified as positive ("positively infected") (Brocchi *et al*., 2006; Dekker *et al*., 2008). The area under the curve (AUC) was calculated, and the criteria proposed by Swets (1988) were used to assess the two tests in terms of diagnostic accuracy. To assess if there was a significant difference between the median M2e responses during the period up to day 28 of the two infection experiments, we used a two-way nested analysis of variance. All analyses used the statistical software package IBM SPSS (version 21), except for the comparison of the two infection experiments, for which we used *R* (version 3.1).

Results

Serological status of vaccinated layer chicks: HI antibodies. The day-old chicks used in this study came

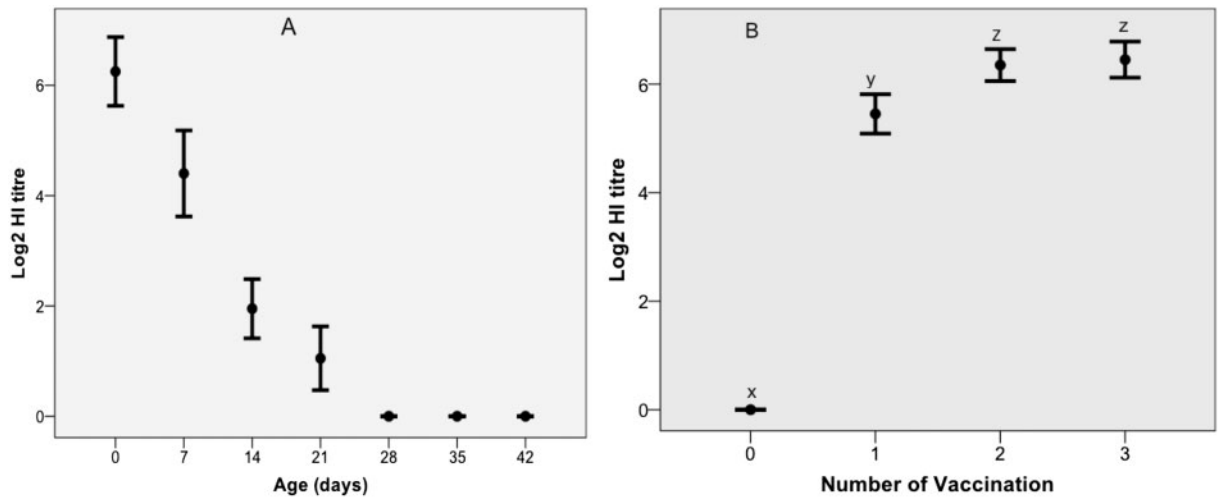

Figure 1. *Mean HI titres of chicks before vaccination (n = 30) (A) and at two weeks after the last vaccination (n = 50) (B). Error bar = 95% confidence intervals of the mean. Different letters above the error bars in (B) indicate significantly different mean titres (*P < 0.05*) as assessed by the Kruskal–Wallis test.*

from a layer breeder flock that was vaccinated against H5N1. As a result, the day-old chicks had high HI antibody titres to H5N1, with the mean titre of the 40 chicks being 6 $\log_2$) (Figure 1A). This titre gradually declined and by 28 days of age had become undetectable (≤2 $\log_2$). From then on, in all chicks (n = 200), the HI antibodies remained undetectable until the time of vaccination, suggesting that the chicks had no accidental exposure to the H5N1 infection during the rearing period.

All birds responded to vaccination with inactivated H5N1 vaccine (Figure 1B). At two weeks after the last vaccination, the mean HI titres of the birds vaccinated twice (group B) and three times (group A) were significantly higher ($P < 0.05$) than the mean HI titre of birds vaccinated once (group D) (6.6 $\log_2$, 6.4 $\log_2$ and 5.4 $\log_2$, respectively). The mean HI titres of birds vaccinated twice and three times were not significantly different ($P > 0.05$).

M2e antibodies. Antibodies to M2e were absent in all sera (n = 280) collected from birds at 1, 7, 14, 21, 28, 35 and 42 days of age in both the sp-M2e and MAP-M2e ELISAs, with the cut-off OD_{420} being 0.10 and 0.03, respectively (data not presented). This negative response was regardless of whether the sera contained high or undetectable maternal HI antibodies. The M2e antibodies remained absent in all birds throughout the rearing period and up to the time of the vaccination at the age of 8, 12 and 16 weeks in groups A, B and D, respectively, and at 20–28 weeks of age in group C.

To determine if multiple vaccinations with inactivated H5N1 vaccine induced M2e antibodies and thereby might influence the specificity of the M2e DIVA, sera of birds vaccinated once, twice and three times were tested with the sp-M2e and MAP-M2e ELISAs, with the cutoff OD_{420} being 0.10 and 0.03, respectively. As shown in Figure 2, M2e antibodies were absent in all vaccinated birds (n = 150) regardless of whether they had been vaccinated once, twice or thrice. Vaccination also did not increase the background ODs in the ELISAs (Figure 2), i.e. the pre- and post-vaccination sera ODs were comparable in both the sp-M2e and MAP-M2e ELISAs, except that the variation of OD in the MAP-M2e ELISA was lower.

Table 1. *M2e antibodies in birds vaccinated once, twice or thrice and those not vaccinated following challenge with H5N1.*

	Experiment 1[a]				Experiment 2[a]			
	No of vaccinations				No of vaccinations			
	3	2	1	0	3	2	1	0
No of birds	8[b]	8	8	6	6	6	6	6
Positive for virus[c]	5/8	5/8	4/8	6/6	6/6	6/6	6/6	6/6
Increased HI titre at 14 dpi	6/6	6/7	6/7	–[f]	4/4	4/4	4/4	-[f]
Seroconverted to sp-M2e at 14 dpi[d]	3/6	5/7	2/7	–[f]	4/4	4/4	4/4	-[f]
Seroconverted to MAP-M2e at 14 dpi[e]	5/6	7/7	5/7	–[f]	4/4	4/4	4/4	-[f]

[a]Experiment 1 – all birds inocu lated with A/chicken/West Java/Sbg-29/2007 H5N1 virus; Experiment 2 – only unvaccinated controls were inoculated with H5N1 and the other birds were infected by the in-contact route.
[b]No of birds at the start of experiment.
[c]Successful virus isolation from either cloacal or oro-pharyngeal swab at between 1 and 8 days post-infection.
[d]Cut-off OD value for sp-M2e ELISA ≤0.1.
[e]Cut-off OD value for MAP-M2e ELISA ≤0.03.
[f]Not examined – dead before 14 days post-infection.

Clinical disease and virus isolation following H5N1 challenge. In the unvaccinated control birds (group C) inoculation of H5N1 virus caused lethargic behaviour in some birds at 2 dpi, with sudden death at 3–5 dpi. The virus was re-isolated from cloacal and oro-pharyngeal swab samples from all birds at 3 dpi. In the unvaccinated control birds there were no apparent differences in the outcome of infection between Experiments 1 and 2 (Table 1).

Vaccination protected the birds, although not completely, against mortality and infection following challenge with A/chicken/West Java/Sbg-29/2007 (Table 1). In Experiment 1, where birds were infected oro-pharyngeally two weeks after the last vaccination, four (17%) birds died between 9 and 14 dpi, two in the group vaccinated thrice, and one in each of the groups vaccinated one and two times. By comparison, in

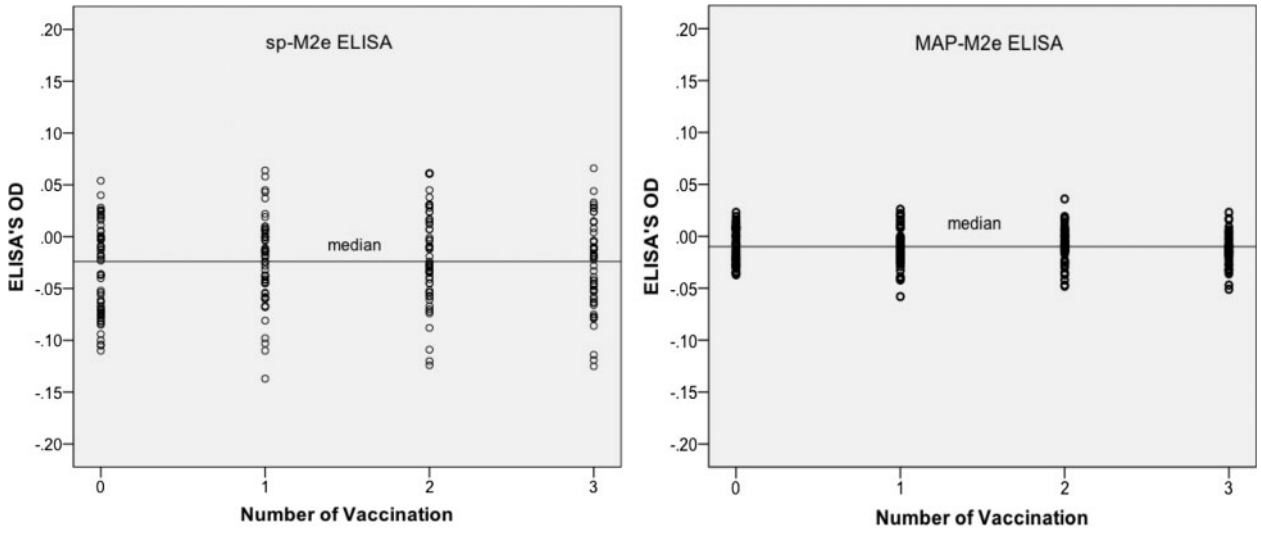

Figure 2. *Vaccination with inactivated H5N1 vaccine did not induce M2e antibodies as detected by (A) sp-M2e ELISA and (B) MAP-M2e ELISA. Four groups of birds, 50 in each group, were either vaccinated once, twice or thrice, or not vaccinated. M2e antibodies were measured in sera collected at two weeks after the last vaccination. Circles represent the OD value of individual birds.*

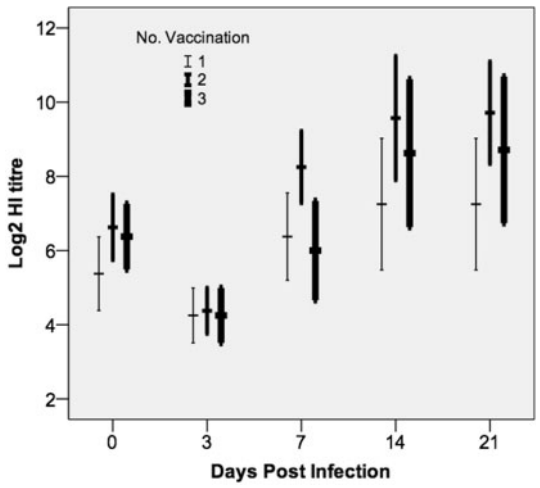

Figure 3. *Mean homologous HI antibody titres in sera of vaccinated birds following challenge in Experiment 1. Groups of eight birds vaccinated once, twice or thrice were challenged at two weeks after the last vaccination with H5N1 strain A/chicken/West Java/Sbg-29/2007 and HI titres determined at the times indicated. Error bar = 95% confidence interval of the mean.*

Experiment 2, where birds were infected by the in-contact route six weeks after the last vaccination, six (33%) birds died between days 7 and 9, two from each of the vaccinated groups. In Experiment 1, H5N1 virus was re-isolated from birds at either 3, 7, 14 or 21 dpi, in total of 50%, 63% and 63% of birds vaccinated once, twice or thrice, respectively. In Experiment 2, H5N1 virus was re-isolated from swabs of 6/18 (33%) at 3dpi and from 18/18 (100%) birds at 7 dpi.

HI antibody response. At the time of challenge with H5N1 virus, the mean HI titres of the birds in Experiment 1 were 5.4 $\log_2$, 6.6 $\log_2$ and 6.4 $\log_2$ in the groups vaccinated once, twice and thrice, respectively (Figure 3). Individual HI antibody titres ranged from 4 to 8 $\log_2$ (result not shown). The HI titres dropped at 3 dpi but rapidly rose again, and at 7 dpi mean HI titres were higher in all groups than at the time of infection (Figure 3). In all vaccinated groups the majority of birds had high HI titres (86%, 86% and 100% in groups vaccinated once, twice and tree times, respectively) (Table 1). There was no apparent association between the HI titres at the time of infection and the re-isolation of the challenge virus. Virus was not re-isolated from some birds with HI titres $\leq$6 $\log_2$ and was isolated from others having HI titres $\geq$7 $\log_2$. Also, no apparent association was detected between virus re-isolation and magnitude of HI titre increase at either 14 or 21 dpi (results not shown). In Experiment 2, all surviving birds had increased HI titres that corresponded with the re-isolation of challenge virus from all birds.

The proportion of vaccinated birds that developed the M2e antibodies. Overall, the M2e antibody response was detected in vaccinated birds challenged with a heterologous H5N1 A/chicken/West Java/Sbg-29/2007 strain but the number of birds that developed the M2e antibodies varied between Experiments 1 and 2 (Table 1).

In Experiment 2, where birds were challenged at 6 weeks after the last vaccination, all surviving birds, 12/12 (100%), developed M2e antibodies regardless of number of vaccinations, and these were detected by both the sp-M2e ELISA and the MAP-M2e ELISA. All birds developed M2e antibodies by 14 dpi and the number of M2e positive birds did not increase thereafter (result not shown). In Experiment 1, where vaccinated birds were challenged at two weeks after the last vaccination, only a proportion of birds developed the M2e antibodies and the number of birds detected as M2e positive were influenced by the test used. With the sp-M2e ELISA 2/7 (29%), 5/7 (71%) and 3/6 (50%) birds were M2e positive at 14 dpi in groups vaccinated once, twice or thrice, respectively. However, the MAP-M2e ELISA detected a greater number of birds that seroconverted, 5/6 (83%), 7/7 (100%) and 5/7 (71%) at 14 dpi in groups vaccinated thrice, twice or once, respectively. In this first experiment, one additional bird, which was from the group vaccinated once, was initially negative at 14 dpi and then became M2e positive at 21 dpi.

The time course and strength of M2e antibody development. The time course and strength of M2e antibody development also differed between Experiments 1 and 2 (Figure 4). In Experiment 1, where vaccinated birds were challenged two weeks after the last vaccination, the M2e antibody was observed in most birds at 14 dpi, then increased and peaked at 16 dpi and from then on decreased reaching negligible levels at 28 dpi (Figure 4). In Experiment 2, where vaccinated birds were challenged at six weeks after the last vaccination, the M2e antibodies were detected at 7 dpi, increased and peaked at between 14 and 21 dpi and decreased thereafter. At 56 dpi the M2e antibodies were still detectable although at a low level. The overall strength of the M2e antibody response, as assessed by the median of the standardized OD response measured at d7, d14, d21 and d28, excluding birds with incomplete data due to premature death, was significantly higher ($P < 0.05$) in Experiment 2 than in Experiment 1. This difference in response was shown with both the sp-M2e ELISA (median OD 0.424 vs. 0.073) and the MAP-M2e ELISA (median OD 0.424 vs. 0.073).

Association between the M2e sero-conversion and the increase in HI antibody titres. A strong association between the generation of M2e antibody and the increase in HI titres after infection was observed in Experiment 2, but was less obvious in Experiment 1. In Experiment 2, all the birds, 12/12 (100%), that generated M2e antibodies had increased HI antibody titres and all were infected (Table 1). In Experiment 1, in the majority of birds (16/20), the presence of M2e antibodies was associated with the increase in HI titres, although some birds with increased HI titres did not develop M2e antibodies (2/20) (Figure 5). No association was observed between the strength of the M2e and HI responses, as some birds with strong HI response (increase in HI titres of 3 $\log_2$ and above) did not develop M2e antibodies.

Comparison of sp-M2e and MAP-M2e ELISA by ROC curve analysis. As shown in Table 1 and Figure 2, while the sp-M2e and MAP-M2e ELISAs both detected M2e antibodies in vaccinated and infected birds, the MAP-M2e ELISA was more sensitive and detected a larger number of M2e positive birds. Also, the variability of ODs among vaccinated sera was lower in the MAP-M2e ELISA. The superiority of MAP-M2e ELISA was further demonstrated by the ROC curve analysis (Figure 6 and Table 2). For this analysis, 450 sera, collected from birds in the vaccinated groups A, B and D, were considered as negative (non-infected), whereas 262 sera collected after the

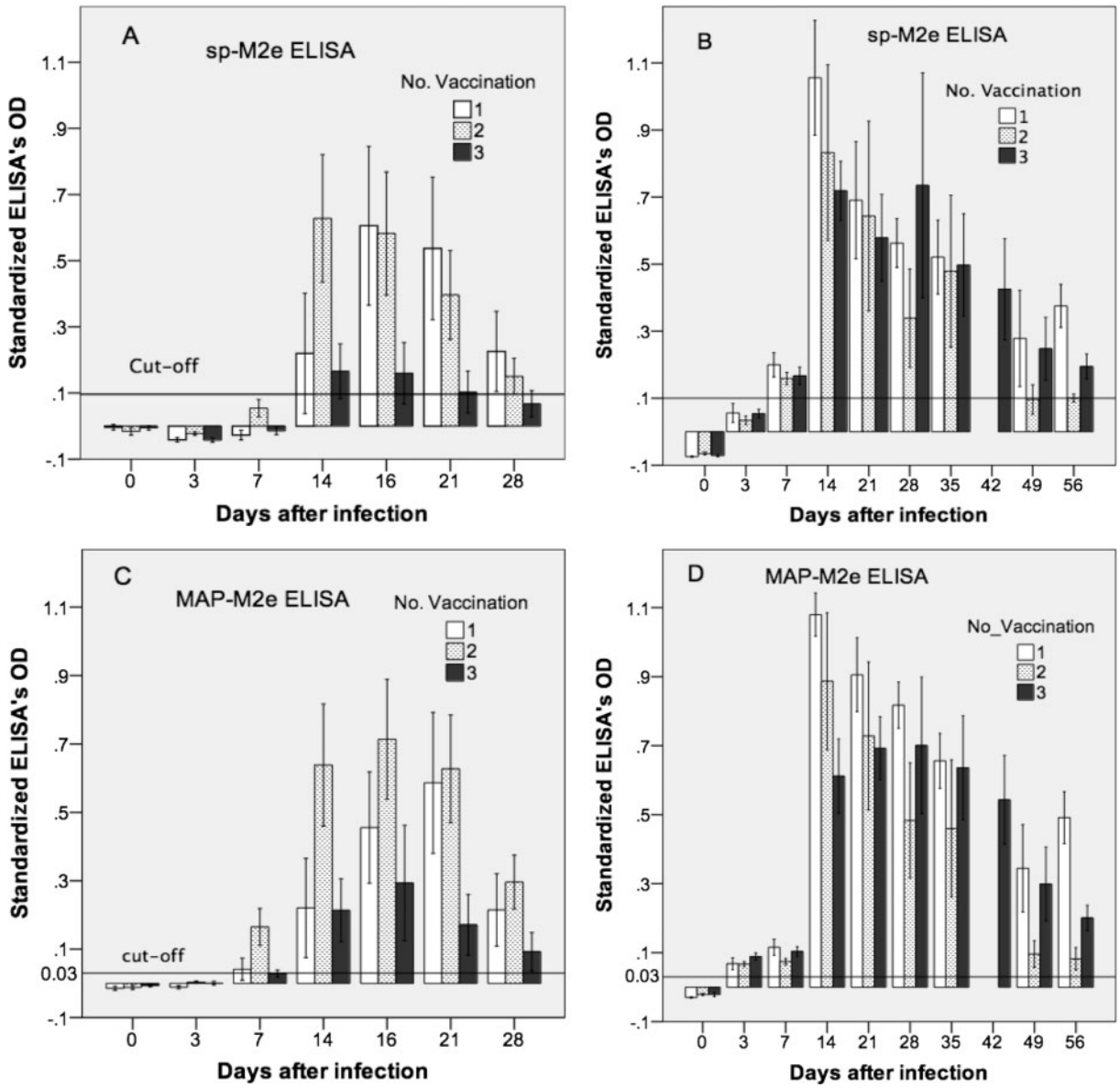

Figure 4. *M2e antibodies in vaccinated birds infected oro-pharyngeally with A/chicken/West Java/Sbg-29/2007 H5N1 virus at two weeks after the last vaccination in Experiment 1 (A & C) or infected by in-contact transmission at six weeks after the last vaccination in Experiment 2 (B & D). M2e antibodies determined using sp-M2e ELISA (A, B) or MAP-M2e ELISA (C & D). Error bars denote standard error of the means.*

challenge (142 sera from Experiment 1 and 120 sera from Experiment 2) were considered as positive (positively infected). The accuracy of the sp-M2e and MAP-M2e ELISAs to detect AIV infection in these sera by ROC curve analysis is depicted by the AUC. The AUC of the MAP-M2e ELISA was 0.940, whereas that of the sp-M2e ELISA was lower at 0.862, in comparison to the ideal value of 1 (Table 2).

Sensitivity, specificity and cut-off value of M2e ELISAs. The sensitivity and specificity of the sp-M2e and MAP-M2e ELISA when different ODs are selected as cut-off values are presented in Table 3. If a specificity of 100% was chosen as the desired characteristic of the M2e DIVA, the lowest OD cut-off point for MAP-M2e ELISA was at 0.03, giving a test sensitivity of 75%. For the sp-M2e ELISA at test specificity of 100%, the lowest cut-off point was at OD of 0.1 resulting in a test sensitivity of 53%.

Discussion

This study confirms that the antibody to the M2e protein can be used for DIVA in commercial flocks vaccinated multiple times using a sensitive test such as the MAP-M2e-based ELISA. The use of a DIVA test has been considered as an important component of control measures in countries with endemic HPAI H5N1 aiming at eventual eradication (Capua & Alexander, 2006). Despite the early work on the potential of the M2e protein for DIVA testing, there have been only a few studies on the M2e antibody response in vaccinated and infected poultry (Lambrecht *et al*., 2007; Kim *et al*., 2010; Marché & van den Berg, 2010; Hemmatzadeh *et al*., 2013).

To use the M2e DIVA test, it is critical to confirm that the M2e antibodies are absent in all poultry vaccinated multiple times with inactivated H5N1 vaccines. We showed that M2e antibodies were absent in birds with high HI titres of maternal antibodies (≥6 $\log_2$), which are usually derived

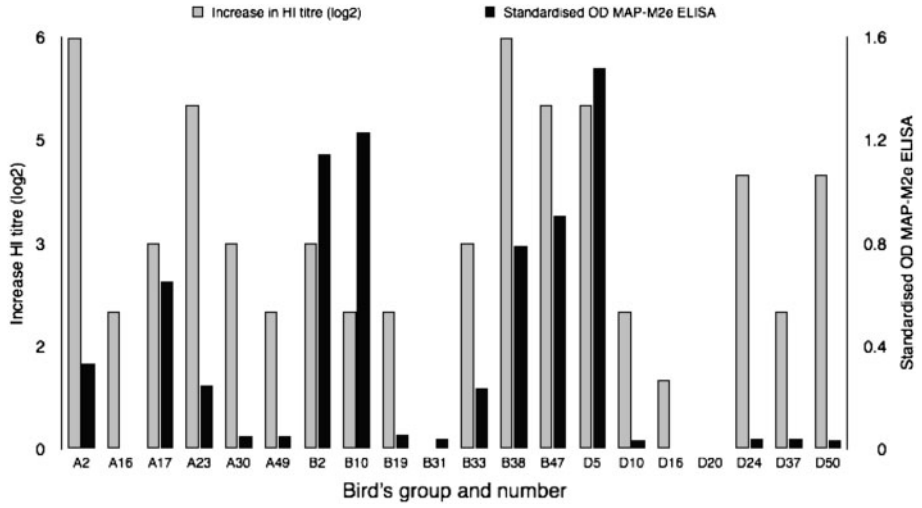

Figure 5. *Agreement between increase in HI titre and strength of M2e antibody response detected by MAP-M2e ELISA in vaccinated birds challenged at two weeks after the last vaccination with H5N1 strain A/chicken/West Java/Sbg-29/2007 (Experiment 1); HI titres were determined against the A/chicken/West Java/Sbg-29/2007. The log_2 values indicate difference in HA titres at time of challenge and 14 dpi. Birds vaccinated (A) three, (B) two and (D) one time with inactivated vaccine Medivac-AI. OD above 0.03 were considered as positive.*

Table 2. *The area under the curves (AUC) for MAP-M2e and sp-M2e ELISA.*

ELISA	Mean	SE	95% CI mean	Diagnostic accuracy[a]
sp-M2e	0.862	0.017	0.829–0.894	Moderately accurate
MAP-M2e	0.940	0.010	0.919–0.960	Highly accurate

[a]Based on the criteria proposed previously where 0.5 ≤AUC <0.7 is considered to be less accurate, 0.70 ≤AUC <0.9 is moderately accurate, 0.9 ≤AUC <1 is highly accurate, and that with AUC =1 is perfectly accurate (Swets, 1988).

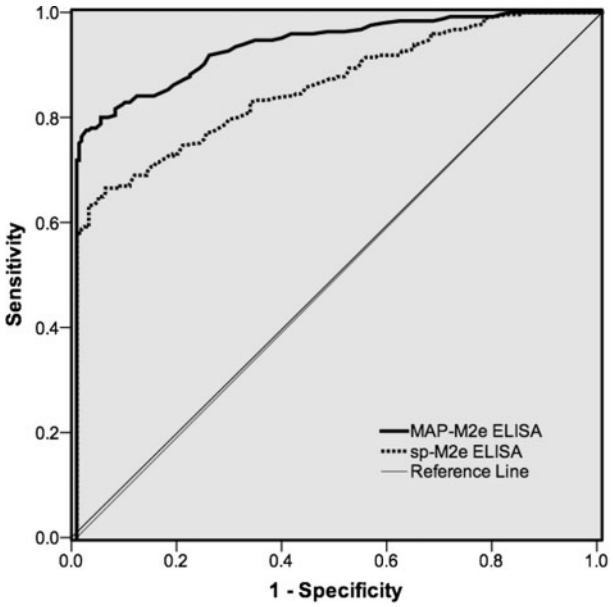

Figure 6. *The receiver operating characteristic (ROC) curves of MAP-M2e and sp-M2e ELISAs. The "ROC space" is defined by false-positive rate, which is equal to 1 minus the specificity, and the true positive rate, which is the sensitivity (x and y axes, respectively). This depicts the relative trade-off between the true-positive and false-positive rates – and is thus effectively the sensitivity versus specificity plot.*

from multiple vaccinations of breeders with vaccines of different origins (Spackman & Swayne, 2013). The M2e antibodies were also absent in vaccinated layers regardless of whether they were vaccinated once, twice or thrice. This is an important finding, as many commercial layer flocks in Indonesia are vaccinated multiple times with inactivated H5N1 vaccines derived by propagation of local H5N1 strains in embryonating chicken eggs. Hence, there is a potential for the M2e protein to be present in such vaccines, originating either from the virion itself, or from necrotic cells of chicken embryos in which the vaccine virus was propagated (Lamb *et al.*, 1985).

The vaccinated layers in our experiments, regardless of whether they were vaccinated once, twice or thrice, were susceptible to challenge with the heterologous A/chicken/West Java/Sbg-29/2007 H5N1 virus, as determined by virus isolation, an increase in HI titres and M2e sero-conversion. Although most of the vaccinated birds had HI titres at the time of challenge ≥5 log_2, and were thus protected against mortalities, there was no association between these HI titres and re-isolation of challenge virus. This result is in agreement with several studies where high HI titres protected chickens against mortality but not against infection with HPAI H5N1 (Swayne, 2006; Abdelwhab *et al.*, 2011; Spackman & Swayne, 2013).

Oro-pharyngeal and in-contact routes of infection with H5N1 were used to ensure efficient challenge of each bird (Experiment 1) and to simulate field challenge (Experiment 2). The importance of the oro-faecal transmission in spread of AIV is well recognized (Sergeev *et al.*, 2013). However, in the dynamics of HPAI infection, other factors such as the host, virus strain, immunity and environment, which all contribute to the outcomes of H5N1 infections, are not well understood (Spickler *et al.*, 2008; Kwon & Swayne, 2010; Hénaux & Samuel, 2011; Pantin-Jackwood *et al.*, 2013; Wibawa *et al.*, 2014). While the route of infection may influence the importance of the M2e antibody response, in this study it did not appear to have major influence on the infection rates in vaccinated and challenged birds. In the vaccinated birds infected by the

Table 3. *Trade-off of sensitivity and specificity of sp-M2e- and MAP-M2e-based ELISA.*

sp-M2e ELISA			MAP-M2e ELISA		
Cut-off OD	Sensitivity (%)	Specificity (%)	Cut-off OD	Sensitivity (%)	Specificity (%)
0.01	75	77	0.01	85	83
0.02	70	86	0.03[a]	75	100
0.04	65	96	0.04	70	100
0.05	60	98	0.06	66	100
0.10[a]	53	100	0.10	54	100

[a]The lowest standardized OD having 100% specificity were chosen to be the ELISA cut-off values.

in-contact route, 33% and 100% became infected by 3 and 7 dpi, respectively, and HI antibody titres developed and peaked at 14 dpi. Comparable results were observed in the birds challenged by direct inoculation.

Overall, the M2e antibody response that was detected in vaccinated birds challenged with H5N1 A/chicken/West Java/Sbg-29/2007 strain agreed with other indicators of infection, i.e. virus isolation and increase in HI titres. The number of birds that developed M2e antibody, as well as the time course and strength of M2e antibody response, were all strongly influenced by two factors: the length of the interval between vaccination and challenge and the sensitivity of the test used for the M2e antibody detection.

In the birds challenged at six weeks after the last vaccination, all developed M2e antibodies that agreed 100% with virus isolation and an increase in HI antibody titre. In these birds, M2e antibodies were detected as early as 7 dpi, peaked between 14 and 28 dpi, and lasted until at least 56 dpi. Most of the birds had the highest OD values at 14 dpi, which did not change significantly at 21 dpi, indicative of a strong M2e response to an infection. All M2e positive birds were detected by both the M2e ELISA tests used. In birds infected at two weeks after the last vaccination, with mean HI titres of 6.1 $\log_2$, the number of M2e antibody positive birds was lower. In these birds the test used had a major influence on the number of M2e positive birds detected. The less sensitive sp-M2e ELISA detected 29%, 71% and 50% of birds in comparison to 71%, 100% and 83%, detected with MAP-M2e ELISA in groups vaccinated once, twice and thrice, respectively. The results obtained by the MAP-M2e ELISA also agreed better with the number of birds that were infected and had increased HI titres following challenge. Importantly, the time course and duration of M2e antibody were noticeably shorter in these birds; they were detected at 14 dpi, peaked at between 16 and 21 dpi and declined to negligible levels at 28 dpi. The strength of M2e antibody response, as indicated by OD values, was higher in Experiment 2 than in Experiment 1, indicating that the timing of challenge was important for the development of M2e antibodies in H5N1-vaccinated flocks. Vaccinal immunity has a major role in limiting the replication of the challenge virus and exposure of the immune system to viral antigens, including the M2e antigen and thus may lead to a weak (as was the case in the Experiment 1) or strong (as was the case in Experiment 2) M2e antibody response. Overall, the level of M2e antibody response tended to be lower in birds vaccinated thrice, in comparison to those vaccinated once or twice. This was most pronounced in Experiment 1 in birds challenged two weeks after the last vaccination. This finding has an important implication for field application of DIVA strategies, as it suggests that a flock vaccinated multiple times and considered fully immune may in fact be susceptible to field H5N1 challenge as early as two weeks after vaccination. In such a case the duration of M2e antibodies is short and limited to a small number of M2e positive birds, resulting in a small window of opportunity for an effective detection of field challenge.

The relatively short duration of the M2e antibodies, at best lasting –seven to eight weeks and detected in birds challenged with H5N1 at six weeks post-vaccination, is in agreement with the time course of M2e antibodies development in H5N1 convalescent human sera. In these cases, M2e antibodies appeared at 14 dpi and were no longer detected at 60 dpi (Khurana *et al*., 2011). The short duration and variable intensity of M2e antibody response could be either due to intrinsically poor antigenicity of the M2e protein or the M2e presentation to the immune system (Kreijtz *et al*., 2011). Such short duration of M2e antibody in an infected host could indicate that there is only a short window of opportunity of about two to seven weeks in which the M2e response can be detected in a vaccinated flock exposed to field H5N1 challenge. This may be a weakness of an M2e-based DIVA test and has implications for a surveillance strategy based on it, as it would require frequent flock testing to detect field exposure. Evaluation under field condition would therefore be important for validation of M2e DIVA test.

The two ELISAs used, sp-M2e and MAP-M2e, were both highly specific and had practically 0% of false positives among the vaccinated sera used. The absence of false positives could be attributed to the choice of buffers used for serum dilution and/or washing, in that they contained high concentration of salts and detergents. This low rate of false positive is an advantage when using sera from commercial birds, which are known to be a cause of a high degree of false-positive results in some M2e ELISAs. Previous studies have reported a significant number of false positives (>5%) in vaccinated chickens, which lowered the test specificity (Lambrecht *et al*., 2007; Hemmatzadeh *et al*., 2013). The use of M2e in the form of recombinant M2e tagged with maltose binding protein as the antigen produced a high "background noise" in the ELISA and a high false-positive rate as some chicken sera recognized the tagged protein (Hemmatzadeh *et al*., 2013). In another study, a lower number of false positives (<4%) was obtained by using synthetic peptide as the ELISA's coating antigen, and treating serum samples by heating at 56°C for 30 min followed by treatment with 12.5% kaolin (Kim *et al*., 2010).

Although the diagnostic specificity of both the MAP-M2e and the sp-M2e ELISAs was high, their diagnostic sensitivity was rather low. This was due to the fact that serum samples collected at 3 and 7 dpi were also categorized as infected although the M2e antibody had not

yet developed. The number of such sera collected prior to the development of M2e antibodies was large, and this was the major contributor to the low diagnostic sensitivity of M2e ELISAs. Using M2e in the form of a four-symmetry branched M2e as the coating antigen in MAP-M2e ELISA significantly increased the test sensitivity in comparison to using the single M2e peptide, as was the case in the sp-M2e ELISA. Most probably the four-symmetry branched form of M2e is closer to the native M2e, which is in the tetramer form (Sugrue & Hay, 1991). Indeed, the tetrameric form of the M2e was shown to increase sensitivity of M2e antibody detection when used as a recombinant antigen in an ELISA (Hadifar *et al*., 2014) and to increase the M2e immunogenicity and antibody responses when used in vaccine formulations (De Filette *et al*., 2008).

Despite the fact the sp-M2e and MAP-M2e ELISAs have a rather low diagnostic sensitivity, both tests – particularly the MAP-M2e ELISA – should be useful for surveillance of HPAI H5N1 infections in vaccinated flocks. Since the surveillance of HPAI H5N1 in poultry is usually carried out at the flock, rather than at the individual level, the sensitivity of the MAP-M2e ELISA at the flock level can be raised by increasing the number of birds sampled.

In summary, the presence of M2e antibodies in vaccinated commercial poultry is a reliable indicator of HPAI H5N1 infection as M2e antibodies are absent in vaccinated birds, even after repeated immunization with inactivated whole virus vaccines. The M2e antibodies can be detected reliably with the MAP-M2e peptide ELISA, which is significantly more sensitive and accurate than the previously reported single M2e peptide ELISA.

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