XI. VACCINATION OF NON-DOMESTIC AVIAN SPECIES AGAINST HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI) VIRUSES

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Introduction

Avian influenza viruses (AIV) are type A influenza viruses and belong to the *Orthomyxoviridae* family. They can be classified according to the antigenicity of its surface proteins haemagglutinin (H) and neuraminidase (N). Currently 16H (H1-16) and 9N (N1-9) subtypes have been described in avian species (Fouchier *et al.*, 2005). Furthermore the subtypes can be classified on the basis of their pathogenicity in chickens after intravenous inoculation.

Highly pathogenic avian influenza (HPAI, formerly termed fowl plague), an acute generalised disease in which mortality in chickens may be as high as 100%, is restricted to subtypes H5 and H7, although most viruses of these subtypes have low pathogenicity, and do not cause HPAI. Low pathogenic avian influenza (LPAI) virus strains cause more variable morbidity and mortality (ranging from sub-clinical to fatal) but are generally associated in poultry with a mild, primarily respiratory disease with loss of egg production (Capua & Alexander, 2004), or mild enteric disease in non-domestic birds. In certain cases (in poultry flocks) the LPAI virus phenotype (of subtype H5 or H7) may mutate into the HPAI virus phenotype by the introduction of basic amino acid residues (arginine or lysine) at the cleavage site of the precursor haemagglutinin (HAO) (Banks *et al.*, 2001), which facilitates systemic virus replication. H5 and H7 subtypes with an amino acid sequence at the HA0 cleavage site comparable to those that have been observed in virulent AI viruses are considered HPAI viruses, even when mortality in chickens is low (Office International d'Epizooties., 2004). However, the two forms of avian influenza (HPAI and LPAI) are distinctly different and should be regarded as such.

Avian influenza viruses have a worldwide distribution and are infectious to all avian species (commercial, domestic and wild), with variable morbidity per virus isolate and species. Aquatic avian species, mainly those of the taxonomic orders Anseriformes and Charadriiformes are considered the main natural reservoir of all avian influenza viruses, including the LPAI ancestral viruses of HPAI strains (Munster *et al.*, 2005; Munster *et al.*, 2007). Waterfowl were generally considered resistant to infection with HPAI virus until 2002. However, in 2002 an outbreak of HPAI H5N1 virus occurred in wild migratory avian species and resident waterfowl (Sturm-Ramirez *et al.*, 2004). Since then, this particular HPAI virus subtype has made an unprecedented spread from South East Asia throughout Asia and into Europe and Africa, with morbidity and mortality not only in domestic poultry, but in more than

130 non-domestic avian species from various taxonomic orders: Anseriformes, Charadriiformes, Ciconiiformes, Columbiformes, Falconiformes, Galliformes, Gruiformes, Passeriformes, Pelecaniformes, Phoenicopteriformes, Strigiformes, Struthioniformes, Psittaciformes, and Podicipediformes (USGS National Wildlife Health Center, 2008). Additionally, this virus strain has has caused mortality in a large number of mammalian species, and has caused 403 human cases with 254 deaths to date (27 January 2009) (World Health Organisation, 2009).

Documented outbreaks of Asian lineage H5N1 HPAI virus in captive non-domestic birds have been limited to 6 cases: Penfold Park, Hong Kong, (People's Republic of China, 2002), Kowloon Park, Hong Kong (People's Republic of China, 2002), Phnom Tamao wildlife rescue centre (Cambodia, 2004), Ragunan Zoo, Jakarta (Indonesia, 2005), Dresden Zoo (Germany, 2006) and Islamabad Zoo (Pakistan, 2007). Large felids with H5N1 infection have been reported in Suphanburi Zoo (Thailand, 2003), and Sri Racha tiger zoo (Thailand, 2004). To curtail these outbreaks, a combination of increased bio-security measures (isolation and quarantine of infected animals, disinfection of the area), feeding of cooked poultry only, treatment of infected animals in quarantine areas, selective culling, extensive surveillance of migratory and captive birds and vaccination were used.

Vaccination

Vaccination is a useful means of reducing the horizontal spread of AIV in poultry (Capua *et al.*, 2004; van der Goot *et al.*, 2005) (Ellis *et al.*, 2004). Vaccination protects against disease and mortality, but does not always prevent infection and virus spread. However, the dose required for infection is much higher, and vaccinated birds shed far less field virus after infection than unvaccinated birds (Brugh *et al.*, 1979; Karunakaran *et al.*, 1987).

Protective antibodies produced in response to infection or vaccination, are directed against the H and N surface proteins. Vaccine-induced antibody responses are species-, dose-, and vaccine strain-dependent, e.g. the antibody responses upon AIV vaccination are generally higher in chickens than in other poultry species (Higgins, 1996). Published minimum serum antibody titres measured by HI test in vaccinated chickens that correlate with protection after challenge with HPAI virus are 1:10 (Swayne *et al.*, 2006), or 1:16 (Ellis *et al.*, 2004; Tian *et al.*, 2005). However, domestic ducks with very low or undetectable antibody titres post vaccination have been shown to be protected from HPAI virus challenge (Middleton *et al.*, 2006; Webster *et al.*, 2006). Duration of protection from HPAI virus challenge may vary between species: chickens for up to 40 weeks after one dose of vaccine, domestic ducks for more than 52 weeks after 2 doses, while domestic geese which received 3 doses were protected for 34 weeks (Tian *et al.*, 2005).

The degree of homology of the H protein will largely affect the level of cross-protection and therefore efficacy of the vaccine (Swayne *et al.*, 2000). A so-called Differentiation of Infected from Vaccinated Animal (DIVA) strategy, with a heterologous vaccine (using the same H subtype as the field virus, but a different N subtype), is recommended to differentiate between vaccinated and field-virus infected animals (Capua *et al.*, 2003). However, in housing systems where birds are not housed permanently indoors (e.g., in zoos), contact with free-ranging birds can result in LPAI virus infections that go by unnoticed, but which may interfere with the DIVA principle.

In the European Union routine vaccination of poultry against avian influenza viruses is currently not practised as this would interfere with stamping-out policies and international

trade agreements. Instead, eradication measures during an outbreak in poultry include (long-term) confinement, large-scale culling and safe disposal of carcasses of all poultry on the infected farm, and, depending on the poultry density in the area and the epidemiological situation, pre-emptive culling of poultry on neighbouring farms and emergency vaccinations (Directive 92/94/EEC). Since 2003, more than 300 million birds have been culled to eradicate HPAI outbreaks.

Vaccination in European Zoos

The standard measures used to prevent and eradicate HPAI virus outbreaks in poultry (long-term confinement and large scale culling) would be detrimental to the welfare, conservation status and breeding programmes of zoo birds, which often are irreplaceable, valuable and endangered avian species (IUCN Red list, http://www.iucnredlist.org/). Directive 2005/94/EC foresees a derogation from culling of birds provided the birds can be brought inside and are subjected to virus detection tests (after the last death/positive finding, 2 tests at an interval of 21 days have to be performed according to the diagnostic manual Decision 2006/437/EC). However, most zoos do not have the capability to suitably confine their entire bird collections for extended time, and many species would not be able to adjust to confinement and increased stress with subsequent welfare problems and increased exposure to pathogens resulting in disease (e.g. aspergillosis and bumblefoot) (McMillian & Petrak, 1989; Redig, 2000; Harcourt-Brown, 1996).

Instead of confinement, vaccination of zoo birds against HPAI virus was allowed as an additional preventive measure (while reducing confinement measures) in Belgian, Dutch and German zoos during an outbreak of HPAI H7N7 virus in poultry in 2003 (Decision 2003/291/EC). Similarly, in 2005, Decision 2005/744/EC allowed vaccination in European zoos against the encroaching H5N1 subtype. These campaigns were subject to rigorous surveillance and control requirements.

Vaccination against HPAI H7N7 in zoos

During the HPAI H7N7 outbreak in poultry in 2003, birds in Dutch zoos were vaccinated twice with six weeks interval using a whole inactivated oil-adjuvanted vaccine, based on influenza virus A/chicken/Italy/473/99 (H7N1), with high homology to the field strain HPAI H7N7 A/chicken/Netherlands/1/03 (97.4% nucleotide and 98.7 % amino acid sequence identity). This resulted in the induction of antibody titres ≥ 40 (used as a correlate of protection in this study) in 81.5% of the vaccinated birds, with an overall GMT of 190. Birds of the taxonomic orders Anseriformes, Galliformes and Phoenicopteriformes showed higher GMT, and larger percentages developed a serum HI antibody titre ≥ 40 than those of the other orders. Furthermore, a decrease in antibody response with an increase in body weight > 1.5 kg was shown. The high agreement between post vaccination antibody titres determined by serum HI test (using the vaccine strain), and VN titres (using the field strain), was used as a further measure of immunogenicity. The broad efficacy demonstrated in a large variety of taxonomic orders illustrated the benefits of vaccination as an additional preventive measure against HPAI virus infection (Philippa *et al.*, 2005).

Vaccination against HPAI H5N1 in zoos

In 2005, the Dutch zoos were the first to implement Decision 2005/744/EC to provide protection against the encroaching HPAI H5N1 subtype. Birds were vaccinated with an inactivated adjuvanted H5N2 vaccine with vaccine doses adapted to mean body weight per

species, using data collected during the H7N1 vaccination campaign. The vaccine strain (A/duck/Pottsdam/1402/86) had a homology of 90% to the HA gene of the H5N1 field strain (A/turkey/Turkey/1/05) on the basis of nucleotide sequence (1530 base pairs, including basic cleavage site), and 92.4% on the basis of amino acids. Vaccination was safe, and proved immunogenic throughout the range of species tested, with some variations between and within taxonomic orders. After booster vaccination the overall homologous GMT to the vaccine strain, measured in 334 birds, was 190 (95% CI:152–236), and 80.5% of vaccinated birds developed a titre of ≥ 40. Titres to the HPAI H5N1 virus followed a similar trend, but were lower (GMT: 61 (95% CI: 49–76); 61%≥ 40) (Philippa *et al.*, 2007).

The breadth of the immune response was further demonstrated by measuring antibody titres against prototype strains of four antigenic clades of currently circulating H5N1 viruses. Antigenic distances to the prototype strains were determined using antigenic cartography (Smith *et al.*, 2004). Antigenic cartography uses the antigenic properties of influenza viruses combination with epidemiological and genetic data, and is used to select virus strains for use as human pre-pandemic (H5N1) vaccine candidates (World Health Organisation (WHO), 2006). Influenza vaccines whose haemagglutinins are antigenically similar to circulating strains provide the highest level of protection from infection in humans (Subbarao, 1999). The birds clustered in two groups based on the breadth of antibody responses. Group 1 (Anseriformes, Galliformes, Phoenicopteriformes, Psittaciformes and Struthioniformes) showed a very broad response to vaccination, with predicted protection against future strains up to 12 antigenic units from the current vaccine. Group 2 (Ciconiiformes, Gruiformes, Pelecaniformes and Sphenisciformes) had low HI antibody titres against the prototype strain of the most antigenically distant clade (A/Indonesia/5/05).

In 2006, a working group of Animal health and Welfare experts was established by the European Food Safety Authority (EFSA)(European Food Safety Authority (EFSA), 2007), to provide a scientific assessment of the preventive vaccination against avian influenza of H5 and H7 subtypes carried out in zoos in Member States (MS). The total number of birds vaccinated, as reported by 12 MS, was 44721. Individual data from 4718 birds (374 species from 19 taxonomic orders) were submitted. Not all of these could be used for every evaluation: pre-vaccination titres could be evaluated for 3039 birds; titres after first vaccination were evaluated for 1429 birds, and post-second vaccination titres for 2296 birds.

Differences in vaccination schedules, doses and routes, differences in methodology and antigens used in the HI tests between laboratories (due to the absence of international reference standards, and the absence of inter-laboratory standardisation of methodology), the use of different vaccines[1] in different taxonomic orders and the sometimes incomplete reporting of results, limited the evaluation of some of the data provided by EU MS. Cut-off points varied with laboratory, and titres considered a measure of adequate immune response were 8, 16, 32, 40, and 64. Most countries used dilution series starting at 4 or 8, therefore results were evaluated for titres 16 and 32 [documented surrogate markers for protection in chickens (Ellis *et al.*, 2004; Office International d'Epizooties., 2004; Swayne *et al.*, 2006; Tian *et al.*, 2005)], and undetectable titres were regarded as 4 for calculation of GMT. In the absence of (and unfeasibility of obtaining) vaccination/challenge data in often endangered

[1]
Vaccine A: H5N9 (A/turkey/Wisconsin/68).
Vaccine B: H5N2 (A/duck/Pottsdam/1402/86).
Vaccine C: H5N2 (A/chicken/Mexico/232/94/CPA)
Vaccine D: H5N9 (A/chickenk/Italy/22A/98).
Vaccine E: H5N9 (A/chicken/Italy/22A/H5N9/1998).

zoo bird species, the evaluation had to be based on extrapolation of serological data from poultry and limited other bird species.

The H5 vaccines registered for poultry in the EU showed differences in efficacy, measured as serum HI antibodies induced by two doses of vaccine (Table 1.). Three of the five vaccines evaluated induced relatively high GMT and high percentage seroconversion in the vast majority of vaccinated birds. The HI titres induced by vaccination showed marked differences between and within taxonomic orders. Both routes of vaccination (i.m. and s.c.) were effective in inducing HI serum antibody responses, and for most avian species the poultry dose was suitable. In some larger species higher doses adjusted to body weight, induced higher serum antibody titres. (e.g. for ostriches a 10-fold increase of the poultry dose (10 x 0.25ml). However, extremely high doses at a single site of injection (e.g. vaccination of ostriches with 10 ml of vaccine) appeared to have a negative effect on the induction of serum antibody titres, and induced local adverse reactions.

There were indications that one vaccination was sufficient to induce high serum antibody titres in at least two taxonomic orders of birds (Anseriformes and Galliformes). However, a second vaccine administration ensured seroconversion in the majority of birds of most species. Limited data indicated that antibody titres persisted in several species for six months after vaccination. Adverse effects and mortality associated with vaccination were low and were mainly attributable to handling stress or trauma. Differences in adverse effects reported from different zoos highlight the importance of proper skilled handling.

Longevity of antibody titres

One year after vaccination with the H5N2 vaccine, birds in Dutch zoos were revaccinated with the same vaccine. Antibody titres one year after the initial two vaccinations and the effect of one booster vaccination at this time were evaluated. In Rotterdam Zoo, 72 previously vaccinated birds could be evaluated for the effect of one booster vaccination (Philippa et al, in press). For 44 birds, serum samples were available from 4 weeks after the initial two vaccinations the previous year, at the time of revaccination, and 4 weeks later. Birds which had been vaccinated with the H7 vaccine two years prior to the H5N2 revaccination were additionally tested for the presence of H7-specific antibodies.

Serum antibody titres of the birds tested in Rotterdam Zoo had clearly decreased in one year time: while 80% of birds had a positive titre (≥ 8) and 68% a high positive titre (≥ 32) after 2 vaccinations, these figures were 61% and 30% respectively one year later. Four weeks after re-vaccination these figures increased to 93% and 77% respectively. Although a larger percentage of these 44 birds had a serum HI antibody titre ≥ 32 after re-vaccination, the GMT was lower than GMT after 2 vaccine doses one year before (88 vs 66).

Birds from 4 out of 8 taxonomic orders did not have a GMT > 5 one year after vaccination, and only one order (Phoenicopteriformes had a GMT > 40. Four weeks after one revaccination 6/8 taxonomic orders tested had a GMT > 40.

GMTs had decreased even further two years after vaccination, as was shown by the H7 specific serum HI antibody titres. As these birds were not revaccinated with an H7 vaccine, the effect of revaccination two years after the initial vaccinations is not known.

Conclusions

Bio-security measures remain the first line of protection of zoo birds against the introduction of AI viruses and should be implemented in zoos. These bio-security measures should include strict hygiene and quarantine measures, but should also exclude the possibility of introducing AI viruses through feed animals such as day old chicks, other poultry or their products. Continuous clinical monitoring of captive and wild birds in zoos should be practiced, for early detection of introduced viruses by wild birds, domestic birds, or their products. Strict bio-security measures will also reduce the risk of subsequent infection of wild birds from zoo birds. Wild birds have been documented to be susceptible to HPAI virus infection, and could potentially play a role in the spread of HPAI virus, although the majority of avian influenza viruses detected in free-ranging birds have been LPAI viruses. If bio-security measures cannot sufficiently protect zoo birds from exposure to HPAI viruses coming from wild birds (based on an overall risk assessment which includes welfare aspects) vaccination with vaccines against HPAI of H5 and H7 subtypes authorised for use in poultry should be used to protect these zoo birds. In designing AI vaccination programmes and schedules for zoo birds, recent data on wild bird migration and prevalence of AI viruses should be taken into account. Vaccination against AI viruses of the H5 and H7 subtypes with current inactivated oil-adjuvanted poultry vaccines is safe and, in most taxonomic orders of zoo birds, effective in terms of inducing HI serum antibody titres. AI vaccines should be administrated in a way that elicits high HI antibody titres in the vast majority of the zoo birds vaccinated, i.e., by adjusting dose to average body weight. Although there are indications that one vaccination might suffice for some species, a second vaccine dose ensures high titres in the vast majority of species. Unless it is demonstrated that one vaccine administration is sufficient, two administrations are recommended. The H5 and H7 vaccines currently registered for poultry in the EU show differences in the performance in terms of HI response in zoo birds after two doses. There appears to be no difference in route of vaccination (s.c. or i.m.), so route can vary depending on the bird species to be vaccinated. In order to maintain high titres in the captive populations in zoological collections, annual re-vaccination seems to be required, as antibody titres decrease significantly in most taxonomic orders, and high titres are seen after a single annual booster dose.

Mortality and adverse effects were low in all zoos evaluated in EU MS, and mainly attributed to handling stress and trauma. Zoos can, and should therefore try to minimise these losses in the execution of HPAI vaccination programmes. To minimise indirect losses due to decreased breeding results, AI vaccination during breeding seasons should be avoided whenever possible. Mortality due to catching and handling stress can be reduced by handling the birds less. Once the efficacy of a vaccination protocol has been validated for certain species using certain vaccines, measurement of post-vaccination HI serum antibody titres should no longer be mandatory by the EU. These birds will then only have to be handled for vaccination, and not 4 weeks later. Further research should be carried out to establish effective vaccination schedules, route, and dose regimen in different zoo bird species. This may, amongst others, lead to a reduction in the number of booster vaccinations needed in certain species. Novel generation vaccines which may be administered in the form of an aerosol (as is used in vaccination of poultry against Newcastle disease virus) may prove to be useful in non-domestic species, and would eliminate the need for handling the birds.

The vaccination campaigns against HPAI virus have focused on protecting birds in zoological collections. However, a large number of mammalian species, including tigers and leopards, have also been documented with HPAI virus infection with recent H5N1 subtypes. There is currently no commercial vaccine available to protect mammals from HPAI H5N1 virus

infection. A recombinant fowlpox-vectored vaccine expressing the H5 gene has been shown to produce high antibody titres against heterologous H5N1 virus antigen in cats after booster vaccination (Karaca *et al.*, 2005), and may prove to be useful in prophylactic vaccination programs of mammals in the future. Until then, these animals have to be protected by excluding the introduction of AIV through raw poultry used as feed.

The broad vaccine efficacy in the different avian taxonomic orders illustrates that vaccination against avian influenza is a useful tool for the protection of non-domestic avian species in zoos, which allows for an alleviation of confinement measures – and is therefore beneficial to the health and welfare of these birds. However, increased bio-security measures in combination with virological monitoring remain imperative in combating outbreaks of HPAI.

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