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

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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.

1. 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 (Charadriformes) 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

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.

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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 [8,9]. 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.

2. Materials and methods

2.1. 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.

2.2. 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].

2.3. 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).

2.4. 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.

2.5. 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