OFFLU AVIAN INFLUENZA MATCHING (OFFLU AIM) GUIDE TO ASSESSING ANTIGENIC CHARACTERISTICS OF AVIAN INFLUENZA VIRUSES

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Scope: This report provides information for national/sub-national level laboratories on how to assess antigenic characteristics of avian influenza viruses.

In the continuous assessment of vaccine efficacy, the challenge of immunized animals represents the gold standard. However, this approach is labour and cost intensive and requires high biosecurity laboratories and animal facilities. Challenge trials should be reserved for cases where there is already evidence of antigenic variation from other sources suggestive of reduced vaccine efficacy. Furthermore, as experimental studies are generally undertaken in specific pathogen free birds, assessment of vaccines in field situations, even in the absence of virulent challenge, but with robust serological assessment is highly beneficial to understand responses in the presence of potential inhibitory factors.

Antigenic cartography involves the use of reference sera raised against reference strains and test viruses but is usually restricted to being undertaken in reference laboratories with the facilities to undertake live virus work. As such, whilst cartography forms a critical analytical aspect of the OFFLU AIM project, it requires samples of live infectious virus to be sent to reference laboratories.

Other methods can also be used to provide evidence of possible antigenic variation in field viruses, including comparative serological tests using sera from vaccinated birds against antigens prepared from field viruses and vaccine antigens in use. Genomic sequencing is being deployed in many countries and can be used to detect changes in known or putative antigenic or glycosylation sites that could affect responses to vaccination. Work in this area on Gs/Gd H5Nx viruses has been conducted for over 15 years (see for example Cattoli et al., 2012) with the explicit aim of monitoring for antigenic evolution rapidly.

Cross-HI testing using sera from vaccinated birds

One of the methods used to assess the antigenic characteristics in places where vaccines are being used is to obtain sera from vaccinated birds and conduct cross hemagglutination inhibition (HI) tests using the vaccine antigen (or a closely related proxy) versus an antigen prepared from a field isolate of interest.

It is recommended that at least 30 serum samples from vaccinated birds are used for the test and vaccine antigen. It is important to note that this method only indicates antigenic relatedness. If a virus demonstrates a marked reduction in titres (at least 4 $\log_2$ differences) then full antigenic characterisation should be undertaken and greater attention should be paid to vaccinated flocks for evidence of infection.

For example: Country X is using a vaccine containing antigen Y and finds a virus in a well-vaccinated flock through evidence of clinical disease or screening sera as part of an active surveillance initiative. Virus Y was then used as an antigen in HI tests using 30 sera from vaccinated birds that had not been exposed to field virus. The average titer for antigen Y was 20 whereas the average titer for samples using the vaccine antigen (Z) was 160. This >3fold difference between mean titers for antigen Y and Z, along with the case history strongly suggests significant antigenic variation is occurring that could impact protection provided by vaccination. In such cases consideration should be given to conducting challenge experiments or undertaking full antigenic characterisation at an appropriately recognised Influenza reference laboratory, such as a WOAH or FAO animal or avian influenza reference laboratory.

Use of gene sequences for assessing possible antigenic changes

Changes in antigenicity are usually the result of genetic changes to the viral haemagglutinin (HA) protein in putative antigenic sites, or loss or acquisition of glycosylation sites.

As full genetic characterisation and antigenic characterisation of A(H5Nx) viruses increases, more information on the significance of specific changes will be obtained. Whilst often, multiple genetic changes are required to effect major antigenic variation (e.g. Cattoli *et al.*, 2011), single amino acid changes may also have a profound effect on protein structure and the induction of glycoprotein reactive antibodies.

Screening for changes in putative antigenic sites can be carried out using sequence data, however, attention must be paid to the haemagglutinin numbering system which is used to align sequences and infer point changes as different publications use different HA numbering (e.g. H5 vs H3). The publication Burke and Smith (2014) provides a list of sites using different numbering systems and a review by Luczo and Spackman (2024) compiles to-date information on putative antigenic sites for H5 and H7 HPAI viruses including references for H3, H5 and H7 numbering.

Where possible, proposed antigenic sites should be assessed with reference to evolving information in the literature and with consideration of defined reference viruses. Interpretation of antigenic changes can be complex and ideally phenotypic data should be available. Some studies are conducted and validated with respect to how the human immune system recognises the antigenic components of these viruses. This may differ to how the chicken, or other avian species recognise these viruses. Therefore, where possible, inference using genetic data should be backed up with phenotypic data or field data. Technical support should be sought from an appropriate FAO or WOAH reference laboratory if required and OFFLU strongly recommend that member states or countries share information and viruses with the OFFLU network, especially if viruses are detected with changes in putative antigenic sites.

Methods for assessing vaccines other than those based on a killed virus antigen

Vectored vaccines can potentially stimulate a greater antibody response as they replicate to generate higher levels of target antigen within the vaccinated bird. However, replicative ability of vectored vaccines may differ depending on the vectors ability to replicate in the vaccinated bird. Examples of different vectors include Herpes virus of turkeys, Newcastle disease virus and Pox viruses. As such, HI titers developed by these viruses may be lower than those produced by killed antigen vaccines, especially against field viruses, making it difficult to assess the levels of protection likely to be afforded. Experimental studies have demonstrated broader cross protection from these vaccines against a range of viruses in some situations and this information should be used as a guide to likely levels of protection (Palya *et al.*, 2018; Niqeux *et al.*, 2023)

Furthermore, because vectored vaccines are based on vaccination with live viral material expressing the target protein of vaccinal importance these vaccines will stimulate cell mediated immune responses as well as humoral responses. However, methods for measuring cell mediated immunity against different vectors remain poorly defined. Where replicative restrictions to vectors in different avian species are identified, options exist to assess vaccination efficacy (e.g., using feather follicles to detect the HVT virus 3 weeks post vaccination (Palya *et al.*, 2018)). Such approaches may provide useful information on the need for revaccination with an alternative vaccine.

Methods harmonised between participating laboratories used for the OFFLU AIM project

Generation of chicken antisera

A standardised panel of H5Nx chicken antisera and homologous antigens were generated at IZSVe and APHA. Antisera were obtained by inoculation of specific pathogen-free (SPF) birds with emulsions of the antigens in water-in-oil adjuvant. The selection of viruses was based on genetic and molecular analyses aimed at identifying strains that are either representative of relevant circulating clades or genetically related to vaccine seed strains, hereinafter referred to as surrogate vaccine seed strains.

For each antiserum, a batch of the homologous virus was generated in SPF embryonated chicken eggs and the allantoic fluid was chemically inactivated with beta-propiolactone (BPL). Batches had a minimum antigen content equal to 128.000 HAU (e.g., 100 ml with a titer of 1:32). Batches underwent innocuity testing by three blind passages in SPF embryonated chicken eggs.

10 SPF chickens per antigen were inoculated via the intramuscular route with 1ml emulsions of BPL-inactivated avian influenza antigen plus Montanide or water-in-oil as an adjuvant (inoculation 1). (Virus/adjuvant [30/70%]).

At 14 days (+/- 3 days) post primary inoculation, birds were boosted (inoculation 2) with the same antigen preparation and procedure. Around 20 days after the primary inoculation, blood was collected from the birds via wing bleeds and used to assess antibody status against the inoculated antigen. If HI antibody titres were insufficient for use in assays (e.g. < 1:16 HAIUs/25µl) birds were boosted again (inoculation 3). Blood sampling, serological assessment and re-inoculation was repeated once more if necessary (inoculation 4).

At a maximum of day 36, all birds were euthanised and blood collected via heart bleeds under terminal anaesthesia. The serum was separated from red blood cells and heat treated at 56°C for 30 min prior to being lyophilized for storage and distribution. For each batch of serum and antigen generated, volumes were shared between WOAH and FAO reference laboratories IZSVe and APHA, ACDP and SEPRL.

Hemagglutination Inhibition (HI) assay

Antigenic properties of avian influenza A H5 viruses were characterised using established harmonised approaches to HI assays at APHA, IZSVe, ACDP and SEPRL using chicken mono-specific antisera. These HI assays were conducted as described in the WOAH Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (WOAH, 2023) with a modification in the steps involving the incubation of chicken red blood cells with antigens (incubation time of 1 hour at a temperature of 4°C). The antigen containing 4 hemagglutinating units was confirmed by back titration. In the HI assay, sera were diluted two-fold from a starting dilution of 1:2 and tested in duplicate. Positive antisera and negative control sera obtained from SPF chickens were also tested for each assay.

Sequencing of viral isolates

Each viral isolate tested in HI assay was sequenced and sequence data was compared with wild type sequence to ensure no egg or cell adaptive changes had taken place in antigenic sites within HA or NA during propagation. Whole genome sequencing was undertaken using Illumina sequencing. Sequencing libraries were run on a NextSeq 550 platform (illumina) with paired end reads. Raw sequencing reads were assembled using custom scripts FluSeqID or denovoAssembly.

Links to OFFLU AIM documents

OFFLU Avian influenza technical activity vaccination page

Concept note OFFLU AIM project - April 2022

OFFLU AIM presentation – February 2023

OFFLU AIM pilot report – October 2023

References

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