Epidemiology-driven approaches to surveillance in HPAI-vaccinated poultry flocks aiming to demonstrate freedom from circulating HPAIV

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A R T I C L E   I N F O

A B S T R A C T

Incursion pressure of high pathogenicity avian influenza viruses (HPAIV) by secondary spread among poultry holdings and/or from infected migratory wild bird populations increases worldwide. Vaccination as an additional layer of protection of poultry holdings using appropriately matched vaccines aims at reducing clinical sequelae of HPAIV infection, disrupting HPAIV transmission, curtailing economic losses and animal welfare problems and cutting exposure risks of zoonotic HPAIV at the avian-human interface. Products derived from HPAIV-vaccinated poultry should not impose any risk of virus spread or exposure. Vaccination can be carried out with zero-tolerance for infection in vaccinated herds and must then be flanked by appropriate surveillance which requires tailoring at several levels: (i) Controlling appropriate vaccination coverage and adequate population immunity in individual flocks and across vaccinated populations; (ii) assessing HPAI-infection trends in unvaccinated and vaccinated parts of the poultry population to provide early detection of new/re-emerged HPAIV outbreaks; and (iii) proving absence of HPAIV circulation in vaccinated flocks ideally by real time-monitoring. Surveillance strategies, i.e. selecting targets, tools and random sample sizes, must be accommodated to the specific epidemiologic and socio-economic background. Methodological approaches and practical examples from three countries or territories applying AI vaccination under different circumstances are reviewed here.

1. Introduction

Globally, an increasing incursion pressure into poultry holdings of highly pathogenic avian influenza viruses (HPAIV) of subtype H5 derived from the goose/Guangdong (gs/GD) lineage is observed. These viruses continue to evolve and spread globally since their first report in 1996 in southeastern China [1]. The most recent epizootic waves sweeping also across Africa, Europe and the Americas [2] challenged previous preventive measures mainly based on biosecurity and put vaccinations against HPAI in these areas into a new focus [3,4]. Use of vaccination against HPAI still is not generally permitted in Europe and North America but several epidemiological scenarios for its eventual application are being considered [5]: (i) Preventive vaccination of

poultry in an area free of HPAIV infections but in danger of incursions. This resembles the situation of the European Union (EU) since 2017. (ii) Emergency vaccination in areas previously free of HPAI but currently under pressure of massive HPAI outbreaks as currently experienced in several EU member states and in the USA. (iii) Vaccination targeting a reduction of circulating virus load in enzootic areas with limited veterinary infrastructure and where prospects of virus eradication within the next 5 years are low. (iv) Vaccination as part of an eradication program in enzootic countries with suitable conditions to achieve eradication. Accordingly, the preference could be to set into practice the concept of free compartments or zones (and surveillance for demonstration of disease freedom).

The ultimate goals of vaccination of poultry against HPAI comprise

(i) the prevention of clinical disease in vaccinated flocks. This will improve animal welfare since suffering through clinical signs induced after infection are abrogated. In addition, economical losses through increased mortality or otherwise impaired production will be reduced. (ii) the prevention of an infection of vaccinated birds following exposure to field HPAIV. Vaccinated flocks bearing a resilient immunity are less susceptible to infection and amplify less virus that is shed over a shorter period which aids in disrupting transmission chains of the virus. Such flocks can remain free from sustained infection, as it is expected that only a few animals are capable of virus replication and these have a low likelihood of onward transmission to other flocks. In contrast, inappropriate vaccination may induce a population status where clinical prevention is largely attained, but prevention of field virus infection and spread is not. In such flocks, healthy-looking vaccinated animals may nevertheless be infected with HPAIV and even though virus shedding by vaccinated infected animals is less pronounced with respect to amplitude (but not necessarily by duration) compared to unvaccinated animals, effective transmission, especially to unvaccinated poultry, cannot be excluded [6–9]. Vaccination alone will not eliminate viruses from places where they are entrenched [10,11].

While high-income countries have so far been coping with repeated HPAIV incursions and spread applying an early detection and stamping out strategy, recent developments of increasing virus incursion pressures starting in wild bird population and subsequently reaching the poultry industry rendered vaccination as a complementary tool to control virus spread into a much brighter focus.

One of the obstacles that have prevented a greater acceptability and wider use of vaccination against HPAI is related to surveillance of vaccinated poultry populations to confirm safety of vaccinated poultry and products thereof. The primary aims of surveillance in such context will be modulated according to the described epidemiological scenarios but will essentially cover the following: (i) *Monitoring vaccine coverage and flock immunity* seeks to ensure vaccine is applied appropriately and results in sufficient coverage and that vaccination induces protective immunity with respect to circulating viruses. (ii) *Early detection of new/re-emerged outbreaks* secures timely instigation of suitable, effective control measures when incidence of infections is low. This is expected during preventive and emergency vaccination campaigns or towards the end of (successful) vaccine-flanked eradication campaigns in enzootic regions. (iii) *Following the trends of incidence* is pivotal in enzootic regions which includes surveillance in agglomeration and distribution hubs of poultry such as live bird markets. As a final goal all surveillance activities will seek to (iv) *demonstrate freedom from HPAIV* infection which essentially comprises the exclusion of infection with HPAIV in vaccinated flocks with geographic (country, region or zone) and/or production-based restrictions (production sectors or compartments thereof).

Due to the very wide range of poultry production and rearing of birds by small holders, in hobby flocks and zoos, a number of stratifications and limitations need to be imposed: This review will essentially focus on industrial/commercial production of chickens (broilers and layers), fattening turkeys and Pekin ducks. This necessarily introduces a number of gaps regarding minor poultry species (quail, pheasants, ostriches, Muscovy and Moulard ducks) and zoo birds which are outside the scope. The impact of the age and production cycles of poultry, i.e. short-vs long-lived populations will be considered though. Impacts of trading traditions via live bird markets and movement control measures such as pre-marketing surveillance will be targeted in sections dealing with practical experience with surveillance in countries or territories previously or actively vaccinating poultry against HPAI.

While the main focus is on HPAI, low pathogenicity avian influenza viruses (LPAIV) cannot be disregarded since in many regions an (enzootic) LPAI background of H9N2 or other non-notifiable LPAIV would affect the use of diagnostic tools, sample sizes and frequency of sampling in perspective to each of the surveillance objectives.

2. Tools and strategies of surveillance

2.1. Technical tools

The most prominent goal of surveillance in AI-vaccinated flocks is to produce robust and reliable data proving the absence of AI virus circulation in those flocks, so as to confirm that no risks of virus spread emanates from such holdings. When dealing with HPAI, *clinical signs* can be used as a marker of infection especially in gallinaceous poultry and, to a lesser extent, due to their increased clinical resistance, in waterfowl [12,13]. However, as vaccination essentially seeks to protect poultry from developing disease upon virus exposure, clinical signs, in particularly mortality, will be curbed anyway, also in gallinaceous poultry species. Thus, the easy slogan "no disease, no virus" unfortunately does not apply. Thus, virus circulation cannot be excluded *ex ante* in healthy vaccinated flocks. Modulated by the efficacy of the vaccine used and the field strain(s) circulating, complete absence, partly inhibited or full-blown clinical signs may ensue upon field strain exposure [14,15]. In addition, more subtle parameters affecting production such as weight gain, egg production, reduced consumption of drinking water or feed, etc., may indicate incursions and virus circulation depending on vaccination status and poultry species [16]. Use of unvaccinated poultry left in an otherwise fully vaccinated flock has been proposed to act as clinically fully vulnerable sentinels which indicate virus incursions by developing full blown disease [17]. Yet, the sentinel system comes with an array of further challenges as detailed below.

Less ambiguous than clinical signs, *laboratory-based parameters* can be used to monitor vaccinated poultry flocks for circulating AI viruses. *Virological examination* of clinical samples enables a direct insight into virus circulation within the flock at the time point of sampling. The most sensitive and, from many angles of view, most versatile tool in this respect are real time RT-PCRs (RT-qPCR) which can be tailored to specific needs, e.g. generic as well as sub- and pathotype-specific detection of AI viruses [18]. Classical methods of virus detection such as virus isolation in embryonated chicken eggs provide the largest possible diagnostic width but require significantly more time than RT-qPCR until results are available.

Serological investigations seek to detect and, ideally, distinguish (in case a DIVA [Differentiating Infected from Vaccinated Animals] vaccine is used) humoral immune responses against AI viruses generated either due to vaccination (using live vector, subunit, or DNA vaccines) or following a productive field virus infection [19]. Essentially, these assays provide retrospective insights into the herd immune status as approximately 2–4 weeks must be expected until measurable, stable antibody titers will have mounted. For some vaccine types (e.g. HVT vector vaccines) and poultry species (e.g., turkey) induction of protection might forerun the mounting of measurable antibody titers [20]. By far the most commonly used matrix for antibody detection is serum but the use of yolk, in laying flocks, might be considered for animal welfare reasons or ease and costs of collection. Working with yolk instead of blood in the laboratory however is much more laborious, hard to automate, results in much more waste and the antibodies have to be extracted from the yolk to be able to use low dilutions as needed for the HI-test. Antibodies also appear with a delay in the yolk compared to serum [21–23] as IgM is not transferred to the yolk.

An array of assays is available ranging from ELISA formats appropriate for high throughput screening to more labour-intensive but highly specific hemagglutination inhibition (HI) and neutralization assays. Blocking and competitive ELISAs, generic or subtype-specific, are species-independent and can be used for various species of hosts. Indirect ELISAs are species dependent and can therefore only be used for specific host species as determined by the conjugate that is being used [24]. Agar gel immunoprecipitation assays can be used for gallinaceous poultry where resources are limited.

Essentially, there is consensus that virological surveillance performed with optimized RT-qPCRs has the highest sensitivity to detect

virus presence in vaccinated flocks [25].

2.2. Surveillance approaches

In general, surveillance can be differentiated as passive and active surveillance which could be used in combinations to serve different surveillance objectives [26]. *Passive surveillance* relies on farmers/veterinarians/traders etc. to report/notify suspicions or to submit samples from these birds. The strength of passive surveillance is that it is more or less continuously in place, the weakness is that its quality can vary widely between farms. Passive surveillance is in particular useful if there is a clearly visible manifestation of infection, e.g. after an incursion of HPAIV in an unvaccinated flock of gallinaceous poultry. For that reason, passive surveillance is less effective in a vaccinated population in comparison to an unvaccinated population. Should infection occur despite vaccination, no, or less clear, clinical signs are expected due to the protective immunity of vaccination. This effect is related to an increased probability of vaccine failure in these cases which is modulated by the combined effects of using an imperfect vaccine (not the best possible match between vaccine and field virus antigens; efficacy) and the inadequate use of such vaccine (vaccination coverage). Still, even in a vaccinated population it is important to include passive surveillance as a surveillance component, because it may help detect vaccine failure due to inadequate administration or emergence of virus strains that escape vaccine induced immunity [27–29]. Passive surveillance can be enhanced by awareness campaigns and compulsory requirements to notify changes in mortality, that could be focused on specific periods, regions or farm types. A DIVA strategy using sentinel birds could also be adopted involving passive surveillance of clinical signs in the sentinel birds. A suspicion from passive surveillance needs to be followed up by collecting appropriate samples for virus detection (see below).

In *active surveillance*, inspection, sampling and testing is prescribed by the protocol of the surveillance programme. Consequently, compared to passive surveillance, the sampling frame is transparent and structured regarding the farms to be included, type and number of samples to be collected and the sampling frequency and the tests to be applied. A disadvantage is that it cannot be applied on a daily basis like passive surveillance. This makes active surveillance most effective for detecting infections in the absence of a clear clinical manifestation and demonstrating freedom from infection, in contrast to passive surveillance that is most effective when there is a clear clinical manifestation of infection. For detecting infection, active surveillance can use continuous collection and analysis of production data (e.g. egg production) that, in case of a suspect signal, should be followed up by specific diagnostic attempts. Active testing for virus in apparently healthy-looking birds is considered not very effective. Nevertheless, collecting dead birds for a pooled (e.g., weekly) testing, the so-called bucket sampling, could be useful to detect infection in flocks in the absence of clear clinical manifestation and in the case of low prevalence in infected flocks resulting in unalarming mortality (EFSA opinion 2017 and [30]. In DIVA-vaccinated poultry populations, active serological surveillance can establish retrospectively whether silent virus spread has occurred in vaccinated flocks at risk for infection. Moreover, serological surveillance in long-lived poultry is useful to demonstrate freedom from infection in a region, zone or compartment as it demonstrates absence of infection in the preceding period.

3. Targets of surveillance

3.1. Verification of vaccine coverage

Vaccination governance is essential as vaccination against HPAI alone has never been successful in eliminating HPAI. Biosecurity, continuous evaluation of vaccination uptake and efficacy, proper surveillance of vaccinated flocks for the freedom or presence of field infections and typing of detected field strains are all required as well,

demanding an active role by the authorities. To be able to perform these tasks, it is required that all holdings that actively vaccinate or keep vaccinated poultry are known (i.e. registered) to the authorities, including location, inclusion in zones or compartments, number and age of birds present at any time, dates of movements and the vaccines and vaccination schemes that have been applied. Based on these data, an epidemiologically relevant surveillance programme comprising, where possible, all vaccinated flocks can then be scheduled.

The same data allow monitoring the vaccination uptake, i.e. the percentage of birds that has truly received the whole dose of vaccine; the uniformity of vaccine-specific serological responses within a flock (i.e. a small coefficient of variation of antibody titers) would indicate achievement of this goal. Generic serological investigations employing blocking ELISAs specific for the NP protein are the appropriate tool if inactivated whole virus vaccines are used and endemic LPAIV infections are excluded from flocks. Serosurveys using HA-subtype-specific ELISAs or HI assays with vaccinal antigens instead would be applicable in case of DIVA vaccines with NP as a negative marker. Positive markers in DIVA vaccines would require specific ELISA tools tailored to the respective marker. However, the concept of positive markers has many disadvantages and is not used in any of the available HPAI DIVA vaccines to date. In poultry populations vaccinated against HPAI in areas with endemic LPAIV such as H9N2 or H6N1, serosurveillance using DIVA serological tests is severely hampered. This is the same for flocks that are vaccinated against non-HPAI strains using inactivated vaccines with whole virus antigens. The LPAIV infections and/or LPAI vaccinations will induce antibodies to the NP protein, resulting in positive serology in the DIVA test. The HI test using the LPAIV antigen will become positive as well but this can't reliably be used to explain the positive serology in the DIVA test as the flock might have suffered from both infections. For HPAI vaccinated flocks in areas with endemic LPAIV, therefore, routine screening by subtype-specific PCR for the HPAIV subtype is the most appropriate method.

3.2. Assessment of vaccine-induced protection

As mentioned above, the efficacy of protection of a HPAI vaccine depends on several factors of which vaccination coverage (2.1) is only one. The antigenic match between the field virus(es) and the vaccine strain(s) determine efficacy to a great deal. The greater the homology of the hemagglutinin of these antigens the more efficacious are the induced antibodies not only in blocking infection (and consequently development of clinical disease), but also in decreasing field virus shedding and transmission within vaccinated flocks [31]. Therefore, in addition to serosurveillance, assessing vaccine coverage (2.1) by complementing HI assays that employ the field virus antigen are essential to validate efficacy. Correlating antibody titer as measured by hemagglutination inhibition (HI) assay with protection is inconclusive can even be contradictory [32]. In general, however, HI titers of $\geq$1:32 against the homologous antigen are interpreted to signal robust clinical resistance but a threshold of >1:8 or 1:16 have been proposed as well [33,34]. Yet, correlating thresholds of HA antigen-specific antibody titers with protection has always been difficult since factors related to species, age, vaccine-type, dynamics of individual titers vs population immunity may interfere with judgement, and choosing the most fitting sample size is tricky [35]: Very often sample size aims at detection at a designed prevalence (e.g., 30 samples from a flock can detect a 10% prevalence with 95% confidence). However, actually an estimate of a prevalence with a specified precision is required here. For example, the aim of vaccination could be to have 80% of the birds at titers leveling or even exceeding the antibody threshold. Collecting sera of 30 birds from a 40k flock and observing 80% positives at this level would merely mean a 95% confidence that the true prevalence will be between 65% and 95. If a higher precision is required to ensure the lower limit is above 80%, sample size will need to increase significantly. In practice, trade-offs between what is feasible to collect and what is ideal have to be found.

In case of doubts about levels, retesting or revaccinating may be further options.

Since the definition of thresholds remains precarious it can be necessary to start such efficacy assessments empirically and subsequently establish baselines in different flocks/holdings/integrations for different vaccine-types and poultry species. Gathering experience over time and developing an understanding for the protection level has proven beneficial e.g. in the Indonesian vaccination programmes (5.1). The final proof to assess efficacy of a specific vaccine against a specific virus would of course be to conduct vaccination-challenge experiments in the target group(s) of poultry (ideally not using SPF poultry but directly derived from the field). As this is clearly not feasible in the field, in particular when HPAIV is encountered, centralized institutions should perform such experiments before licensing vaccines for general use. Emergence or incursion of antigenically altered virus variants in a region with vaccinated flocks would require repetition of these experiments with the altered strain(s).

3.3. Early detection of new outbreaks in vaccinated flocks

Early detection here is defined as surveillance of health indicators and HPAIV infection in defined population(s) to increase the likelihood of timely detection of new or re-emerged outbreaks before significant spread within the flock occurs and definitely avoiding spread between flocks so as to keep the reproduction number R_o between farms below 1 [26,36].

Passive surveillance is mainly based on the daily (clinical) monitoring, by the farmer, of clinical signs in a flock and reporting of aberrant observations. Acute mortality, apathy, respiratory problems, reduced food or water intake, swollen head and wattles, and drops in egg production have been frequently reported in affected poultry, including backyards [37–39]. The farmer's awareness of these clinical signs is the basis for reporting suspicions. These clinical signs are clearly observed in unvaccinated gallinaceous poultry whilst mild (subclinical) disease, with less clear clinical signs, is usually observed in unvaccinated waterfowl or vaccinated gallinaceous poultry. However, as stated before, it is still important to include passive surveillance as a surveillance component in vaccinated populations, because it may help detect vaccine failure due to inadequate administration or emergence of virus strains that escape vaccine induced immunity. To enhance the efficacy of passive surveillance in industrialized farms, reporting thresholds – based on the conditions of the production sector in a specific country or region – could be determined and used as guidance for farmers to detect and report suspicions of HPAIV infections (Gonzales and Elbers 2019, [16,40–42]. These thresholds have been so far determined for unvaccinated populations in certain areas and with certain prevalences of co-infections only. Similar studies would be desirable, to establish clinical indicators of infection in vaccinated flocks. In these flocks, mild disease could be expected, hypothetically similar to that caused by LPAI infections in chickens. In such scenario, consistent monitoring of production parameters has also been shown to be useful for detection of infection (Gonzales and Elbers 2018, Beltran-Alcrudo et al. 2009, [42]. When unvaccinated sentinels are used to detect infection in vaccinated flocks, monitoring of a wider range of clinical signs in sentinel birds may prove to be more effective than using flock level detection thresholds. Key for the use of sentinels is their location in the barn. Empirical observations in Indonesia (5.1) indicate that locating the sentinels close to the barn's air outlet may increase the chance of the sentinels becoming infected and thereby detecting infection. Further practical details on the use of sentinels for surveillance are gathered in the section describing the practical experience in Hong Kong (5.2). Finally, it should be noted that detection of clinical signs is not a specific indicator of HPAIV infections, and any report needs to be followed up with laboratory confirmation.

Active surveillance strategies for detection of infected vaccinated flocks may be based on sampling and detection of virus genome. This will allow detection of actively infected flocks and its efficacy will mainly depend on the infection dynamics in the flock (prevalence of infected and dead birds in time), the number of samples taken and the frequency of sampling (sampling interval). If sampling is too early or towards the end of the outbreak, when the prevalence is low, the chance of detection is likely to be low, unless large sample sizes are taken or flocks are re-sampled at a predefined frequency.

Sampling strategies could vary from random selection of birds to be sampled to targeted testing of dead and apparently sick birds which are more likely to have been infected and therefore improve the likelihood of detection. So-called bucket sampling strategies where pools of samples from dead (normal mortality + HPAI disease mortality) and/or sick birds are tested in a predefined frequency could be effective [43] and cheaper than random sampling and testing of individual birds. With some knowledge of the expected transmission dynamics of HPAIV in vaccinated flocks [44–46] and diagnostic test performance, simulation models can be used to determine the best sampling frequency [47,48]. When using unvaccinated sentinels in a flock, it is advisable to test sick and dead sentinels as soon as possible.

In addition to testing sick and dead birds, in scenarios of subclinical infection with unnoticed (disease induced) mortality, environmental samples could be a less invasive, effective and more economical alternative (Hood et al. 2019). Dust samples – in the form of swabs or boot socks taken from floors, walls, feeder troughs, nests, cages, or fans as well as air, water and drinker biofilms have been used to sample commercial farms [49,50]; Lopes et al. 2019) and live bird markets (LBM) [51] (see also section on Indonesia 5.1). The non-invasive nature of these samples for testing allows sampling with higher frequency and sample size (to ensure good area coverage of the barn) improving thereby the sensitivity for early detection [49]. It is unclear to date whether such sampling has been actually conducted in practice in vaccinated herds.

Molecular characterization of virus variants detected in a vaccinated population is essential to exclude or confirm the emergence of mutants that gained in fitness because of amino acid substitutions in those critical epitopes that induce neutralizing antibodies. T cell epitopes of course could be affected as well but seem to reside largely outside the HA and NA proteins and therefore might reveal a higher degree of conservation [52]. The risk of emergence of these escape mutants is high in populations with patchy or otherwise incomplete immunity. Apart from being generated *in situ*, such variants or new viruses representing antigenically disparate clades can be introduced by infected wild birds or by uncontrolled trading with endemic areas where no vaccination is practiced.

4. Follow the trends of infection in infected regions/vaccinated areas and in places at risk of incursion

When HPAI vaccination is applied in an infected region, surveillance could be applied to follow the trends of infection in such regions or in parts of the population that may be at increased risk of incursions. In the absence of a clear objective towards testing for demonstration of freedom from infection, low cost active monitoring could be considered in addition to passive surveillance of outbreaks. This could include, for example, constant periodic environmental surveillance in LBM [53] or in Europe/US slaughter plants only receiving vaccinated birds if/once vaccination is allowed. Although this does not give precise data on the infection level in a region, it will provide a trend over time and will also demonstrate the virus evolution over time. In case a region or a compartment is aiming to move towards disease freedom, a more accurate view on the course of the number of infected farms is needed. Serosurveys using a DIVA test are most suitable for that goal in case no interfering LPAIV infections occur. A downward trend in the (sero) prevalence of infected flocks would indicate that vaccination is accompanied by reduced virus spread and thus effective. This has been described for the Indonesian situation (5.1) where lately the

environmental prevalence of H5N1 in LBMs has been declined year by year, while H9N2 becomes the more dominant AI virus. An upward trend would indicate the opposite and would require adjustment of the vaccination programme, which could include updating the vaccine (better match with circulating field virus), improved vaccination scheme, better vaccination surveillance or combinations thereof. To follow a trend, collection of the serum samples could best be stratified according to the poultry sectors present in the region/zone/compartment. Depending on the regional situation and type of poultry, sampling could also take place at the slaughterhouse or at time points used for other checks such as the check of the *Mycoplasma gallisepticum* status. Serosurveys in a region or zone can best be done according to a two-stage sampling design, where first farms are selected and next a sample of birds within those farms is tested [54] (Box 1). When serosurveys are done at farm level, seropositive results could be followed up with virological testing using either samples taken from selected birds or environmental samples (see virosurveillance section). This is particularly useful in the final phase of vaccination programmes, when the prevalence is already low.

Serosurveillance is favoured in specific epidemiological situations and regions where other options, e.g. mass testing via PCR, are not feasible. When applying serological tools, the species of poultry needs to be considered in order to select the correct assays (blocking and competitive versus species-specific ELISAs) and/or to apply the correct pretreatments, e.g. for use in HI assays. In addition, the likelihood of co-presence of other endemic LPAIVs needs to be considered. Serological DIVA assays usually target antibodies to the nucleoprotein (NP) of AI viruses. The NP protein does not harbor epitopes that are relevant for virus neutralization, and NP-specific antibodies do not induce protection. Therefore, the majority of currently available DIVA vaccine products disposes of the NP protein which, consequently, can be used as negative marker in DIVA concepts. Flocks vaccinated with NP-deficient vaccine will not mount NP-specific antibodies. NP-specific antibodies detected in such flocks, therefore, must have been induced by an incursion and spread of field AI viruses. Since NP protein is a major immunogenic viral protein, there is a high likelihood, at least in naïve poultry flocks, to mount a measurable response detectable by commercial ELISA. Although some DIVA vaccines have been used abundantly in the field (e.g. in Egypt or Bangladesh), no reports on the efficacy of NP-specific ELISAs as a tool have been published yet. The usefulness of this approach is of course compromised when simultaneously other LPAIV circulate endemically. Since large areas of Asia, the Middle East and north Africa are currently plagued by entrenched H9N2 infections, any H9N2 infection in HPAIV-vaccinated poultry will induce a positive result in the NP ELISA or by AGPT which, in the context of DIVA, would be interpreted as failure of vaccination/infection. Other negative markers such as the NS-1 and M protein or disparate neuraminidase proteins have been validated as well with variable success, mainly due to incomplete seroconversion of field virus-infected poultry or insensitivity of available assays. Thus, no commercial solutions except for the NP DIVA system are available to date. Examples from the practice of serological DIVA testing with sentinel systems are listed in section 5.2.

In addition to monitoring trends of the presence of HPAIV, it is required to regularly perform whole genome sequencing (WGS) of viruses or at least the HA genome segment of viruses detected in vaccinated flocks to keep track of virus evolution. In combination with antigenic cartography this would inform vaccine optimization needs and prevents the vaccination programme becoming ineffective due to the emergence of escape mutants.

5. Demonstrate freedom from HPAIV in vaccinated flocks

This is a most difficult task and the choice of surveillance tools and strategies will largely depend on the epidemiological situation. If it is confirmed by monitoring of vaccination coverage and protection (2.1, 2.2) that vaccination is effective, incursions of HPAI virus are expected to result in minor outbreaks affecting a few birds per flock only [8]. It is unlikely that these low numbers will be detected by RT-qPCR surveillance given the routine random sample size which is used and affordable (an example of needed sample size is shown in box 2). Consequently, negative outcomes of surveillance based on clinical manifestation or reduced production is insufficient to confirm absence of field virus in

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Box 1
Estimating prevalence

To follow a trend of infection in a region or a compartment the percentage of HPAIV infected farms needs to be estimated over time. A proportion of farms in the region/zone/compartment needs to be selected and within each farm a proportion of the birds is tested for antibodies against field virus. Web-based tools are available to help calculate the numbers farms to be selected and the number of birds within a farm to be sampled (e.g. Epitools https://epitools.ausvet.com.au/prevalencess). To calculate the number of farms to be tested in a compartment of the selected region the following information is required:

- *a priori* estimate of the percentage of infected farms in the region/compartment,
- the desired precision of the final result,
- the desired level of confidence.

The *a priori* percentage estimate of infected farms could follow from a previous testing round, but if this is not available it is advised to assume 50% as it will result in the highest number of farms to be tested and thus does work also for all other prevalences. If a 95% confidence is desired and a 10% precision, countrywise that would mean 97 farms were to be tested. Should 50% of those farms be infected as an outcome, the conclusion is that there is 95% confidence that the true prevalence of infected farms would be between 40% and 60%. This calculation assumes an infinite number of farms in a region and a herd sensitivity and herd specificity of 100% each for the diagnostic test used. Available knowledge of these items can then be included in further calculations.

For stage 2 (selecting the number of birds to be tested within a farm) a design prevalence has to be agreed on beforehand, that is the prevalence that should be detected with a specified confidence (e.g. 95%). The chosen confidence level is synonymous with the herd sensitivity (while the herd specificity is the probability that an AI free farm will be designated as such). Very often an arbitrary low prevalence is assumed, but one could also assume a seroprevalence that would normally be obtained should the virus continue to spread within a farm. Assuming that in case of infection the seroprevalence would mount to 10% at least, this would imply that we would need collect 30 samples from birds randomly distributed across the farm. Also here, extra complexity can be brought in by taking account of the number of houses on the farm and the quality of the test to be applied.

Box 2
Testing for freedom from infection

Designing a sampling frame for demonstrating freedom from infection can make use of the Epitools software as well (section 3). However, in this case the minimal proportion of infected farms expected to be present in a region or a compartment, should AI be present, needs to be agreed upon as the design prevalence, in addition to a desired confidence level. As an example, it is assumed that in an infected region/compartment the prevalence of infected farms would be at least 1% and the desired confidence level is 95%: This would require testing of 299 farms. In case none of these farms would turn out positive, it would be 95% certain that the prevalence of infected farms is below 1%. The procedure to deciding the numbers of animals to be tested in a farm is the same as in box 1 and similarly, extra complexity can be brought in including numbers of farms in a region, population sizes, test qualities etc.

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such situations.

Serology does not have these generic limitations as the serological profile of a flock reflects the cumulative incidence of infection at sampling. In vaccinated poultry populations in areas with no enzootic LPAIV such as H9N2 or H6N1, serosurveillance using DIVA tests is appropriate to demonstrate freedom from HPAIV. To enhance surveillance efficacy, risk-based surveillance can be used, including an overrepresentation of poultry farms more at risk for infection (e.g. free-range poultry) [47]. Slaughterhouse sampling can be used to reduce the costs associated with sample collection. The latter obviously requires a good registration for linking of the slaughtered bird products to a farm.

In addition to a serum sampling frame, a strategy for dealing with positive serological findings needs to be in place as the specificity of the test used is lower than 100% and, consequently, the high number of samples to be tested will certainly result in some false positives. Seropositives, however, will prompt immediate and severe restrictions including prevention of trade in products from that farm and recalling all eggs (if it were a layer farm) delivered since the last negative test. Therefore, DIVA-directed serosurveillance essentially requires a confirmatory serological DIVA test and a protocol for additional sampling for virological testing if the result of the confirmatory test is also positive. In case of confirmed positives, the situation would need to be examined on a case by case basis. If the proportion of positives on a farm is high, it is likely that massive virus spread (HPAIV or an unrelated LPAIV) has occurred in the farm. Virological testing will be performed, but might turn out negative because the outbreak has already stopped. In that case the farm itself is not an acute risk anymore, however it is advisable to test farms that have been exposed either in the neighbourhood or by contacts to trace ongoing spread between farms. In case one or a few single serological reactors are confirmed in a farm, the farm should be revisited for virological and serological testing to identify possible ongoing within farm transmission. If the latter is not found, the farms

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Box 3
Decision tree on using surveillance tools (blue) to test for presence of HPAIV in vaccinated flocks.
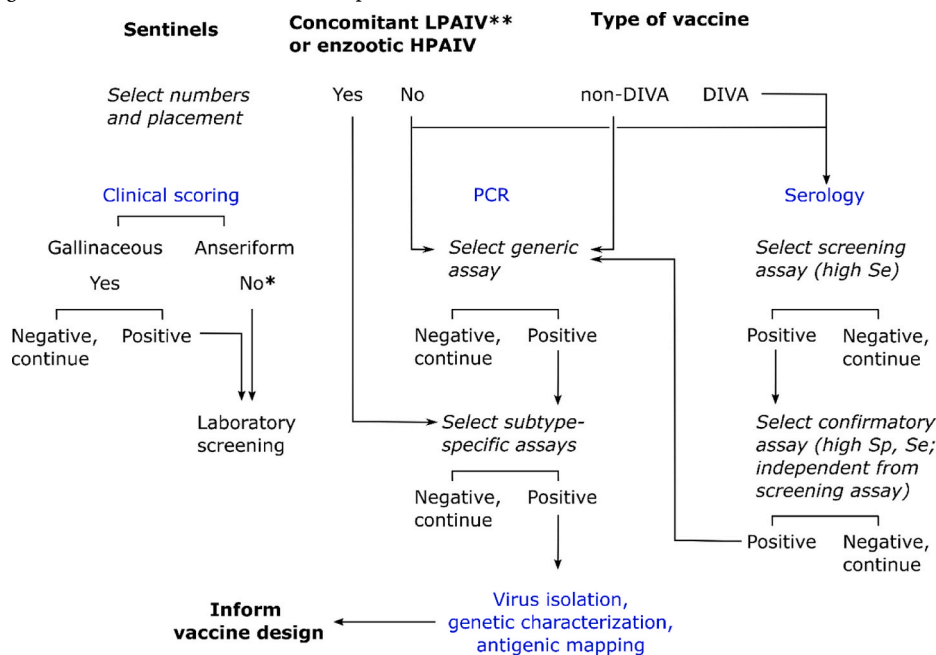
* Susceptible anseriform poultry may also show clinical signs and increased mortality depending on the HPAIV strain. For example, HPAIV H5N8, clade 2.3.4.4b, has been noticed for its high virulence in ducks [55].

** Samples are expected to come not only from birds but also from the environment. The use of environmental samples may of interest particularly for regions with concomitant LPAIV circulation.

can be considered free from infection. Finally, to substantiate freedom from infection, when perfect specificity is assumed (all seropositive results are followed up until definitive virological confirmation) all sampled flocks need to be confirmed as non-infected. Alternatively, environmental samples, particularly in areas with enzootic LPAI infections, could be tested for AIV-specific RNA. PCR-positive results, however, do not necessarily indicate the presence of infectious virus in that environment or in the flock which is housed in this environment as the decay of viral infectivity is faster than that of viral RNA. Therefore, positive results will need to be followed up by sampling individual birds to confirm presence of infectious virus as explained above. It remains to be clarified whether (and what kind of) environmental samples can complement surveillance in order to reduce sample size.

A summary of diagnostic tools applied for surveillance and the steps to follow when selecting which tools to use up to confirmation of either active virus infection or freedom in a vaccinated flock is provided in box 3.

6. Practical experience with surveillance of vaccinated flocks

In sections 1-4 the theoretical aspects and backgrounds of the objectives, tools and options to design and target optimized surveillance programmes in HPAI-vaccinated flocks have been reviewed. The putative complexity of such programmes is substantial. Implementing such programmes in practice may pose unexpected obstacles and calls for flexible responses according not only to the particular epidemiological situation but also taking into account specific cultural traditions of poultry rearing and trading as well as the overall economic status of a country. Section 5 reflects on experiences from the practice of vaccination and surveillance in Indonesia, Hong Kong Special Administrative Region of China, and Italy. Vaccination in Indonesia has been implemented in the frame of enzootic HPAIV infections for almost two decades; Hong Kong is practicing, in a geographically comparatively small area, continuous preventive blanket vaccination and Italy has used emergency vaccination, limited in time and space, to combat severe secondary infections in a densely populated poultry area (DPPA), following sporadic HPAIV incursions.

6.1. Indonesia

Avian Influenza H5N1 virus of clade 2.1. of the Gs/Gd/1/96 lineage had been detected in backyard poultry as early as mid 2003 in Central Java/Indonesia [56]. Very soon it became apparent that the HPAI problem extended beyond the backyard poultry sector. Since the start of the epizootic in 2003, millions of poultry have died due to HPAI or depopulation during control activities. To the end of 2008, 31 out of 33 provinces were affected and the livelihoods of people dependent on the poultry industry have been threatened.

It was not until 2004 that vaccination campaigns started. Both local and imported vaccines have been introduced and distributed through animal health companies to sector 1-3 farmers (1 – industrial vertical integrations of poultry production, 2 - medium, and 3 -small scale commercial poultry enterprises), while government procurement focused on mass vaccination in sector 4 (backyard poultry) through provincial and district governments but has been stopped following critical reviews in 2008. Vaccination (and surveillance) in industrial and commercial sectors was not financially supported by the government. In 2004, at least 12 commercial vaccines were licensed, the majority of
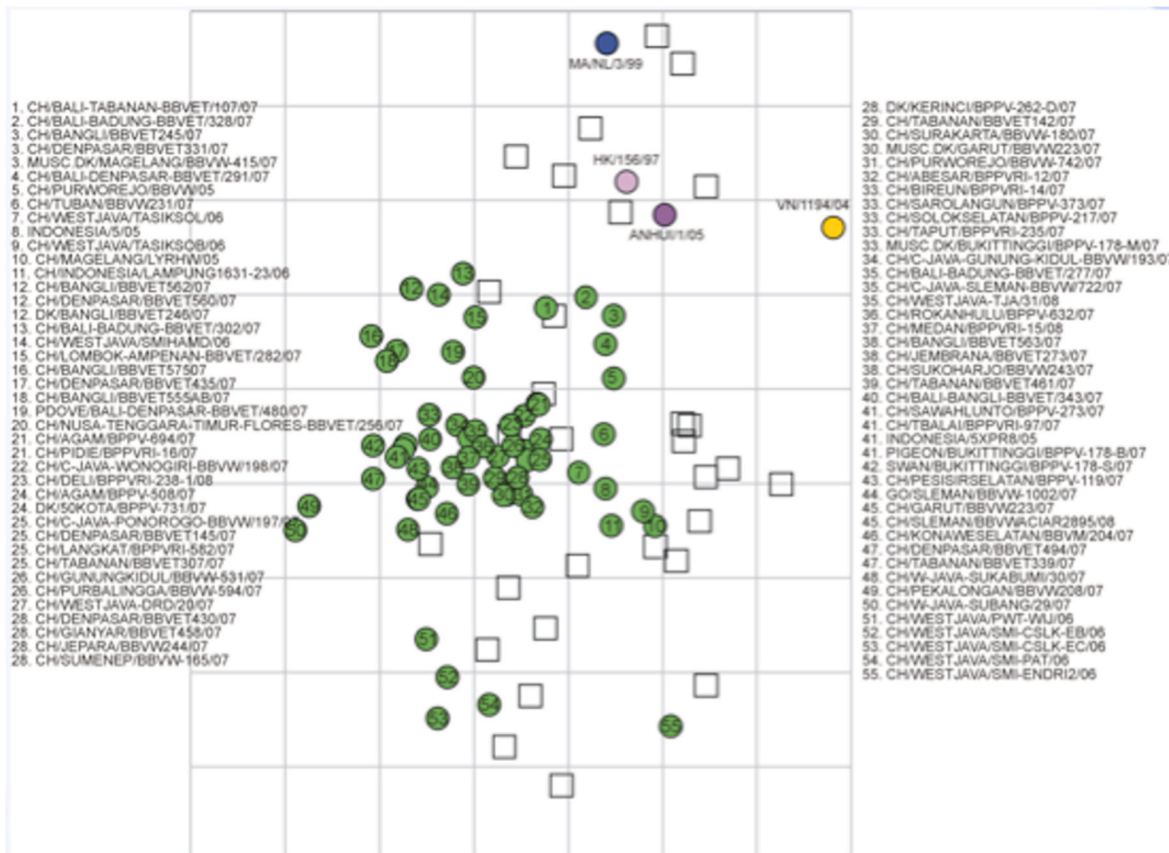

Fig. 1. Antigenic distances between classical heterologous vaccines and Indonesian H5N1 AIV isolates (2003–2009, map: courtesy of Japfa Comfeed Indonesia and Erasmus MC: Ron Fouchier, Stefan van Vliet, I Wayan Wisaksana Yasa, and Teguh Prajitno). The outlier viruses at the bottom of the antigenic map were derived from heavily but inappropriately vaccinated long-lived poultry, such as breeder and layer chickens where escape mutants start to circulate. For complete vaccination coverage, a bivalent vaccine, consisting of isolates from two clusters is required.

them based on highly heterologous H5 vaccine viruses. Soon it was realized that these vaccines and the vaccination schemes might be suboptimal as variant strains of HPAIV emerged, particularly in long-lived poultry (breeders, layers) in West Java, that were capable of vaccine evasion (Fig. 1).

Guided by FAO, a National Strategic Work Plan for the "Progressive Control of Highly Pathogenic Avian Influenza in Animals" was developed in 2006 in Indonesia to put in place an emergency structure and a systematic program that addressed the control of the AI epizootic in animals by monitoring AI variants in Indonesian poultry and defining an effective and sustainable vaccination strategy in 2007 [57]. In this frame it was concluded that vaccination of backyard poultry had little beneficial effects and future actions should be concentrated on sectors 1-3 [58]. In an endemic situation as faced in Indonesia the primary goal of vaccination is to enhance the immunity threshold of the poultry population and the suppression of virus shedding and circulation. DIVA vaccination schemes and use of unvaccinated sentinel birds was deemed impractical and ineffective. Yet, the possibility of an emergence of antigenic variants posed a constant threat: The OFFLU project on "Monitoring AI virus variants in Indonesia Poultry and defining an effective and sustainable vaccination strategy", launched in 2007, addressed concerns on vaccine efficacy, as HPAI outbreaks in poultry sectors 1-3 increased and none of the vaccines registered in Indonesia was found effective against some outlier strains such as A/Ck/PWT-WIJ/2006 [35]. It was understood that key to define an effective vaccine was not vaccine potency, but the antigenic relatedness of the vaccine virus to the circulating virus. A network of national and international collaboration achieved retrieval of circulating viruses, their genetic characterization, antigenic cartography, and finally efficacy testing in animal experiments. Rapid detection of antigenic variants and accurate measuring the antigenic distance to the vaccine was found to be essential for identifying efficacious vaccines, that can counteract virus evasion from vaccine immune response [59].

The antigenic map as shown in Fig. 1 depicts three clusters of AI virus variants, that have evolved between 2003 and 2007. A large proportion of circulating viruses still resides in the centre of the map, showing, however, already three antigenic units' distance from the classical heterologous vaccines used. In 2008, variant strains predicted to evade vaccine protection were isolated in West Java from industrialized, large-scale production of commercial chicken breeders and layers which had been heavily immunised. Accordingly, Indonesian authorities decreed that the identified virus variants of 2007/2008 were to be used to develop new vaccine seed, and since 2011 only AI vaccines, which incorporated the official vaccine seeds have been licensed for use in Indonesia.

In line with these decisions, the Government of Indonesia, with the assistance of FAO/OFFLU, has developed an innovative surveillance network ("Influenza Virus Monitoring", IVM) involving veterinary diagnostic laboratories, both private and public, for antigenic and genetic characterization of HPAIV H5N1 and web-based presentation of results thereof: In sector 1 (large-scale industrialized and vertically integrated farms including slaughterhouses), serological and PCR monitoring data of flocks were obtained by the industry on a regular base to obtain the self-declared freedom from HPAI in a confined compartment (compartmentalization; [60]. Beyond the use of vaccination, the focus of compartmentalization is on implementation of biosecurity, movement control, surveillance, early warning system, risk analysis and contingency and emergency plans in case of an outbreak. All activities within free compartments must be traceable in a transparent manner, and management and production systems are constantly inspected, controlled and verified through internal or government inspections. Certified AI-free compartments are allowed to export from their establishments even if they are located in an enzootic region. The AI-free compartment status is granted for 1 year and is subject to continuous review and inspections.

In sector 2-3, government-controlled monitoring programmes were conducted in commercial poultry sent to live bird markets (LBM; retail) and collector yards (CY; wholesale). The final leg of poultry production in Indonesia is the distribution of birds from farms to centralized LBMs in urban areas. LBMs provide live poultry that is mostly slaughtered at the markets and sold to the consumer. Approximately 70–80% of poultry meat in Indonesia is distributed this way. Surveillance activities implemented in LBMs and CYs revealed a much higher prevalence of HPAIV H5N1 compared to poultry producing establishments, indicating that HPAIVs must spread extensively during the trading process [51,61]. Lack of compensation in case of a HPAI H5N1 outbreak, forces many small holders to cull their infected poultry into CYs and will inevitably also contaminate transport vehicles and fomites. The connectivity of LBMs and CYs as a factor leading to an enzootic status of HPAIV H5N1 in the poultry marketing chain cannot be denied. Biosecurity interventions alone are unlikely to counter the effect of a constant virus influx from farms. Consequently, a PCR-based LBM surveillance program has been developed and implemented since 2014. Environmental samples are in focus of this programme. In 2015, LBM environmental sampling revealed that clade 2.3.2.1c introduced in 2012, became the dominant H5N1 strain in Indonesia and has completely replaced clade 2.1.3.2. a. Lately the environmental prevalence of HPAIV H5N1 in LBMs has been declining year by year, while LPAIV H9N2 became the dominant AI virus in Indonesia.

Vaccination campaigns in sector 4 poultry have been abandoned in Indonesia.

Along the development of the vaccination and surveillance campaigns in Indonesia decentralization has been recognized as the most important challenge. In Indonesia, the autonomous district governments are mainly responsible for controlling HPAI, based on national guidelines. Thus, the implementation of surveillance, biosecurity and other control measures, as well as technical understanding, skilled resources and funding did vary considerably at the district and provincial levels. Consequently, it was and still is difficult to systematically evaluate the efficacy of vaccination on a nationwide scale. To date, Indonesia has still considered itself enzootic for HPAIV H5N1. However, the majority of the Indonesian integrated poultry industry has managed to become H5N1-free by implementing the AI-free vaccination, compartmentalization and zoning program in long-lived birds, i.e. breeders and layers, including hatchery and slaughtering facilities. Short-lived broilers cannot be protected by vaccination. The incidence of clade 2.3.2.1c has declined from 2016 to 2021 by implementing vaccination using matching vaccines and stringent biosecurity in the commercial poultry sectors. Initial programs such as vaccination of backyard poultry, restructuring of the poultry industry and modernizing the poultry marketing chain, have not been sustained and thus did not contribute to the control of HPAI H5N1 in Indonesia. As a most important lesson learned, vaccination intervention programmes must follow virus evolution closely [62].

6.2. Hong Kong Special Administration Region, China

Currently there are 29 licensed chicken farms in Hong Kong, with the holding capacity varying from 10,000 to 162,300 chickens and the total maximum holding capacity in all chicken farms as approximately 1.3 million chickens. Since the first H5 HPAI outbreak hit Hong Kong in 1997, the Government has implemented a series of preventive and control measures, with a view to reducing the risk of AI at all levels of the live poultry supply chain [63]. Sporadic H5 HPAI outbreaks have occurred at the farm level since 1997 until the compulsory vaccination campaign was fully implemented in 2003. Since then, only one H5 HPAI outbreak was reported in 2008, which was also the latest outbreak at the farm level. Culling of the live chickens (summing up to a total of approximately 3,500,000 heads of poultry) on farm had been conducted as the major control measure for these outbreaks.

In view of the ongoing AI threats on public health and animal health, the Agriculture, Fisheries and Conservation Department (AFCD) of the

Hong Kong government had implemented compulsory preventive H5 AI vaccination campaigns for all local chicken farms starting from 2003 after a 12-month trial programme commenced in 2002. The field trials and laboratory challenge studies indicated that vaccinated birds were protected against H5N1 HPAI virus challenge and shed significantly less H5N1 virus; and vaccination was able to control virus shedding in flocks during field outbreaks [64]. Considering the constant mutations of AI viruses, AFCD has been closely monitoring the AI epidemiology in the region as well as the latest AI vaccine development to timely update the stipulated AI vaccine being used in local chicken farms in order to ensure the effectiveness of the vaccination programme (Table 1). Since January 2018, in view of the continuous threat of H7N9 AI viruses in the region, the compulsory AI vaccination campaign has been expanded to cover both H5 and H7 subtypes, and it was proposed lately to further expand to cover two antigenic variants of H5 viruses in 2022.

The H5/H7 AI vaccination strategy implemented in local chicken farms with the use of unvaccinated sentinel chickens is summarized as below:

(1) For each batch of chickens, at least 60 chickens would be selected as sentinel chickens, which should not be vaccinated with H5/H7 vaccine.
(2) First dose of H5/H7 vaccination at 8–10 days old.
(3) Second dose of H5/H7 vaccination 4 weeks after the first dose (i. e. at around 36–40 days old).
(4) For chickens aged 120 days old or more (eg. breeders), a booster dose of H5/H7 vaccine is required, followed by further booster shots once every 6 months or whenever the antibody titre of vaccinated chickens in the same batch failed in routine serosurveillance.

AI surveillance in vaccinated chickens in Hong Kong is conducted aiming to ensure that chickens are free of H5/H7 zoonotic avian influenza prior to chicken sale to maintain public health and food safety. In Hong Kong, chicken farms apply "batch-in-batch-out" management for each batch of chickens introduced, while each chicken farm could keep multiple batches of chickens at the same time. Passive surveillance, including regular inspections to all local chicken farms by AFCD officers and mandatory reporting of abnormal death of chickens over stipulated thresholds by farmers, are in place for monitoring health status of chickens on farms. In addition, sentinel chickens are used and placed in-between the vaccinated flocks for early detection of AI virus incursions [68]. Acceptable AI testing results of the sentinel chickens and the vaccinated chickens are the pre-requisites for approval of market sale. Each batch of chicken/farm are subject to the following H5/H7 AI active surveillance activities:

(1) Random sampling of oropharyngeal and cloacal swabs from sentinel chickens (n = 30) of pre-sale chicken batch are collected for H5 & H7 AI PCR test.

(2) Random sampling of blood samples from sentinel chicken (n = 30) of pre-sale chicken batch are collected for H5 & H7 AI serological test by hemagglutination inhibition (HI).
(3) Random sampling of blood samples from both vaccinated (n = 30) and sentinel chickens (n = 30) are collected for AI vaccination efficacy evaluation and silent infection checking (H5 & H7 HI titers), respectively, on each chicken batch 4 weeks after second AI vaccination.
(4) Random sampling of blood samples from both vaccinated (n = 30) and sentinel chickens (n = 30) are collected from breeder chicken batch for H5 & H7 serological test by HI test on a regular basis.
(5) Random environmental swabs sampling (n = 110) on a regular basis for AI virological testing.

Market sale will only be approved if ≥ 70% of samples from vaccinated chickens show H5 and H7 HI titers ≥1:16 (revaccination would be required for the concerned batch of chickens if this requirement is not met); no samples of unvaccinated sentinel chickens have H5 and H7 titer ≥1:16; and all sentinel chickens have negative PCR results for subtypes H5 and H7 AI virus.

In addition to the farm level, the Hong Kong government has also been implementing a multi-level surveillance system along the live poultry supply chain, including wholesale LBM and retail LBM. Furthermore, AFCD is also conducting very extensive AI surveillance activities on wild birds to detect novel AI viruses, such as dead bird surveillance and environmental sampling from Nature Reserves. If any HPAIV is detected in these surveillance activities, the AFCD veterinary laboratory would perform genetic analysis on the AI virus as well as isolate the virus for vaccine matching to determine the protectiveness of the currently using AI vaccine against the field virus for any timely actions to be taken at the farm level, as well as optimization of the AI laboratory testing methods [69]. Nevertheless, there are certain concerns when considering the feasibility and applicability of similar surveillance system in other places, including:

(1) Frequent official farm visits (eg. on a weekly basis) are being conducted to all local chicken farms in Hong Kong for animal health monitoring and other AI surveillance activities given the small poultry production scale, which may not be acceptable in terms of manpower, resources, time, etc., for other places with a larger geographic area and with more farms.
(2) The Hong Kong government fully covers the cost of the AI surveillance system, which may not be affordable in larger places with a much larger poultry production scale given the intensiveness of the surveillance activities.

The need for H5/H7 AI vaccination in Hong Kong has been reviewed regularly over the years, and it is expected that H5/H7 AI vaccination would continue to be implemented in local chicken farms in the near

Table 1
Summary on the history of AI vaccine composition introduced to local chicken farms [65–67].

Year of introduction	Vaccine introduced	Strains of seed viruses (clades)
2003	H5 Intervet Nobilis; monovalent	A/duck/Potsdam/1402-6/1986 (H5N2, European LPAIV)
2012	[a]H5 Re-5/H5 Re-6; monovalent	H5 Re-5: A/duck/Anhui/1/2006 (H5N1, clade 2.3.4)
		H5 Re-6: A/duck/Guangdong/S1322/10 (H5N1, clade 2.3.2.1)
2016	[a]H5 Re-6 + Re-8; bivalent	H5 Re-6: A/duck/Guangdong/S1322/10 (H5N1, clade 2.3.2.1)
		H5 Re-8: A/chicken/Guizhou/4/2013 (H5N1, clade 2.3.4.4g)
2018	[a]H5 Re-8 + H7 Re-1; bivalent	H5 Re-8: A/chicken/Guizhou/4/2013 (H5N1, clade 2.3.4.4g)
		H7 Re-1: A/pigeon/Shanghai/S1069/2013 (H7N9)
2019	[a]H5 Re-11 + H7 Re-2; bivalent	H5 Re-11: A/duck/Guizhou/S4184/2017 (H5N6, clade 2.3.4.4h)
		H7 Re-2: A/chicken/Guangxi/SD098/2017 (H7N9)
2022	[a]H5 Re-13 + H5 Re-14 + H7 Re-4; trivalent	H5 Re-13: A/duck/Fujian/S1424/2020 (H5N6, clade 2.3.4.4h)
		H5 Re-14: A/whooper swan/Shanxi/4-1/2020 (H5N8, clade 2.3.4.4b)
		H7 Re-4: A/chicken/Yunnan/SD024/2021 (H7N9)

[a] Developed by the National Avian Influenza Reference Laboratory of Harbin Veterinary Research Institute.

future. Recently, however, the existing practice of using sentinel chickens in local chicken farms in Hong Kong has been ceased starting from October 2022 onwards after critical review with other complementary measures proposed to be implemented, which include, among others:

(1) Conducting AI PCR testing on oropharyngeal and cloacal swab samples from vaccinated chickens to replace the testing for sentinel chickens;
(2) Additional routine AI surveillance on dead chickens found on farm during regular inspections; and
(3) Enhancing environmental surveillance for AI monitoring on farms by increasing frequency and optimizing sample types to be collected.

Such change to cease the use of sentinel chickens in local chicken farms has taken various views into account: The AI surveillance and monitoring mechanism using unvaccinated sentinel chicken has been in place in local chicken farms since 2002 primarily for the detection of sustained silent infection in vaccinated flocks if it were to occur. It was expected that infected sentinel chickens would die shortly after infection given HPAIVs of the H5N1 subtype usually cause death in infected unvaccinated chickens when compulsory preventive H5 AI vaccination campaigns initially introduced. In addition to observing for unusual mortality in sentinel chickens, sentinel chickens are also tested to assess infection status just before vaccinated chickens are sent to the market for sale in case unvaccinated sentinel chickens were infected but not showing any clinical signs of disease. The mechanism was adopted when vaccination was first introduced to Hong Kong, where there were limited cost-effective options available for surveillance and monitoring, in particular that the use of vaccination meant conventional serological tests could not be used in identification of H5 infection to detect silent infection and differentiate from vaccination, i.e. DIVA, at that time. Nevertheless, since the introduction of sentinel chickens in 2002, none of these chickens has been detected as being virus-positive based on the results of virological surveillance. The only farm outbreak during this period was detected in 2008, via an increased mortality in chicken (in the house involving both sentinel and vaccinated chickens) instead of routine sentinel virological and/or serological surveillance. On the contrary, sentinel chickens were tested positive serologically in multiple occasions every year, leading to suspension of the concerned chicken farms for thorough disease investigation of the flocks. However, none of these investigations resulted in the detection of HPAIV by further virological testing, yet caused unnecessary disruptions to the poultry trade due to suspension. Those positive serological results were possibly caused by mixing up of vaccinated with sentinel chickens, or misvaccination of sentinel chickens due to farm management issues of the respective chicken farms; and the coincidental use of other LPAI vaccines (e.g. H9 AI vaccine) or the presence of other LPAIVs (e.g. H3N8 or H9N2) infections on farms, which may induce cross reactivity between antibodies against different AIVs or other non-specific serological reactions which are known to occur with HI tests. In fact, the trade had raised concerns from time to time on the management challenges in keeping unvaccinated sentinel chicken among vaccinated flocks, especially under manpower shortage situation (e.g. during COVID-19 pandemic), as well as possible amplified risks of AI outbreaks due to the presence of unvaccinated chickens on farms.

The successful prevention of HPAIV infections in local chicken farms, with only one outbreak in 2008, over the past 20 years has reasonably indicated the effectiveness of vaccination in the prevention and control of HPAI in Hong Kong. With 20 years' experience of implementing AI vaccination in Hong Kong, it is considered that silent infection is unlikely to occur in well vaccinated flocks, with good antibody response against a well-matched vaccine antigen to the circulating field strains (Sitaras eta l., 2016). The limited shedding by these vaccinated chickens is very unlikely to result in sustained transmission, while the presence of

unvaccinated sentinel chickens on farm may on the contrary pose a higher introduction and transmission risk of HPAIV at the farm level. Considering the main purpose of surveillance is to detect silent infection in vaccinated flocks, testing of unvaccinated sentinels for evidence of HPAIV is an insensitive method given any infected chicken would shed virus for a very short period before developing clinical signs and die. As a matter of fact, the only HPAI outbreak in Hong Kong at the farm level since the compulsory AI vaccination campaign was implemented in 2003, occurred in 2008 and was detected via an increased mortality in chickens in one farm involving both sentinel and vaccinated chickens as mentioned above. Hence, routine dead chicken testing may be a more sensitive method for detection of HPAI outbreaks in vaccinated flocks. The concerned farm had modified its management practices so that sentinel chickens were all housed together that would have facilitated introduction and transmission of the virus in this unvaccinated group of chickens. Experimental study later also revealed that the H5 HPAI virus causing the outbreak was an antigenic variant that the AI vaccine using at that time could not provide sufficient protection against the infection [70]. Given the technology advancement for diagnostic testing in the past 20 years, AFCD's experiences with sentinel chickens and greater knowledge regarding behaviour of H5 and H7 AI viruses in vaccinated flocks, it is considered that there is no longer a need to retain sentinel chickens for surveillance purposes. Equivalent AI surveillance information could be obtained without them by alternative AI surveillance approaches.

6.3. Italy

Several epizootic waves of both HPAIV and LPAIV affected Italy since 1999 (Table 2). The majority of these outbreaks were detected in a DPPA along the Po Valley in northeastern Italy, where approximately 70% of the national poultry production is concentrated reaching densities higher than 10,000 birds/km^2 [71]. The area is also characterised by the presence of large wetlands and wintering sites for migratory waterfowl. The high density of poultry farms and their interconnection via personnel and vehicles might hamper the promptness of control measures once the AI virus has been introduced in the domestic sector, and may lead to massive economic losses [72].

Following the devastating HPAI epidemic in 1999–2000 and the consecutive H7N1 LPAI outbreaks, the Italian Veterinary Authorities defined a set of contingency measures to be put into force in case of further detection of AI viruses in the domestic poultry sector. Besides enhancing biosecurity at the farm level, banning of movement of vehicles, poultry and personnel, and surveillance activities, also emergency vaccination was accounted to prevent the infection and limit the spread of any potential H5 and H7 viruses circulating in the industrial poultry sector [73].

Emergency vaccinations were approved by the European Commission (Commission Decision 2000/7217 EC) to limit the spread of LPAIV in the DPPA during the 2000–2001 and 2002–2003 epidemics (Table 2). The approach was to apply a DIVA strategy, using heterologous strains in inactivated, adjuvanted whole virus vaccines. Long-lived poultry

Table 2
Avian influenza epizootic waves in Italy since 1999.

Year	Virus Subtype	Pathogenicity	No. outbreaks
1999	H7N1	LPAI	199
1999–2000	H7N1	HPAI	423
2000–2001	H7N1	LPAI	78
2002–2003	H7N3	LPAI	388
2004	H7N3	LPAI	28
2005	H5N2	LPAI	15
2007	H7N3	LPAI	17
2013	H7N7	HPAI	7
2017–2018	H5N8	HPAI	83
2021–2022	H5N1	HPAI	317

Table 3
Avian influenza vaccination campaigns in Italy.

Start	End	Type of Vaccination	Vaccination protocol	Vaccine strain
November 2000	September 2001	Emergency	Monovalent	A/ck/PK/95-H7N3
December 2002	October 2004	Emergency	Monovalent	A/ck/IT/1999-H7N1
October 2004	December 2006	Preventive	Bivalent	A/ck/Italy/22A/98-H5N9
				A/ck/Italy/1067/99-H7N1
September 2007	March 2008	Emergency	Bivalent	A/mallard/It/4810-79/04-H7N4
				A/ck/It/22A/98-H5N9
			Monovalent	A/ck/Italy/AG-473/1999-H7N1
				A/ck/Italy/1067/99-H7N1

were vaccinated at holdings that applied all-in/all-out regimens [74]. According to the epidemiological situation, the local veterinary authorities could require the vaccination of turkey, guinea fowl, and chicken breeders. A specific vaccination protocol was implemented for each species, and monitoring activities were applied to assess the vaccination coverage and the circulation of AI viruses. Coverage was assessed with the aim of detecting non-immunised flocks, assuming that immunisation was effective on 90% of the vaccinated farms (with a level of confidence of 95%). Sera of at least 20 birds per farm were tested to assess the vaccine coverage.

In each farm subjected to vaccination, 1% of the housed birds (with a minimum of 100 individuals) were kept as unvaccinated sentinels to assess the circulation of AI viruses. Sentinel birds needed to be identifiable and homogeneously distributed in all the farm units, with at least 50 sentinels per shed. Blood samples were taken every 45 days from at least 10 sentinel birds per vaccinated farm (95% probability to detect a positive bird if the within-holding prevalence is equal to or greater than 30%). During the 2000–2001 and 2002–2003 epidemics only 89 vaccinated flocks resulted having positive sentinel birds (1 in 2000–2001 and 88 in 2002–2003; [75].

In July 2004, Italy proposed and obtained authorization from the EC to implement a preventive vaccination campaign, as provided for in the Council Directive 2005/94/EC, in effect at the time. The vaccination relied on a bivalent vaccination program, to be effective on both H5 and H7 viruses (Table 2). The preventive vaccination was in place when an H7N3 LPAIV was introduced and circulated among meat turkey flocks in the province of Verona from September to December 2004, and in April 2005 when the introduction of an H5N2 LPAIV was detected in meat turkeys in the provinces of Brescia, Cremona, and Mantua [73]. The two epidemics mainly involved vaccinated birds (n = 27/28, 96.43% and n = 13/15, 86.67% for the 2004 and 2005 epidemics respectively), where vaccination was not duly applied (i.e. poultry was administered less than three vaccination as scheduled; [75]. Both emergency and preventive vaccination strategies proved to be effective in reducing viral circulation and, in association with restrictions, surveillance measures and enhanced biosecurity, they supported the control and eradication of LPAIV infections [76]. Although it did not completely prevent the infection of immunised flocks, preventive vaccination showed a greater capacity to reduce the transmission of the disease than emergency vaccination, likely due to the high overall vaccination coverage at the beginning of the epidemic [77].

The introduction of an H7N3 LPAIV in commercial turkeys in 2007, after previously circulating in rural and hobby poultry, required the application of a new emergency vaccination plan, to prevent the reintroduction of the virus in the DPPA. Compulsory vaccination was enforced on all meat turkey holdings, laying hen farms that apply the all-in/all-out measures, capon farms, and, upon request by the local veterinary services and after informing the European Commission, breeder farms. The vaccination relied on a bivalent heterologous subtype vaccine (H7N4 and H5N9), mainly used in laying hens, and a monovalent heterologous subtype (H7N1) (Table 3) [78]. Monitoring activities were planned to assess the vaccine efficacy. An intensive surveillance program aimed at promptly detecting AI virus-infected flocks. Samplings were collected also in unvaccinated flocks, located both inside and outside the vaccination area.

7. Summarizing conclusions

The recent developments towards a massively extended circulation, in space and time, of HPAIV in wild birds and in the poultry industry in many regions worldwide have moved vaccination into focus as a complementary prevention tool in major parts of the globe. HPAI vaccination alone has never been successful in controlling HPAIV. Biosecurity, continuous evaluation of vaccination uptake and efficacy, proper surveillance of vaccinated flocks for the freedom from field infections and typing of detected field strains to inform vaccine design are all equally required. Since vaccination may not fully stop infection and transmission of HPAI virus and is likely to suppress expression of clinical signs in vaccinated flocks, the surveillance strategies, suited to the epidemiological situation of a country (zones and compartments therein) and to the type(s) of vaccine(s) used, need to be carefully planned and executed. In a population of vaccinated flocks, active surveillance components (eg. serosurveys of vaccinated flocks to monitor herd immunity or assess vaccine coverage, environmental sampling at LBM) have higher relevance for effective detection of HPAI virus or proving freedom [79–81]. However, passive surveillance still plays an important role, since it may help early detection of vaccine failures resulting in vaccinated infected flocks showing clinical signs of infection.

Declaration of competing interest

None.

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