



Murdoch
UNIVERSITY

MURDOCH RESEARCH REPOSITORY

This is the author's final version of the work, as accepted for publication following peer review but without the publisher's layout or pagination.

The definitive version is available at

<http://dx.doi.org/10.1016/j.vaccine.2008.08.008>

James-Berry, C.M., Foong, Y.Y., Mansfield, J.P., Vind, A.R., Fenwick, S.G. and Ellis, T.M. (2008) Evaluation of a positive marker of avian influenza vaccination in ducks for use in H5N1 surveillance. *Vaccine*, 26 (42). pp. 5345-5351.

<http://researchrepository.murdoch.edu.au/8334/>

Copyright: © 2008 Elsevier Ltd.

It is posted here for your personal use. No further distribution is permitted.

Evaluation of a positive marker of avian influenza vaccination in ducks for use in H5N1 surveillance

Cassandra M. James, Yvonne Y. Foong, Josephine P. Mansfield, Azita Rezazadeh
Vind, Stanley G. Fenwick, Trevor M. Ellis

School of Veterinary and Biomedical Sciences, Faculty of Health Sciences, Murdoch
University, South Street, Perth 6150, Western Australia, Australia

Abstract

Control measures for H5N1 avian influenza involve increased biosecurity, monitoring, surveillance and vaccination. Subclinical infection in farmed ducks is important for virus persistence. In major duck rearing countries, homologous H5N1 vaccines are being used in ducks, so sero-surveillance using H5- or N1-specific antibody testing cannot identify infected flocks. An alternative is to include a positive marker for vaccination. Testing for an antibody response to the marker would confirm approved vaccine use. Concurrent testing for H5 antibody responses would determine levels adequate for protection or indicate recent infection, with an anamnestic H5 antibody response requiring further virological investigation. In this study, we have evaluated the use of a TT marker in ducks given avian influenza vaccination. Wild or domestic ducks were tested for antibodies against TT and all 463 ducks were negative. High levels of TT-specific antibodies, produced in twice-TT vaccinated Muscovy ducks, persisted out to 19 weeks. There was no interference by inclusion of TT in an inactivated H6N2 vaccine for H6- or TT-seroconversion. Thus TT is a highly suitable exogenous marker for avian influenza vaccination in ducks and allows sero-surveillance in countries using H5N1 vaccination.

Abbreviations

AI, avian influenza; DIVA, differentiating infected from vaccinated animals; FAO, Food and Agricultural Organisation; HA/H, haemagglutinin; HI, haemagglutination inhibition; HPAI, highly pathogenic avian influenza; LPAI, lowly pathogenic avian influenza; NA, neuraminidase; NP, nuclear protein; NS1, non-structural; OIE, World Organisation for Animal Health; PBST, phosphate-buffered saline/Tween 20; s.c., subcutaneous; S.E.M., standard error of the mean; TT, tetanus toxoid; WHO, World Health Organisation

Keywords

Avian influenza vaccines; Ducks; Virus surveillance

Introduction

Avian influenza (AI) is an enormous global threat, both to the poultry industry and human public health, with an economic loss of over US\$ 10 billion estimated for H5N1 outbreaks [1]. Wild waterfowl (Anseriformes) and shorebirds (Charadriiformes) worldwide are natural reservoirs of influenza A viruses. Domestic ducks are generally susceptible to AI virus infection, but until the emergence of the H5N1 viruses, in Asia in 2002, ducks generally showed no clinical disease. These recent H5N1 viruses can cause severe disease in ducks with high mortality and both sick and clinically normal ducks infected with these viruses shed high virus loads from the cloaca and the oropharynx [2]. In many of the countries that have experienced the recent H5N1 epizootic, domestic ducks mix closely with terrestrial poultry especially in small village farms, households and live poultry markets. Recurrent outbreaks in these countries have been linked to unapparent infections in domestic ducks.

In countries like Vietnam, China and Indonesia, with large duck populations and persistent H5N1 disease, AI vaccines are increasingly being used as a tool in control programs for highly pathogenic avian influenza (HPAI) viruses in domestic ducks as well as other commercial and backyard poultry [3]. On a flock basis, vaccination can help break the infectious cycle, in combination with vigilant monitoring and strong biosecurity measures [4]. However, domestic ducks can be infected sub-clinically with other circulating AI virus strains. Some of the AI viruses of low or high pathogenicity that are currently circulating in areas that have experienced H5N1 outbreaks include H5N1, H5N2, H6N1, H7N1, H7N2, H7N3 and H9N2 [5]. Vaccination complicates AI sero-surveillance using commonly available diagnostic tests, as for example in China, Vietnam and Indonesia, where duck flocks are being vaccinated with killed H5N1 vaccines, vaccinated and naturally infected birds will both produce H5-specific antibody.

Vaccination to control AI should be part of a science-based control strategy that includes suitable monitoring of all flocks at risk including vaccinated flocks [6]. As part of this approach, various strategies have been developed for differentiating infected from vaccinated animals (DIVA) where AI vaccination is used to enhance virus surveillance, prevent unnecessary culling of birds, and regulate poultry vaccination, movement and trade [7].

Although several DIVA strategies based on the specificity of antibody responses have been devised, they have serious limitations, especially for testing duck species in countries like China, Vietnam and Indonesia [8] and [9]. Ducks in these countries are currently being vaccinated with reverse genetics-derived killed H5N1 vaccines that are relatively cheap and have shown good potency against field viruses. However, this does not allow the use of the

heterologous neuraminidase (NA) type of DIVA strategy. Circulation of other AI viruses in these countries will generate nuclear protein (NP)-specific and non structural (NS)1-specific antibody responses that complicate use of NP- and NS1-specific tests to monitor H5N1 infection. Also any HxN1 viruses (e.g. H6N1, H7N1) circulating will complicate use of N1 antibody testing if a heterologous NA vaccination DIVA strategy is in place. At present the NS1-specific tests have not been fully validated for use in an AI monitoring system and the heterologous NA DIVA approach has only been validated for low pathogenicity AI virus surveillance in chickens and turkeys [7].

Considering the nature of the domestic duck industry in countries like China, Vietnam and Indonesia, and the difficulty with being able to identify vaccinated ducks, we have considered an alternative strategy that could be used for sero-surveillance in ducks when vaccination is officially used as part of a H5N1 control program. This involves inclusion of an exogenous antigen in the vaccine that can be used as a positive marker for vaccination. Testing for an antibody response to the marker would confirm that the approved vaccine has been used and a concurrent test for H5 antibody would determine if adequate H5 antibody responses were present in the flock to give an indication of vaccine efficacy and the effectiveness of vaccine delivery. If high H5 antibody responses were present relative to the normal vaccine response curve this could indicate recent infection and trigger further virology investigation, enabling a novel DIVA strategy. The positive identification of vaccinated birds would allow the authorities to regulate vaccination especially in small commercial flocks, village and backyard poultry, where record keeping and biosecurity is poor.

We have previously evaluated tetanus toxoid (TT) as an exogenous marker for AI vaccines in chickens and showed that there is no interference with TT or AI H-specific antibody responses in TT- and AI-co-vaccinated chickens [10]. The TT marker was selected on the basis that chickens are highly resistant to tetanus (chicken toxic dose is 350,000 times the equine toxic dose per gram body weight) [11], are not routinely vaccinated with TT, and naturally existing antibodies to TT are absent in chickens from a variety of sources. Furthermore, the antigen is of relatively low cost to manufacture, has minimal regulatory and market acceptance issues and development of an accurate and relatively inexpensive antibody test to the antigen is possible [10]. In this study, we evaluate the levels of naturally acquired antibodies to TT in wild and domestic ducks from Australia, the immunogenicity of TT in Muscovy ducks, and interference by TT on H6- or TT-seroconversion in ducks given separate TT and H6N2 AI vaccines or combined TT/H6N2 vaccines. Our data supports the suitability of the TT marker for AI sero-surveillance in ducks.

Materials and methods

Birds

Muscovy ducklings (6–8 weeks old) were obtained from small breeder farms in the outer metropolitan area of Perth, Western Australia. For one vaccination study, ducks were kept in inside pens with straw strewn on a concrete floor at the Department of Agriculture Research Station at Medina, Western Australia, and in the other separate study, ducks were housed in outdoor pens with soil floors at the Animal House, Murdoch University. Animal experimentation was given prior approval by Animal Experimentation Ethics Committees of the Department of Agriculture of Western Australia and Murdoch University, which comply with guidelines from the National Health and Medical Research Council, Australia.

Serum was obtained from wild Plumed Whistling ducks trapped at Kununurra, north Western Australia, and waders (Pacific Black and Maned ducks) from south Western Australia (provided by Dr. Cheryl Johansen, The University of Western Australia). In addition, serum was obtained from TT-unvaccinated female Hy-Line Brown layer pullets (6–7 weeks old) sourced from Altona Hatchery Pty. Ltd. (Perth, Australia) and used as negative controls in the competitive ELISA for the screening studies.

Virus

Low pathogenicity avian influenza (LPAI) A/Coot/Perth/2727/79 H6N2 virus, isolated from a Eurasian coot (*Fulica atra*) in Perth, Western Australia (kindly provided by Professor John Mackenzie, Curtin University), was propagated according to OIE protocols [12]. Briefly, H6N2 was cultured using 9- to 11-day-old embryonated, specific pathogen-free (SPF) fowl eggs [10]. Harvested allantoic fluid containing H6N2 virus was inactivated with formalin 0.1% (v/v) for 65 h at 37 °C, and virus inactivation confirmed by embryonated fowl egg inoculation. The HA titre of the inactivated H6N2 AI virus stock (2^7 HA units) was determined using the OIE standard protocol [12] as previously described [10].

Vaccination

Ducks were either vaccinated once or twice (at weeks 0 and 4) by the subcutaneous (s.c.) route with 1 mL of vaccine in the dorsal midline at the lower end of the nape of the neck near the anterior dorsal thorax. Vaccines consisted of pre-vaccine formulation TT (total protein estimation 30 mg/mL, Pfizer, Melbourne, Australia) mixed 1:1 (v/v) with Montanide™ ISA-70 VG (Seppic, France) as previously described [10]. Inactivated whole virus H6N2 vaccine

(2^7 HA units in allantoic fluid) with Montanide™ ISA-70 VG adjuvant was also administered as a water-in-oil emulsion to control groups of ducks. In separate studies, ducks were either vaccinated with doses of TT and inactivated H6N2 as two separate injections delivered at the same site or co-vaccinated with doses of TT mixed with inactivated H6N2, delivered as a single injection (water-in-oil emulsion).

Blood collection

Blood samples were collected by venipuncture from wing or leg veins at week 0 (pre-bleed) and at specified weeks post-vaccination. Blood was collected into glass or serum clot activator-treated plastic vacutainers (Starsedt, Germany) and serum separated after clot retraction was stored at 4 °C prior to use in assays and transferred to –80 °C for long-term storage.

Determination of TT antibody levels by competitive ELISA

Levels of TT-specific antibodies in duck sera were determined by competitive ELISA. Immunosorbent ELISA plates (Greiner BioOne, Germany) were coated overnight at 4 °C in a humidified chamber with formalin-inactivated, purified TT antigen (0.012 µg/100 µL, List Biological Laboratories Inc., CA, USA) in 0.05 M carbonate–bicarbonate buffer, pH 9.6. Plates were washed 6 times with phosphate buffered saline pH 7.6/0.05% (v/v) Tween 20 (PBST) using a 12-well Immuno™ Wash instrument (Nunc International, Australia). Primary duck antibody was diluted 1/10 in PBST/4% (w/v) dried skim milk powder and 100 µL per well was added in duplicate to the plates which were then incubated at 37 °C for 1 h. Plates were washed 6 times with PBST before addition of goat anti-TT IgG (1/3200 dilution in

PBST/4% (w/v) dried skim milk powder, 100 µL/well) (Accurate Chemical & Scientific Corp., NY, USA) for one hour at 37 °C. After further six washes with PBST, chicken anti-goat F(ab')₂ conjugated to HRP (1/20,000 dilution in PBST/4% (w/v) dried skim milk powder, 100 µL/well) (Chemicon, CA, USA) was added to each well and incubated for one hour at 37 °C. Plates were again washed six times in PBST before addition of 100 µL TMB One solution (Promega Corp., WI, USA) for 5 min. The reaction was terminated with 50 µL/well of 2 M sulphuric acid and after 15 min, plates were read at 450 nm with 630 nm reference wavelength using a spectrophotometer (Bio-Rad Model 680, CA, USA). The level of positive TT-specific antibodies was calculated using the formula:

$$\% \text{ Inhibition} = 100 - [100 \times (\text{Mean OD test serum} / \text{Mean OD negative control serum})]$$

The negative controls used for the seroprevalence studies were duplicates of pre-bleeds from five normal control Hy-Line Brown chickens. Pre-bleeds from domestic Muscovy ducks were used as species-matched negative controls for all subsequent vaccination studies of Muscovy ducks. Data was expressed as % inhibition level for individual serum samples in the seroprevalence study or mean % inhibition + standard error of the mean (S.E.M.) for the vaccination studies. Serum samples were considered positive for TT-specific antibodies if the value for inhibition was above 30%. This cut-off was chosen based on clear distinctions between levels of antibody observed in TT-unvaccinated birds compared to TT-vaccinated birds.

Determination of TT-specific and influenza virus-specific IgY antibody titres by indirect

ELISA

Duck IgY subclass responses to TT and whole influenza virus were determined by indirect ELISA using purified TT (1/2400, List Biological Laboratories, Inc., CA, USA) or formalin inactivated whole H6N2 virus (1/100 allantoic fluid containing virus) as coating antigen in 0.05 M carbonate–bicarbonate buffer, pH 9.6 (4 °C overnight), respectively. Each antibody step involved incubation at 37 °C for 30 min and plates were washed six times with PBST prior to antibody incubations. Duck sera were serially 2-fold diluted in PBST/2% (w/v) dried skim milk powder (starting at 1/200 dilution). The secondary antibody, mouse anti-duck IgY heavy chain (1/250, SeroTec, UK) and the tertiary antibody, goat anti-mouse IgG conjugated to horseradish peroxidase (1/20,000, Santa Cruz Biotechnology) were similarly diluted in PBST/2% (w/v) dried skim milk powder. Controls included quadruplicates of the negative control (pooled pre-bleeds diluted 1/400 from 12 unvaccinated Muscovy ducks). Endpoint titres were determined as the last dilution of serum with an OD 450 nm greater than the mean OD 450 nm of control sera + 3 S.D.

Haemagglutination Inhibition (HI) assay

H6-specific antibody titres were determined by HI assay using procedures described in the WHO Manual on Animal Influenza Diagnosis and Surveillance [13]. Pre-incubation of the duck serum with receptor destroying enzyme (Denka Seiken Co. Ltd., Japan) was conducted to remove non-specific inhibitors of haemagglutination. The antigen used was the homologous H6N2 virus (A/Coot/Perth/2727/79) at a dilution determined by haemagglutination (HA) test to contain 4 HA units. The HI titre was determined as the highest dilution of serum giving complete inhibition of haemagglutination and the data are expressed as mean antibody titres (\log_2) + S.E.M.

Statistical analysis

The two-tailed unpaired Student's *t* test assuming unequal variance between means was used to assess statistical significance between groups (**P* < 0.05, ***P* < 0.01).

Results

Natural seroprevalence to tetanus toxoid (TT) in wild and domestic ducks

The presence of naturally acquired antibodies to TT was determined by competitive ELISA using sera obtained from wild and domestic ducks from various geographical locations (Fig. 1). Wild Plumed Whistling ducks from Kununurra in the Northwest region of Western Australia (Fig. 1A, *n* = 236) and waders (Pacific Black and Maned ducks) from the Southwest region of Western Australia (Fig. 1B, *n* = 59) showed negligible levels of serum inhibition. Indeed, only 3/295 samples from wild ducks showed greater than 20% inhibition, screened at a 1/10 serum dilution. Furthermore, all serum samples from domestic Muscovy ducks locally farmed in Western Australia (Fig. 1C, *n* = 98), including those subsequently used for vaccination studies (*n* = 70), were negative for antibodies to TT. In summary, all 463 ducks were found to be seronegative for naturally acquired antibodies against TT.

Antibody response to TT in Muscovy ducks vaccinated with TT

Initially, groups of domestic Muscovy ducks were vaccinated with a range of TT doses (equivalent to 0.1, 0.3, 1.0, 3.0, and 10 mg total protein) emulsified in alum [10] to examine the immunogenicity of TT in ducks. The TT vaccine was administered by the s.c. route at weeks 0 and 4 (*n* = 8/group) and serum antibody levels to TT determined by competitive

ELISA. Prior to vaccination, all Muscovy ducks ($n = 40$) were negative for antibodies against TT. Muscovy ducks did not mount a strong antibody response until 2 weeks following the second vaccination (data not shown). No significant differences were observed between the TT vaccine dose groups, although birds vaccinated with 0.3 mg TT elicited the highest antibody levels at week 6 post-vaccination.

Antibody responses in ducks co-vaccinated with TT and inactivated H6N2 AI vaccines

We selected the TT vaccine doses of 0.1, 0.3 and 1.0 mg for assessment of antibody responses in Muscovy ducks co-vaccinated with TT and whole inactivated H6N2 AI vaccines ($n = 10$ /group). Since whole inactivated AI vaccines are conventionally administered as water-in-oil emulsions, both antigens were mixed with the mineral oil, Montanide ISA-70 VG, and delivered by the s.c. route, as two separate injections given both at weeks 0 and 4. Antibody levels to TT and H6 antigens were determined by competitive ELISA, and HI assay, respectively. Prior to vaccination, all ducks ($n = 30$) were negative for TT- and H6-specific antibodies (Fig. 2). The levels of TT-specific antibodies produced in co-vaccinated ducks were not strongly positive until after the second vaccination (Fig. 2A). There was a significantly higher TT-specific antibody response observed in ducks given 0.1 mg TT/AI compared to 0.3 mg TT/AI vaccine at week 6 post-vaccination ($P < 0.05$). However, there were no significant differences in antibody levels to TT at other timepoints, regardless of the TT vaccine dose.

H6 antibody titres, determined by HI assay, were found to be low in TT and AI co-vaccinated ducks until 2 weeks following second vaccination (Fig. 2B). However, at week 6 post-

vaccination, no significant differences were observed between the H6 antibody titres from birds in any of the groups regardless of the TT vaccine dose.

Antibody responses in ducks vaccinated with a combined TT and inactivated H6N2 AI vaccine

Next, a divalent vaccine composed of both TT and whole inactivated H6N2 antigens emulsified with mineral oil (Montanide ISA-70 VG) was prepared, using three separate doses of TT (0.15, 0.3 and 0.6 mg), as described previously [10]. The vaccine was delivered subcutaneously as a single injection to Muscovy ducks ($n = 24$, 8 ducks per group). Control groups were vaccinated with either 0.3 mg TT vaccine or inactivated H6N2 vaccine alone ($n = 8$ /group). Some ducks in each of the TT only, AI only and 0.3 mg TT/AI-vaccinated groups ($n = 4$ /group) and all birds in the 0.15 mg and 0.6 mg TT/AI vaccine groups ($n = 8$ /group) were given a second vaccination 28 days following priming to assess the efficacy of single versus double vaccination. Prior to vaccination, all ducks ($n = 40$) were seronegative for antibodies against TT by ELISA (Fig. 3A and B) and H6 by HI test (Fig. 3C and D). Note that the AI vaccine only group remained negative for antibodies against TT throughout the 19-week study (Fig. 3A and B). Likewise, the TT vaccine only group remained H6 seronegative for the duration of the experiment (Fig. 3C and D).

Following a single vaccination, the level of TT antibodies found in ducks given the combined TT/AI vaccine compared with the TT vaccine alone (both 0.3 mg TT) showed no significant differences with peak levels observed at 8 weeks post-vaccination (Fig. 3A). At 19 weeks post-single vaccination ducks showed declining TT antibody levels. However, after a second

vaccination, birds in the TT/AI vaccine dose groups showed peak levels of TT-specific antibodies 4 weeks later (at week 8 post-priming), which then slightly declined by week 19 post-vaccination (Fig. 3B). With the exception of birds in the 0.15 mg TT/AI group at week 2, which produced significantly higher TT-specific antibody levels than the TT control group ($P < 0.01$), no significant differences in levels of TT-specific antibodies were found between the TT/AI vaccine groups and the TT only group at any other time point, regardless of TT dose. Notably, the 0.3 mg TT/AI group produced a high level of TT antibodies throughout the experiment, which was significantly higher than the 0.15 mg TT/AI and the 0.6 mg TT/AI vaccine groups at week 8 ($P < 0.05$). Moreover, twice-vaccinated ducks produced significantly higher TT antibody levels than ducks given a single vaccination at week 6 for the TT only group ($P < 0.01$) and at weeks 6 and 8 post-vaccination for the 0.3 mg TT/AI group ($P < 0.01$).

Low titres of H6-specific antibodies, as determined by HI assay, were produced at week 2 post-single vaccination in ducks with no significant differences found between the AI only and the 0.3 mg TT/AI vaccine group (Fig. 3C). At week 4, a low titre of H6-specific antibodies was observed in the 0.3 mg TT/AI group only. H6-specific antibodies were not detected beyond week 4 in AI-vaccinated birds given a single dose of vaccine. In contrast, markedly higher H6 antibody titres were observed in ducks given a double vaccination with AI and the different doses of TT/AI vaccines compared to ducks given a single vaccination (Fig. 3D, $P < 0.05$). However, H6-specific antibody titres significantly decreased by week 19 post-vaccination. At weeks 6 and 8 post-vaccination, no significant differences between H6 HI antibody titres were detected for any of the TT/AI vaccine dose groups compared with the H6N2 vaccine control group.

Duck IgY subclass responses to TT and H6N2 in birds immunised with combined TT and inactivated AI vaccines

We also analysed the abovementioned double-vaccinated ducks from the 0.3 mg TT only, AI only, and 0.3 mg TT/AI-vaccinated groups for IgY subclass titres against TT or inactivated whole influenza virus (H6N2) by indirect ELISA. Following double vaccination, TT-specific IgY titres increased over the 19 weeks post-vaccination (Table 1). Apart from week 8, where the TT control group produced significantly higher titres of TT-specific IgY compared to the TT/AI group ($P < 0.05$), no other significant differences were found between these vaccine groups at any other time point.

Whole H6N2 virus-specific IgY titres increased over the weeks post-vaccination with peak titres observed at week 6 for both the AI vaccine and the TT/AI vaccine group, similar to the data from the HI assay. The TT/AI group produced significantly higher viral IgY titres than the AI control group at week 2 ($P < 0.01$), however at week 8, significantly lower IgY titres ($P < 0.05$) were found in the TT/AI vaccine group compared to the AI control group. No significant differences in IgY titres were found at weeks 4, 6 and 19 between the two vaccine groups. Ducks in the AI vaccine group produced significantly higher viral IgY titres at weeks 6 and 8 when given a double vaccination compared to a single vaccination ($P < 0.01$, data not shown). Furthermore, birds in the 0.3 mg TT/AI vaccine groups produced significantly higher viral IgY titres at weeks 6, 8 and 19 when given a double vaccination compared to a single vaccination ($P < 0.05$, data not shown).

Discussion

Globally, the H5N1 epizootic still continues and the recent increase of outbreaks in existing and newly infected countries is of great concern for public and animal health and has some countries on constant alert [14]. The Food and Agricultural Organisation (FAO), World Health Organisation (WHO) and World Organisation for Animal Health (OIE) have come together to make recommendations on the control of AI and have stated that in many regions of the world, the control of AI is being jeopardised by inadequate control programs. Control of H5N1 HPAI by stamping out involves detection of infection and systematic culling of infected animals within a certain geographical radius [15]. However, culling of poultry is very costly, resulting in economic losses in the order of millions of dollars and loss of animal protein supply in the diet of poorer farmers and villagers in developing countries. In developing countries, where there is inadequate or no compensation, many farmers will simply not advise authorities of outbreaks within their domestic flocks in order to prevent mass culling of uninfected birds. AI control is being managed increasingly in the poultry industry by use of H5 AI vaccination under government-endorsed programmes [16] and [17]. Vaccination as a tool for the control of AI in poultry assists in slowing virus spread, as vaccinated birds shed lower levels of virus for a shorter period than unvaccinated birds, and limited gene reassortment occurs between species including ducks, geese, chickens and quails in live markets [18]. However, vaccination complicates surveillance of outbreaks of HPAI H5N1, especially in ducks, by serological means as both vaccinated and virus-infected birds produce specific antibody to H5. To improve monitoring of H5N1 control and effective vaccination, vaccination programs should incorporate post-vaccination surveillance [19] and [20].

Control of H5N1 AI infection in domestic ducks is considered to be an important component of the overall control of this epizootic with the duck being considered the “Trojan horse” facilitating silent spread of the H5N1 virus in the environment and live bird markets [21]. Although post-2002 H5N1 viruses can cause severe disease in ducks with high mortality, some infected ducks remain clinically normal and both sick and clinically normal ducks infected with these viruses shed high virus loads from the cloaca and the oropharynx. H5N1-infected ducks may shed virus for 10–20 days [2] and [21]. Massive populations of ducks exist in countries with persistent H5N1 infection, such as China, Vietnam and Indonesia, where duck farming is closely integrated with rice production and is vital for provision of both food and income. H5N1 influenza virus was shown to be prevalent among ducks raised in “open” houses, free-ranging (grazing) ducks, and backyard ducks in Thailand [22]. A critical factor for HPAI persistence has been shown to be free-grazing ducks in wetlands used for double-crop rice production, which feed year round in rice paddies [23]. Without clinical signs, it is unlikely that the farmer will recognise influenza virus infection in affected ducks and thus constant bird-water, bird-bird and bird-human interactions promote transmission of virus over large geographical areas, greatly increasing human and domestic poultry exposure [24]. Influenza virions shed by ducks into water have been known to stay viable for extended periods of time, allowing greater numbers of birds to come into contact with virus-contaminated waters. Depending on humidity, salinity and pH levels, influenza virions in water have been shown to survive for 32 days at 4 °C [25]. Another issue of concern is that all 16 HA and 9 NA AI subtypes circulate in ducks which are usually sub-clinically infected and the duck can be a host that facilitates reassortment of viral gene segments resulting in the evolution of new H5 variants of unknown consequences.

In countries like China, Vietnam and Indonesia, vaccination of ducks and other poultry in small commercial farms, villages and households is practised with inactivated H5N1 vaccines. Flock health and vaccination record keeping is poor and there is minimal biosecurity. A cost-effective surveillance system is needed and should be aimed at determining that vaccinated duck flocks have reached target levels of post-vaccinal antibody response and that H5N1 viruses are not continuing to circulate within flocks. An alternative surveillance strategy involving use of an exogenous positive marker of vaccination would provide a powerful investigative tool for post-vaccination surveillance by veterinary authorities. Our approach involves inclusion of TT as an exogenous antigen in the vaccine that can be tested for as a positive marker to confirm that ducks have been vaccinated with an approved AI vaccine, independent of whether the flock has been infected with H5N1 virus. Serological screening of ducks for the TT marker by veterinary authorities will provide more accurate data on the H5 vaccine coverage and by concurrent H5 antibody testing, the effectiveness of the H5 vaccination program on a flock or village basis can be determined. The ELISA to measure TT antibody and the H5 HI test are inexpensive, high-throughput routine serological tests that can be performed simultaneously in veterinary laboratories. If high H5 antibody responses were present relative to the normal vaccine response curve this could indicate recent infection and trigger further virology investigation of the flock.

In this study a total of 463 wild and domestic ducks that had not been vaccinated were tested for TT-specific antibodies and none were seropositive. We have also screened 1779 chickens in Australia, Hong Kong and China, and all were seronegative for TT antibodies without prior TT vaccination [10]. These birds were from various geographical locations and habitats, including farmed and backyard chickens. This finding was critical to the subsequent evaluation of the potential of TT antigen as an immunological marker of vaccination. In the

present study, we found that TT vaccination in Muscovy ducks elicits TT-specific antibodies upon two vaccinations at weeks 0 and 4, with Montanide ISA-70 VG (water-in-oil emulsion). Although, TT-vaccinated Muscovy ducks were slow to mount a peak antibody response to TT, TT-specific antibody titres peaked at 8 weeks post-vaccination and were sustained out to week 19. Moreover, ducks twice vaccinated, with the divalent vaccines composed of both TT and inactivated H6N2, showed no significant differences in TT antibody levels between any of the TT/AI dose groups and TT only vaccine group. In addition, there was no interference by TT in these twice-vaccinated ducks on seroconversion to H6. Taking the results together, optimal vaccination was achieved in Muscovy ducks that received two vaccinations of the 0.3 mgTT/AI dose vaccine. We conclude that TT is a suitable exogenous vaccine marker for use with AI vaccines in ducks allowing for post-vaccination sero-surveillance.

Note that the TT antibody response in ducks, vaccinated with the in-house TT/H6N2 vaccine, persisted at a sustained level out to 19 weeks (the duration of the study) using both competitive ELISA and indirect ELISA but that the H6 HI response declined at 8 weeks and was negligible at 19 weeks post-vaccination. Field use of TT marker with killed H5 vaccines would establish normal antibody response curves for TT and H5 antibody in uninfected flocks and determine a profile of the ratio of TT antibody to H5 HI titre post-vaccination. Superimposed H5N1 infection in a flock would generate anamnestic H5 antibody responses, which would alter the TT/H5 ratio and provide information on whether a natural boost to immunity due to exposure to an H5 virus had occurred in flocks vaccinated with the marker vaccine. Further virology investigation of this flock would then be warranted.

Investigation of mortalities in H5-vaccinated duck flocks could also incorporate testing for evidence of antibody response to the TT marker. If dead ducks in vaccinated flocks were confirmed to have H5N1 HPAI, this would indicate that either the vaccination coverage in the flock was incomplete, or the vaccine was not protecting against the circulating H5N1 virus. Testing of sick or recently dead ducks and their cohorts for TT antibody has the potential to rapidly determine if the vaccine is not protective, or that the vaccination program in the flock is sub-optimal.

In conclusion, there is a paramount need for improved vaccine control programs for AI in poultry, especially in ducks, which may not show clinical signs of infection. Cost-effective monitoring procedures for vaccinated duck flocks are needed especially in countries with large duck populations and recurrent H5N1 outbreaks. Our research has highlighted the suitability of TT as an exogenous marker for AI vaccination in Muscovy ducks and indicated the optimal dose of the TT marker antigen required to give sustained TT antibody responses in ducks vaccinated with a TT/H6N2 vaccine. Such a positive marker of vaccination can be utilised in a surveillance strategy permitting veterinary authorities to provide more accurate data on the H5 vaccine coverage and the effectiveness of the H5 vaccination program on a flock or village basis in countries using H5N1 vaccination of ducks as part of their H5N1 control program.

Acknowledgements

We greatly appreciate the gifts of the Montanide adjuvant from Sandy Tall (Tall Bennett Group, NSW, Australia) and the pre-vaccine formulation of TT from Pfizer (Melbourne,

Australia). We thank the staff at the Department of Agriculture of Western Australia for the use of their facilities. We are grateful to John Mackenzie, Cheryl Johansen and John Curran for their kind provision of the wild duck sera and also to John Mackenzie for the H6N2 AI isolate. We thank Ian Robertson, Mohammed Yazid Abdad, Tze Hoong Chua, and Songhua Shan for technical assistance. This work was supported with funding from Stone Ridge Ventures Pty Ltd. and Murdoch University.

References

- [1] Macan-Marker, Vietnam: new outbreak undercuts record on avian flu, *Imaging Our Mekong*, Released 12 January 2007. <http://www.newsmekong.org>.
- [2] K.M. Sturm-Ramirez, T. Ellis, B. Bousfield, L. Bissett, K. Dyrting, J.E. Rehg, *et al.* Re-emerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks *J Virol*, 78 (2004), pp. 4892–4901
- [3] C.W. Lee, D.L. Suarez Avian influenza virus: prospects for prevention and control by vaccination *Anim Health Res Rev*, 6 (2005), pp. 1–15
- [4] D.E. Swayne Vaccines for list A poultry diseases: emphasis on avian influenza *Dev Biol*, 114 (2003), pp. 201–212
- [5] L.D. Simms, J. Domenech, C. Benigno, S. Kahn, A. Kamata, J. Lubroth, *et al.* Origin and evolution of highly pathogenic H5N1 avian influenza in Asia *Vet Record*, 157 (2005), pp. 159–164
- [6] D.A. Halvorsen The control of H5 or H7 mildly pathogenic avian influenza: a role for inactivated vaccine *Avian Pathol*, 31 (2002), pp. 5–12
- [7] K.B. Grogan, D.A. Halvorson, R.D. Slemons Avian influenza vaccines: focusing on H5N1 high pathogenicity avian influenza (HPAI). Special Publication No. 26 Council for Agricultural Science and Technology, Ames, IA (2007) p. 18–9
- [8] D.L. Suarez Overview of avian influenza DIVA test strategies *Biologicals*, 33 (2005), pp. 221–226
- [9] I. Capua, D.J. Alexander The challenge of avian influenza to the veterinary community *Avian Path*, 35 (2006), pp. 189–205

- [10] C.M. James, Y.Y. Foong, J.P. Mansfield, S.G. Fenwick, T.M. Ellis Use of tetanus toxoid as a DIVA (differentiating infected from vaccinated animals) strategy for sero-surveillance of avian influenza virus vaccination in poultry *Vaccine*, 25 (2007), pp. 5892–5901
- [11] W.A. Hagan, D.W. Bruner *The infectious diseases of domestic animals with special reference to etiology, diagnosis, and biologic therapy The pathogenic sporebearing anaerobic bacteria* (4th ed.) University Press, Comstock, Cornell (1961) p. 325–34 [Chapter 15]
- [12] World Organisation for Animal Health. *Manual of diagnostic tests and vaccines for terrestrial animals*; 2004 [Chapter 2.1.12]
http://www.oie.int/eng/norms/mmanual/A_00037.htm.
- [13] WHO. *WHO manual on animal influenza diagnosis and surveillance*. Global Influenza Program, Geneva, Switzerland; 2002 [WHO/CDS/CSR/NCS/2002.5].
- [14] FAO/ECTAD, *Avian Influenza: FAO in Action No. 7*, Dec 2007
<http://www.fao.org/avianflu>.
- [15] A. Martinot, J. Thomas, A. Thiermann, N. Dasgupta *Prevention and control of avian influenza: the need for a paradigm shift in pandemic influenza preparedness* *Vet Record*, 160 (2007), pp. 343–345
- [16] I. Capua, D.J. Alexander *Avian influenza infections in birds—a moving target* *Influenza Resp Vir*, 1 (2006), pp. 11–18
- [17] FAO/OIE/WHO technical consultation on the control of avian influenza. *Animal health special report*; 2004
http://www.fao.org/ag/againfo/subjects/ed/health/diseases-cards/avian_recomm.html.
- [18] I. Capua, S. Marangon *Vaccination for avian influenza in Asia* *Vaccine*, 22 (2004), pp. 4137–4138
- [19] OIE/FAO. *International scientific conference on avian influenza-Paris (France), Recommendations*; 2005 <http://www.oie.int/eng/AVIAN-INFLUENZA/guidelines>.
- [20] I. Capua, S. Marangon *Control of avian influenza in poultry* *Emerg Infect Dis*, 12 (2006), pp. 1319–1324
- [21] D.J. Hulse-Post, K.M. Sturm-Ramirez, J. Humberd, P. Seiler, E.A. Govorkova, S. Krauss, *et al.* *Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia* *Proc Natl Acad Sci*, 102 (2005), pp. 10682–10687
- [22] T. Songserm, R. Jam-on, N. Sae-Heng, N. Meemak, D.J. Hulse-Post, K.M. Sturm-Ramirez, *et al.* *Domestic ducks and H5N1 influenza epidemic, Thailand* *Emerg Infect Dis*, 12 (2006), pp. 575–581

- [23] M. Gilbert, P. Chaitaweesub, T. Parakamawongsa, S. Premashthira, T. Tiensin, W. Halpravidh, *et al.* Free-grazing ducks and highly pathogenic avian influenza, *Thailand Emerg Infect Dis*, 12 (2006), pp. 227–234
- [24] A. Hampson Avian influenza: a pandemic waiting in the wings? *Emergency Med Aust*, 18 (2006), pp. 420–429
- [25] WHO. Review of latest available evidence on potential transmission of avian influenza (H5N1) through water and sewage and ways to reduce the risk to human health. Updated 10/10/2001
http://www.who.int/water_sanitation_health/emerging/h5n1background.pdf.

Fig. 1. Natural seroprevalence for antibodies against tetanus toxoid in wild and farmed ducks. (A) Wild Plumed Whistling ducks from Kununurra, Northwest region of Western Australia ($n = 236$). (B) Waders (Pacific Black ducks and Maned ducks) from Southwest regions of Western Australia ($n = 59$). (C) Farmed domestic Muscovy ducks from Perth, Western Australia ($n = 98$, 6–8 weeks old). Levels of TT-specific antibodies in ducks are shown as percentage serum inhibition for each bird assayed in the competitive TT ELISA as described in Section 2 using purified TT antigen (List Biological Laboratories Inc.). Negative controls included pre-bleeds from normal unvaccinated Hy-Line Brown chickens for screening wild ducks (A and B) and pre-bleeds from normal Muscovy ducks (C). Farmed ducks were raised on deep litter or soil and fed commercial poultry with a mixture of vegetables.

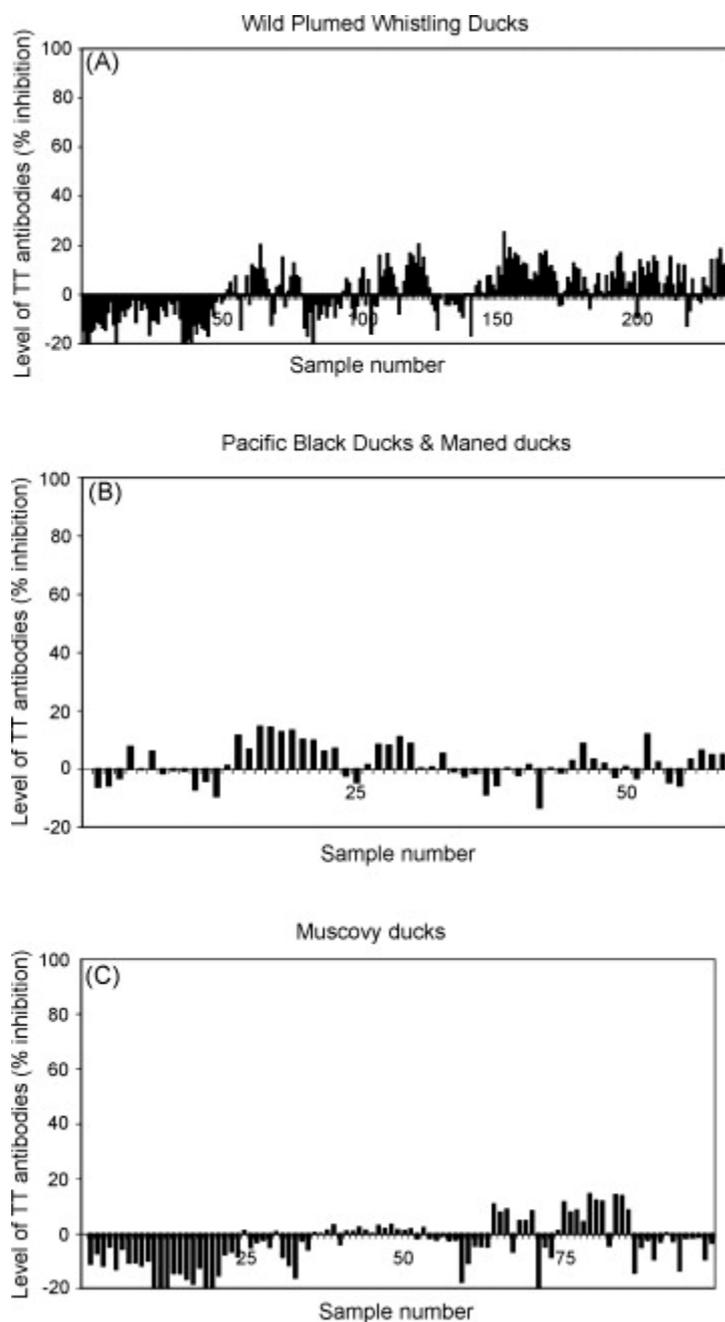


Fig. 2. Antibody responses to TT and H6 in ducks co-vaccinated with TT and inactivated H6N2 AI vaccines prepared as a water-in-oil emulsion. (A) Serum antibody levels to TT determined by competitive ELISA using purified TT antigen (List Biological Laboratories Inc.) and expressed as mean % inhibition + S.E.M. using pooled normal Muscovy duck sera as negative controls (pre-bleeds, $n = 5$). (B) H6-specific antibody titres determined by HI assay and expressed as mean \log_2 + S.E.M. The TT vaccines were composed of 0.1, 0.3 and 1.0 mg TT mixed with Montanide ISA-70 VG adjuvant and delivered as a water-in-oil emulsion. The AI vaccine was formalin-inactivated H6N2 influenza virus emulsified with Montanide (as described in Section 2). The two vaccine preparations were separately injected in each bird by the s.c. route at weeks 0 and 4 ($n = 7-10$ ducks per group). * $P < 0.05$ denotes a statistical difference between TT-antibody levels in the 0.1 mg TT/AI and 0.3 mg TT/AI groups.

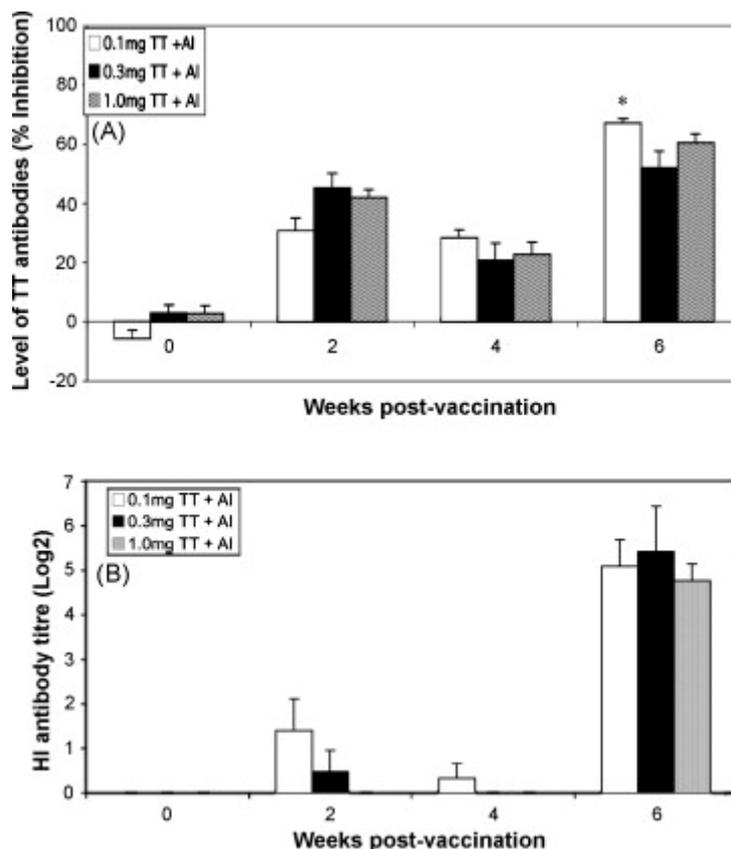


Fig. 3. Antibody responses to TT and H6 in ducks vaccinated with a combined TT and inactivated H6N2 AI vaccine. (A) Single vaccination and (B) double vaccination. Levels of TT-specific antibodies in Muscovy duck sera were determined by competitive ELISA using purified TT antigen (List Biological Laboratories Inc.) and expressed as mean % inhibition + S.E.M. using pooled normal duck sera (pre-bleeds) as negative controls ($n = 12$). (C) Single vaccination and (D) double vaccination. H6-specific antibody titres determined by HI assay and expressed as mean \log_2 + S.E.M. Vaccines were delivered as water-in-oil (Montanide ISA-70 VG) emulsions by the s.c. route and consisted of 0.3 mg TT, inactivated H6N2 influenza virus, and 0.15, 0.3 or 0.6 mg TT doses combined with inactivated H6N2 virus. * $P < 0.05$, ** $P < 0.01$.

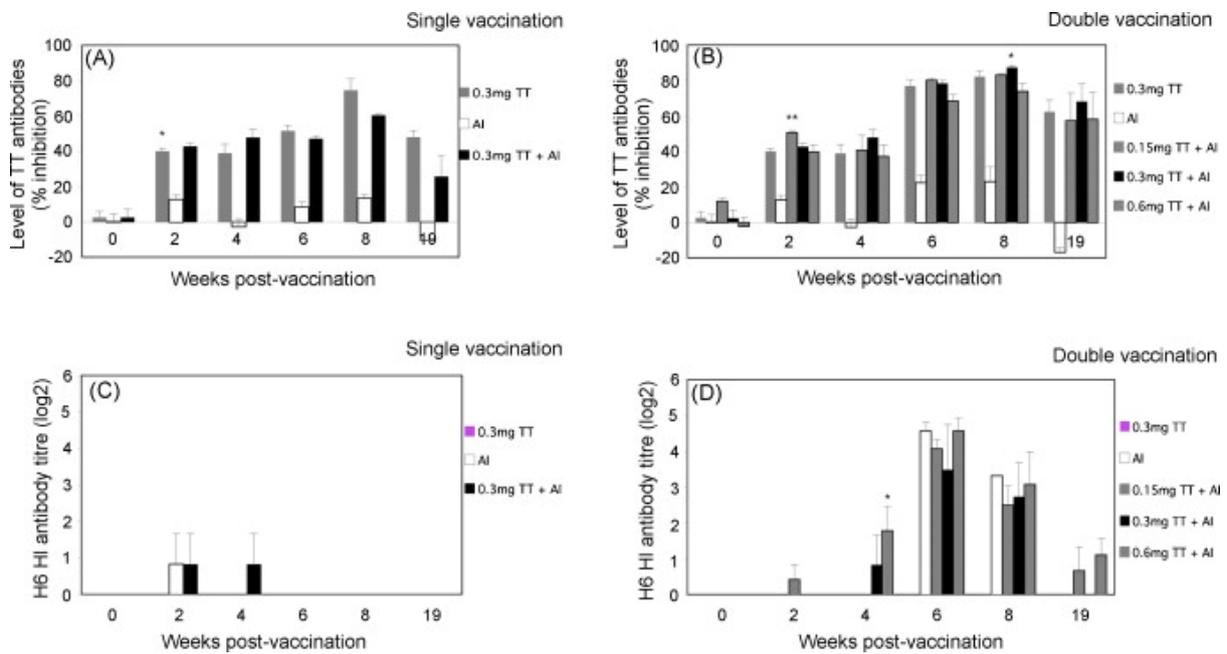


Table 1. IgY subclass antibody responses to tetanus toxoid (TT) and whole H6N2 avian influenza virus in ducks twice-vaccinated with a combined TT and inactivated H6N2 AI vaccine

Weeks post-vaccination ^a	TT-specific IgY titres ^b		H6N2-specific IgY titres ^c	
	TT vaccine only	TT/AI vaccine	AI vaccine only	TT/AI vaccine
2	3.82 ± 2.21	7.89 ± 0.25	8.14 ± 0.29	9.89 ± 0.25**
4	5.73 ± 1.91	7.64 ± 0.00	8.64 ± 0.00	9.14 ± 0.29
6	6.23 ± 2.09	8.64 ± 0.00	11.14 ± 0.29	10.64 ± 0.40
8	9.64 ± 0.41*	8.14 ± 0.29	10.89 ± 0.25*	8.89 ± 0.48
19	9.64 ± 0.58	8.64 ± 0.00	9.64 ± 0.58	10.64 ± 0.00

^aMuscovy ducks were twice vaccinated by the s.c. route with water-in-oil (Montanide ISA-70 VG) emulsions containing 0.3 mg TT, inactivated H6N2 virus, and 0.3 mg TT/AI ($n = 12$, 4 ducks per group).

^bTT-specific antibody titres in sera determined by indirect ELISA using purified TT antigen (List Biological Laboratories Inc.) and expressed as mean endpoint titres + S.E.M. using pooled normal duck sera (pre-bleeds) as negative controls ($n = 12$).

^cViral-specific antibody titres determined by indirect ELISA using inactivated whole H6N2 virus antigen and expressed as mean endpoint titres + S.E.M. using pooled normal duck sera (pre-bleeds) as negative controls ($n = 12$).

* $P < 0.05$.

** $P < 0.01$.