Journal of Vaccines & Vaccination
ISSN: 2157-7560
Berry, J Vaccines Vaccin 2013, 4:1
DOI: 10.4172/2157-7560.1000168
Letter to Editor
Open Access

Vaccine Control of Avian Influenza H5N1 in Poultry: Need for a Positive Marker

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Abstract

Highly pathogenic avian influenza (HPAI) H5N1 virus strains have emerged as zoonotic viral pathogens over the last decade and have eluded our serious attempts of control in domestic poultry by vaccination, with numerous countries continuing to have epidemic waves. Although the biology and genomics of H5N1 influenza viruses are well characterized so far, viral outbreaks still occur in domestic poultry, posing a dangerous threat of human transmission. There are two main types of contemporary inactivated vaccines, namely whole virus vaccines and virus vaccines engineered by reverse genetics, both of which are administered with adjuvant to hatchlings and optimally require a booster. However, determinants of vaccine efficacy need to be considered distinctly in chickens versus ducks on a country basis. There is a critical need for detection of infection and vaccination of domestic poultry to control potentially deadly but silent infection in vaccinated flocks. A positive vaccine marker strategy using tetanus toxoid offers advantages for more effective control programs of HPAI including improved capacity for early detection of virus outbreaks and indisputable data for surveillance of vaccinated flocks in vaccination control programs for backyard and village poultry, highly desirable in endemic regions.

Keywords: Avian influenza; Control; Poultry; Vaccination; Surveillance; H5N1; Virus outbreak; Pandemic influenza

Highly pathogenic avian influenza (HPAI) H5N1 virus outbreaks continue to occur in poultry [1]. Although human cases are sporadic and rare, they can be severe with a sustained 60% mortality rate worldwide. In many cases the risk factors include contact with sick and dying domestic poultry. Many countries that previously declared themselves H5N1 virus-free have now decreed regions a crisis zone following identification of variant H5N1 viruses, which adversely impact global trade in live poultry and poultry products and render current poultry vaccines ineffective [2]. Moreover, virus outbreaks in poultry may be hidden as low pathogenicity avian influenza (LPAI) virus infections and vaccinated birds can be sub clinically infected upon exposure to field virus. Disease levels tend to be high in countries with poor veterinary systems and influenza-prone farming systems, including backyard farms, mixed poultry and pig farms, often in close proximity to wild ducks and other influenza virus reservoirs [3]. Control measures encompass culling poultry around the outbreak site, intensive surveillance campaigns, poultry market closures and poultry movement bans. However, illegal trade of poultry across borders has enabled movement of HPAI H5N1 virus despite biosecurity measures for people and vehicles moving to and from premises containing poultry. Control strategies emphasize vaccination of poultry in some nations where stamping out alone is ineffective. Such poultry vaccines offer a powerful tool to mitigate the high risk of transmission of avian influenza virus to birds and people by reducing virus shedding [4,5]. However, effective vaccination strategies must overcome late onset of immunity, poor efficacy in ducks, mismatch between field and vaccine strains due to emergence of escape virus variants and limited delivery to remote regions. Since 2003, ten clades of H5N1 virus have emerged despite vaccination attempts and cross-protection in poultry now varies widely according to the phylogenetic relationship between seed virus used in vaccines and circulating virus. Global surveillance for detecting virus outbreaks, monitoring how viruses are evolving, understanding risk factors that enable them to spread, and keeping bird vaccines and diagnostics up to date will reinforce the vaccination strategy of control programs for universal coverage of the large, fragmented global poultry industry. However, besides both vaccination and surveillance as critical factors to drive programs to successfully combat disease, both practical and effective options need to be adopted by countries to build strong national responses.

Currently, conventional vaccine preparations of inactivated whole viruses in oil emulsions are preferred options for use in poultry [6,7]. Vaccination of day-old chicks in the hatchery is a common practice as this is less labour-intensive. However, a booster vaccine is often required to optimize vaccine efficacy. Generally, HA-specific antibody responses are induced and a rise in titre to at least 16 correlates with protection in chickens. A rapid four fold-rise in antibody titre of vaccinated birds would be unexpected and suggests an anamnestic response to recent infection. However, classical inactivated vaccines have limited efficacy against variations in field virus, especially when antigenic variance does not produce cross protective H5 HI titres against the vaccine strain. Improved surveillance for detecting H5N1 virus outbreaks in vaccinated flocks as early as possible is needed to prevent spread and human fatalities. Tracking virus outbreaks has been difficult in vaccinated flocks, as vaccinated birds do not show early clinical symptoms with silent infection that can be devastating [8,9]. Vaccination also complicates monitoring of infection by serology as detection of virus infection via H5 seroconversion is unable to Differentiate Infected from Vaccinated Animals (DIVA). Thus conventional vaccine use greatly limits the value of blood testing in an eradication program, especially in the case of LPAI viruses. Various strategies using serology of antigen markers to detect vaccination

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Received December 06, 2012; **Accepted** January 27, 2013; **Published** January 29, 2013

Citation: Berry CM (2013) Vaccine Control of Avian Influenza H5N1 in Poultry: Need for a Positive Marker. J Vaccines Vaccin 4: 168. doi:10.4172/2157-7560.1000168

and infection in vaccinated flocks have been devised to overcome this problem [10].

A DIVA strategy that has been developed for use in Europe exploits a H5N2 vaccine containing a heterologous NA antigen to the circulating H5N1 avian influenza virus [11]. The heterologous NA DIVA approach is based on detection of animals with positive anti-N1 antibody responses before a detectable rise in the flock H5 antibody levels but the anamnestic response in vaccinated poultry to H5 will develop more rapidly than the primary antibody response to N1. Thus, a primary antibody response to the different N subtype to that given in the vaccine takes a longer time to develop than a recall memory antibody response to the H5 antigen, allowing unmonitored virus spread throughout the flock. Current guidelines for serological DIVA testing in Europe, requiring only small sample sizes of 5-10 birds tested for NA antibody, would only provide assurance of detecting infection at the 95% confidence level if over 30% of poultry in the flock have been exposed to field virus [12]. This would likely take at least 3 weeks from introduction of infection to allow detection (one week for the virus to infect sufficient numbers of birds in the flock and a further 2 weeks for development of primary antibody responses in infected birds), a time when infection may no longer be active in the flock [13]. Not all exposed vaccinated poultry will develop antibodies to N1 in the virulent field strain due to limited replication in vaccinated animals [14]. Use of homologous NA antigens in vaccines, particularly for ducks, precludes use of the anti-N1 DIVA strategy. Other DIVA approaches for poultry based on negative markers include anti-NS1 and anti-NP antibody testing for recombinant live virus vector vaccines [15], including rHVT-H5, rNDV-H5 and rFPV-H5. Recently, a novel DIVA approach was developed using HPAI H5N1 virus-like particles [16]. Several disadvantages include likely contamination of whole virus vaccines with NS protein, reducing the value of using detection of anti-NS1 antibodies as a negative marker. Also, anti-NP antibody testing cannot be used if other avian influenza viruses are circulating in the test population, an uncommon situation for Asia and Egypt.

A positive avian influenza marker, unlike the abovementioned negative marker DIVA strategies offers an improved range of capabilities for vaccination and infection surveillance. We have previously used a positive marker strategy for H5N1 vaccination of poultry and have evaluated vaccination of chickens and ducks for protective efficacy against H5N1 virus infection in the laboratory. We selected tetanus toxoid (TT) as the positive marker, an exogenous antigen independent of serotype and relatedness of circulating virus and vaccine strains. Birds are naturally seronegative to TT but when TT is incorporated into poultry vaccines for H5N1 virus, vaccinated chickens seroconvert [17]. Evidence of vaccination in chickens by a simple ELISA test was associated with high specificity and sensitivity. Importantly, protection afforded by the avian influenza virus TT marker vaccine was equal to the homologous avian influenza virus vaccine in chickens [18]. This positive marker strategy has the potential to be used for detection of silent infection in vaccinated flocks, demonstrated by the development of a rapid anamnestic response to H5 antigen in vaccinated chickens exposed to HPAI H5N1 virus. We have observed stable antibody responses to the TT marker lasting out to one year post-vaccination in chickens with no significant difference in titre between six and 53 weeks post-vaccination, in contrast to a decline in antibody titre to AIV over the year post-vaccination.

Significantly, vaccination of ducks is problematic, as they are a natural host to a range of influenza viruses and since infection is often sub-clinical, little incentive exists for farmers to prevent infection in their flocks. Thus anti-NS1, anti-NP and anti-M antibody testing as a DIVA approach cannot be used in ducks. Furthermore, the anti-N1 DIVA strategy has not been evaluated in ducks despite ducks being the largest poultry market in China and Vietnam. We validated the TT marker approach in ducks and found that H6N2/TT vaccination induced TT seroconversion in twice-vaccinated Muscovy ducks, which persisted out to 19 weeks in contrast to a decline in antibody titre to AIV after 6 weeks post-vaccination [19]. Evidence of TT marker vaccination in ducks was performed using a competitive ELISA and a vaccine dose double that used for chickens, as ducks are well known to be poor antibody responders. Ducklings receive H5 vaccination in the hatchery, with maternal immunity waning by 3 weeks, and optimally require a boost for antibody responses to H5. In our laboratory study, Peking ducks vaccinated with a commercially available inactivated H5N2 whole virus vaccine (Nobilis) produced detectable H5 HI antibodies by 3 weeks post-vaccination, which steadily increased in titre over a five-week period. Importantly, protection against HPAI H5N1 virus afforded by the avian influenza virus marker vaccine (TT/H5N2) was equal to the homologous avian influenza virus vaccine (H5N2) in ducks and did not interfere with antibody responses to influenza virus antigens contained in the vaccine [20]. Therefore, evaluation studies of the TT marker for avian influenza vaccination encompassed LPAI H6N2 and HPAI H5N1 vaccinated ducks in Australia and Hong Kong SAR, respectively [19,20]. Moreover, the TT marker has the potential to be used for detection of silent infection in vaccinated ducks, with the development of an anamnestic response to H5 in vaccinated ducks infected with HPAI H5N1 virus [20]. Eradication of H5N1 virus from ducks requires widespread vaccination, boosters of larger antigen doses, preferably before ducks are sent out to graze, and thus segregation of poultry species for control programs [21].

In the field, H5/TT positive marker antibody ratios could be used to assess whether avian influenza virus marker-vaccinated birds have been exposed to field virus post-vaccination, as an anamnestic response will likely occur to H5, influenced by antigen diversity between vaccine and circulating viruses. If infection travels quickly through the flock, it is likely that sufficient poultry will have developed an anamnestic response to allow detection of a shift in the flock H5/TT antibody ratio before 30% of the flock would have developed an anti-N1 antibody response according to a N1-DIVA approach. Evaluation of our field trials using chicken farms in Hong Kong SAR, demonstrated successful use of the TT marker in a standard inactivated whole H5 avian influenza virus vaccine (made in-house with formulated components provided by Intervet). Batches of chickens on five chicken farms were routinely vaccinated either with inactivated water-in-oil adjuvanted TT/H5N2 vaccine (n=120 each farm) or H5N2 vaccine (n=12 each farm) at 8-10 days and 36-38 days of age. Antibody responses in chicks were pre-tested before vaccination and after the first and second vaccination with a final bleed o f chickens at 64 days of age. Pre-vaccination testing for H5 HI titres of thirty 8-day old chicks, which were imported as 1-day old chicks from mainland China from parent bids vaccinated with H5, on each farm showed considerable variation in the level of maternal H5 antibody titres greater than 16 (ranging from 0-60%). However, by the second bleed of these H5 HI positive birds, 86% were H5 HI antibody negative suggesting that maternal antibody had waned. The H5 HI antibody titres following the first and second vaccination with the TT/H5N2 and H5N2 vaccines were variable between the five farms but reached the national target of at least 70% of the batch responding after the two-dose vaccination course with an HI≥20titre. All of chickens tested in this field study had no pre -existing antibody to TT, confirming our earlier published reports in chickens [17,18] and no antibody responses

to TT were found in the H5N2 vaccinated chickens on the farms. As expected, the birds vaccinated with the TT/H5N2 vaccine mounted an antibody response to the TT marker. Future field evaluation studies are needed to conclusively indicate that there is no interference to H5 antibody responses from the TT marker in the combined vaccine although our pen-side studies showed no significant interference with H6 antibody responses from the TT antigen in a combined LPAI TT/H6N2 vaccine [17] and no abrogation of protection in H5N2 vaccine/H5N1 challenge studies of chickens [18]. Outbreak investigations of anti-TT antibodies could provide vital information on whether the flock is vaccinated and the H5/TT antibody ratios could provide data on whether vaccination has been effective and the duration of exposure of the flock. We also demonstrated that anti-TT and anti-H5 antibody titres could be assessed in dead vaccinated birds [17]. This is useful to determine whether a vaccinated bird had died in the flock and the H5/TT antibody ratio provides data on the level of vaccination and possible emergence of an escape variant against which the vaccine would have reduced efficacy. Post-vaccination surveillance of birds seronegative to a positive marker could be used to determine whether poor response to vaccination is due to non-vaccination or infection rather than vaccination, especially in ducks, and could be performed before birds are sent to market using a simple and inexpensive pen-side test to assess antibodies to H5 and TT. A flock trial evaluation study designed for either chickens or grazing ducks specifically for Asian versus European markets is required as no HPAI virus challenge studies have as yet been reported with any DIVA strategy.

Although other positive vaccine markers, such as GFP, have been proposed for avian vaccines, the TT positive marker offers improved capacity for early detection of an avian influenza virus outbreak in the flock, high sensitivity and high throughput testing, low cost and virus strain clade-independence. Further advantages of the TT marker vaccine include indisputable serological data for surveillance programs on whether poultry have been vaccinated in the past and on the vaccination status of flocks at the time of an outbreak without dependence on dubious certification for compliance with vaccination. Current problems associated with existing post-vaccination surveillance programs include records of purported vaccination as opposed to actual vaccination, vaccination failure due to virus strain mismatch or failure to vaccinate where birds are mistakenly missed out, and extent of infection in areas where vaccines are being used. Three scenarios are envisaged for surveillance of H5N1 virus outbreaks in poultry. Firstly, if sentinels are positive to the TT marker and H5 then this indicates that they have been inadvertently vaccinated. Secondly, if vaccinated poultry are negative to the TT marker but H5 positive then this indicates probable vaccine failure and subsequent exposure to field virus. Thirdly, if vaccinated poultry are positive to the TT marker and H5 positive, then the ratio of H5/TT antibodies would assess whether there has been possible exposure to field virus, warranting further detailed virus investigations. Targeted surveillance in pre-slaughter testing of birds could determine vaccination from natural infection empowering investigation of H5 virus circulation in bird markets. Also, border control for smuggled birds could incorporate positive marker serology to check for vaccine-approved animals. Importantly, virus outbreaks in vaccinated flocks can be monitored to identify if the field strain is overcoming vaccine-induced immunity to the H5 antigen, indicating likely emergence of an antigenic variant against which existing vaccines are less effective. Although the TT marker vaccine approach does not represent a true DIVA strategy, it is nonetheless a profoundly useful tool for evaluation of the vaccination process, which is crucial in control programs for HPAI H5N1 virus in smallholder poultry sectors. The TT positive marker vaccine could potentially provide epidemiological data for important decision making on a more focused "risk-based' vaccination campaign to achieve the goal to ultimately reduce the scope of vaccination and provide countries with an exit strategy.

In conclusion, vaccines are effective countermeasures and worthy investments for control of HPAI H5N1 virus in domestic poultry, especially with the lack of immunity in humans to H5-possessing influenza viruses. Use of updated vaccines to emerging H5N1 influenza viruses circulating in poultry and increased surveillance in regions likely to have H5N1 virus outbreaks in flocks using a positive marker approach improves monitoring of flocks in control programs. A positive marker vaccine strategy using TT for poultry, which targets protective efficacy of vaccination coupled with bio-surveillance of vaccinated birds and virus outbreaks in flocks, provides both a practical and effective option for vaccination campaigns against H5N1 avian influenza virus. Future scope of the introduction of such a marker AIV vaccine into high risk areas would depend on demonstration of the benefits without interference with international trade on a country by country basis through future field trials.

Acknowledgments

I wish to thank both past and current members of my laboratory team that contributed to research on H5N1 virus vaccines over the past six years; Dr. Trevor Ellis, Prof. Stan Fenwick, Ms Josie Mansfield, Dr. Yvonne Foong, Dr. M. Yazid Abdad, Ms Azita Rezazedeh Vind, Dr. Tze-Hoong Chua, Ms Hege Jacobsen at Murdoch University and Dr. Deb Middleton and her team at the Australian Animal Health Laboratories. I sincerely thank Dr. Les Sims and Prof. William J. Penhale for insightful discussion.

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