SCIENTIFIC OPINION

Vaccination of poultry against highly pathogenic avian influenza – Part 2. Surveillance and mitigation measures

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Abstract

Selecting appropriate diagnostic methods that take account of the type of vaccine used is important when implementing a vaccination programme against highly pathogenic avian influenza (HPAI). If vaccination is effective, a decreased viral load is expected in the samples used for diagnosis, making molecular methods with high sensitivity the best choice. Although serological methods can be reasonably sensitive, they may produce results that are difficult to interpret. In addition to routine molecular monitoring, it is recommended to conduct viral isolation, genetic sequencing and phenotypic characterisation of any HPAI virus detected in vaccinated flocks to detect escape mutants early. Following emergency vaccination, various surveillance options based on virological testing of dead birds ('bucket sampling') at defined intervals were assessed to be effective for early detection of HPAIV and prove disease freedom in vaccinated populations. For ducks, virological or serological testing of live birds was assessed as an effective strategy. This surveillance could be also applied in the peri-vaccination zone on vaccinated establishments, while maintaining passive surveillance in unvaccinated chicken layers and turkeys, and weekly bucket sampling in unvaccinated ducks. To demonstrate disease freedom with > 99% confidence and to detect HPAI virus sufficiently early following preventive vaccination, monthly virological testing of all dead birds up to 15 per flock, coupled with passive surveillance in both vaccinated and unvaccinated flocks, is recommended. Reducing the sampling intervals increases the sensitivity of early detection up to 100%. To enable the safe movement of vaccinated poultry during emergency vaccination, laboratory examinations in the 72 h prior to the movement can be considered as a risk mitigation measure, in addition to clinical inspection; sampling results from existing surveillance activities carried out in these 72 h could be used. In this Opinion, several schemes are recommended to enable the safe movement of vaccinated poultry following preventive vaccination.

KEYWORDS
avian influenza transmission, highly pathogenic avian influenza (HPAI), poultry, surveillance, surveillance strategies

CONTENTS

SUMMARY

Background and Terms of Reference

During the past three highly pathogenic avian influenza (HPAI) epidemiological years, multiple HPAI virus subtypes have co-circulated in Europe, particularly in the autumn and winter months. The virus has been increasingly detected in migratory waterfowl birds, resulting in an increased risk of infection in poultry. Therefore, it is necessary to explore the potential of the application of vaccination of poultry in addition to the already applied measures to prevent and control HPAI infections. Given this situation, the European Commission asked EFSA to:

ToR 3 – Assess suitability and effectiveness of the reinforced surveillance set out in the Delegated Regulation (EU) No 2023/361 and of alternative surveillance strategies to be implemented upon HPAI vaccination of poultry establishments. The assessments should target, in particular, surveillance for early detection of HPAI, demonstration of freedom from HPAI infection, also considering WOAH standards of surveillance.

ToR 4 – Assess suitability of the restriction and risk mitigation measures set out in the Delegated Regulation (EU) No 2023/361 and of alternatives to be applied in vaccinated establishment and zone, with the aim of preventing the spread of disease with the movements of vaccinated poultry and their products.

Data and Methodologies

ToR 3

Methods to assess surveillance strategies

An overview of the legal framework of the Delegated Regulation (EU) No 2023/361 was provided and alternatives were defined. This regulation provides rules for the implementation of surveillance during and after emergency protective vaccination (hereafter referred to as 'emergency vaccination') and preventive vaccination. The former requires laboratory surveillance with virological testing carried out every 2 weeks and reinforced clinical surveillance; the latter requires passive surveillance with weekly virological testing of a representative sample of dead birds and active surveillance with monthly clinical and serological/virological testing. The WOAH recommendations were also considered, which require all vaccinated establishments to be tested (virological and serological) to demonstrate the absence of HPAI circulation and the effectiveness of the vaccination programme.

Four different alternative surveillance strategies for each vaccination scenario (emergency and preventive) were considered and key attributes such as the performance indicator, sampling frequency, duration, poultry population to be sampled, diagnostic methods were defined according to the objective of the surveillance, i.e. in emergency vaccination (E): early detection (**E1**), assessment of vaccination effectiveness (**E2**), demonstration of freedom from HPAI in a vaccinated establishment (**E3**) and demonstration of freedom from HPAI in the vaccinated zone (**E4**); in preventive vaccination (P): early detection (**P1**), assessment of level of immunity induced by vaccination (**P2**), demonstration of freedom in a vaccinated establishment (**P3**) and in the vaccinated area (**P4**).

Strategy E1, E3 and E4 took into account the ability to detect and remove an infected establishment before it transmits the infection to, on average, one or more other establishments. The between-flock reproduction number given surveillance (R_s) was used as a measure of transmission and the surveillance was considered effective for early detection when leading to $R_s < 1$. Transmission parameters and case fatality for both vaccinated and unvaccinated populations (chicken layer, duck and turkey flocks) were previously determined in Part 1 of the Scientific Opinion on vaccination of poultry against HPAI; mortality at day of reporting suspicion was calculated based on daily mortality data recorded by farmers in the Netherlands and France. Mortality thresholds that could trigger reporting suspicion by farmers were defined based on available literature and the efficacy of using such thresholds for early detection was tested in this Opinion. By employing a deterministic compartmental SEIRD model, graphical representations were provided, illustrating the expected number of infectious birds over time during an outbreak and the expected daily mortality for unvaccinated and vaccinated flocks of chicken layers, ducks and turkeys, whereas, for the assessment, a stochastic model was used. It was assumed that an infected vaccinated flock would remain undetected by passive surveillance until the infectiousness level reaches that estimated for an unvaccinated flock and, the model considered that introduction of infection can take place at any moment between two adjacent sampling events. One thousand simulations of HPAI outbreaks in vaccinated flocks were carried out and used to quantify infectiousness of a flock given detection by passive surveillance only and the expected infectiousness of a flock that escapes detection by the evaluated alternative surveillance strategy (this detection should take place before passive surveillance). The two values were then related to the expected between-flock reproduction number R_h, which was derived from the analysis of the epidemics in France, Italy and the Netherlands, made in Part 1 of the Scientific Opinion on vaccination of poultry against HPAI, to finally calculate the expected between-flock reproduction number given surveillance (R_s). R_s was defined as the reference measure to compare different surveillance strategies, encompassing different sample types (live or dead birds), sizes and frequencies at flock level. A combination of sample size and sampling interval was considered effective only if the probability to escape detection reached levels lower than 0.01 for more than 95% of the outbreak simulations and $R_s < 1$.

Strategies P1, P3 and **P4** were assessed using scenario tree models to estimate the sensitivity of the surveillance system to demonstrate freedom and to early detect HPAI in a preventive vaccination scenario. This analysis provides an estimation of the probability that the surveillance in place is capable of detecting the infection in the unit of interest, given a certain prior HPAIV prevalence. The tree model traces all pathways by which a single unit can be detected as infected by the system. It includes a number of risk nodes and probability nodes, and the ultimate outcome is the probability that the outbreak is detected (P_{pos}) given the sensitivity of testing at flock level (F_{se}).

Surveillance strategies E2 and **P2** were addressed descriptively. Vaccine effectiveness, as the ability of a vaccine to protect from infection under field conditions, can be assessed by observation of the occurrence of HPAI outbreaks in a country where vaccination is implemented. Ways to assess vaccination effectiveness or determine the level of immune response after vaccination were proposed, specifying the data to be gathered and potential analyses for estimation. For emergency vaccination, an assessment of the radius and of the surveillance to be done in the **peri-vaccination zone** was carried out based on data and results from a previous EFSA Opinion. The assessment of the type and duration of surveillance to be conducted **after cessation of vaccination** was based on evidence previously collected in Part 1 of the Scientific Opinion on vaccination of poultry against HPAI and qualitatively assessed for the current Opinion.

Diagnostic methods

The different diagnostic tests that can be used as part of HPAIV surveillance were described: classical virological methods (virus isolation), molecular virological methods (conventional RT-PCR and real-time RT-PCR (RT-qPCR)), rapid antigen detection systems and serological methods (agar gel immunoprecipitation (AGID)), haemagglutination inhibition (HI) and ELISA technology. For each of the described methods, a qualitative assessment, according to the objective of the surveillance, was conducted. Serological data from experimental trials conducted on ducks, turkeys and chickens performed by AI-NRLs were obtained from France (ducks), Italy (turkeys), the Netherlands (chickens) and Belgium (chickens). Those data were analysed with the aim to assess the HI titres in vaccinated birds according to the vaccine(s) used for immunisation, the HI titres in experimentally vaccinated-infected birds and the performance of the ELISA assays used for the detection of antibodies against the nucleoprotein (NP) in birds vaccinated with DIVA compatible vaccines following infection. Also, the monitoring of clinical signs and the possible use of sentinels were qualitatively assessed.

ToR 4

Risk mitigation measures

According to Delegated Regulation (EU) No 2023/361, different risk mitigation measures can be implemented following emergency and preventive vaccination. Following vaccination, vaccinated animals and their products are subject to prohibition of movement. For emergency vaccination, the competent authority may allow the movement of animals if these are subject to compulsory killing or if conditions for moving set out in Delegated Regulation (EU) No 2020/687 are fulfilled. For preventive vaccination, by way of derogation, the competent authority may allow the movement of vaccinated animals and their products according to the conditions laid down in Delegated Regulation (EU) No 2023/361. EFSA assessed qualitatively those measures and identified and assessed the provision of demonstrating freedom as an alternative or complementary approach to guarantee a safe movement of poultry and their products.

Uncertainty analysis

The uncertainty analysis was performed following the procedure detailed in the EFSA guidance on uncertainty analysis in scientific assessments. The sources of uncertainty associated with the data available and the methodology used were listed and discussed, but their impact on the assessment was not quantified.

Assessment

ToR 3

Diagnostic methods

Sterile immunity state is the ideal outcome of an efficient vaccination campaign, but it is usually not achieved on a population base. However, vaccination should elevate the threshold of an individual animal to become infected and reduce virus excretion in amplitude and time compared to unvaccinated ones. To detect smaller amounts of virus at a lower prevalence, it is therefore necessary that during active surveillance of vaccinated establishments, highly sensitive diagnostic tests tailored to the epidemiological situation are employed.

Surveillance to detect HPAIV outbreaks or to prove freedom from disease in a vaccination zone can rely on a number of classical or molecular virological methods for virus detection, as well as serological methods for the detection of antibodies indicating previous exposure, which can be used alone or in combination depending on the specific objective of the surveillance activity.

Since vaccination may reduce the window of viral shedding and lower viral replication titres, the ability of the virological tests to detect viral circulation may diminish. Therefore, due to inferior sensitivity compared to molecular methods and the slower turnaround time, **virus isolation** is not recommended for screening during active surveillance in vaccinated flocks. It can only be considered as an alternative to molecular methods in exceptional circumstances (e.g. unavailability of reagents for molecular testing); it would however be essential to allow phenotypic characterisation of the virus once the outbreak has been confirmed by a molecular method.

Molecular techniques are generally used for HPAIV diagnosis directly from clinical specimens of birds. RT-qPCR offers rapid turn-around time, added cross-contamination prevention and is applicable to high throughput analysis. The outstanding analytical sensitivity of RT-qPCR also allows pooling of clinical samples. In flocks vaccinated with live vectored H5 or H7 vaccines, interpretation of RT-qPCR may be compromised, since active replication of the vaccine would be taking place in different tissues and might return positive results to assays designed to detect the H5 or H7 specific molecular signatures shared between vaccines' inserts and field viruses. To overcome this issue, specific RT-qPCRs tailored to avoid such interference may be required to distinguish residual vector vaccine inserts from HPAI field virus. Analysis of environmental samples employing the same assays could be carried out, as the effort of individual animal sampling is considerable and may itself provide an obstacle to vaccination, while collection of environmental samples may provide an easier handling. At the time of writing, however, evidence that environmental sampling could replace animal-based sampling in surveillance activities to guarantee early detection of HPAI outbreak at the design prevalence of 5% with 95% confidence currently foreseen in Delegated Regulation (EU) No 2023/361 is lacking due to a deficiency of independent systematic studies.

Next-generation sequencing (**NGS**) are high speed techniques that allow generation of large volumes of data and have many applications in research and diagnostic settings. In terms of surveillance purposes, NGS has a very high value in further characterisation of isolated AI viruses.

Rapid antigen detection systems are methods for the detection of viral antigen which are usually based on enzymatic immunoassays or immunochromatography (i.e. they use a monoclonal antibody against AIV nucleoprotein or other highly conserved regions of structural proteins). They produce results very rapidly and can be carried out on the poultry premises, but they have low sensitivity and, consequently, are prone to produce false-negative results with the smaller amounts of virus expected in vaccinated flocks. Therefore, they are currently not considered useful for active surveillance in vaccinated flocks.

The different vaccine technologies exhibit variances in their ability to stimulate preferentially humoral or cell-mediated immune response. The type of vaccine used for immunisation can therefore affect character and magnitude of the specific immune response, which in turn may affect its detectability via serological methods. At present, there are no scalable methods to measure cell-mediated immunity; instead, available tests are all based on antibody detection. For example, agar gel immunodiffusion (**AGID**) tests can detect the presence or absence of antibodies against any influenza A viruses (since they all share antigenically similar nucleoprotein and matrix antigens) in a subtype independent manner. However, several reasons (e.g. restriction to certain species only, generation of qualitative results, low sensitivity, virus proteins not involved in the protective immunity are targeted) limit their use for surveillance purposes. Thus, **ELISA** technology has replaced AGID as a generic serologic screening assay. Quantitative assessment of humoral immune response still relies on haemagglutination inhibition assays (**HI**). Clinical examination alone is not a reliable indicator in surveillance of vaccinated flocks, as it has been demonstrated that, despite the variations in protection after vaccination, absence of clinical signs and increase in mortality are poor indicators of viral circulation. However, in field conditions, a certain number of animals may show clinical signs and there may be an increase in mortality. So, these compromised animals deserve greater attention in surveillance investigations (i.e. bucket sampling strategies). The use of unvaccinated sentinels in vaccinated flocks is highly problematic, since, for correct and successful implementation, optimal mingling and identification of sentinels and vaccines is required, which has been proven difficult to establish in practice. In addition, fully susceptible sentinels may provide a door for viral entry.

Results from analysis of **serological data** indicate that monitoring of vaccine-induced immunity can be performed by HI from 3 to 7 weeks post-vaccination, if antigens antigenically close to the vaccine are used. Similarly, to assess past infection in immunised birds, antigens antigenically close to the field HPAI strains should be used. An increase in HI titres (up to 9 $\log_2$) have been observed 14 days post-infection when sera from vaccinated and PCR-confirmed infected animals were tested using challenge strains.

ELISA assays targeting antibodies against the nucleoprotein were evaluated in vaccine trials performed by different NRLs to assess their suitability for a DIVA testing. Results have showed that antibodies against the NP of HPAI become detectable in vaccinated and infected animals starting from 7 to 14 days post-infection in all tested species and with all vaccines compatible with such DIVA strategy. Diagnostic sensitivity and specificity of indirect ELISA assays have been found to be higher compared to those of competitive ELISA assays, and thus should be favoured if compatible with the species that has to be tested.

Surveillance

Surveillance results for **early detection** at **establishment level** in case of **emergency vaccination (E1)** are reported grouped by poultry species and different surveillance strategies (passive reporting, 'mortality thresholds' and active surveillance). Different combinations of sampling type, sampling intervals and samples sizes were assessed and those leading to a very low probability of escaping detection, were considered effective. The surveillance strategies include sampling all

dead birds (up to a number of 5, 10, 15 and 20) collected within 48 h before sample submission at different sampling intervals. Consequently, there is no minimum sample size: If fewer dead birds are present, all the dead birds available are tested. For flocks **≥ 3000 chicken layers**, molecular testing all dead birds up to a number of 5 every week proved to be effective for early detection; longer sampling intervals can be considered if higher numbers of dead birds are sampled and tested (up to a number of 10 every 2 weeks or 15 for a 3-week interval). For flocks **< 3000 chicken layers**, none of the surveillance strategies assessed resulted effective. In those flocks, the level of confidence of early detection can be increased combining the test of all up to a number of 10 dead birds weekly, with molecular testing of live birds every 2 weeks.

For flocks of **2500 ducks**, the strategy of molecular testing all dead birds up to a number of 5 every week showed to be effective for early detection. The strategy of virological or serological testing 60 live birds every 2 weeks would also be effective for early detection, together with the strategy based on 'mortality thresholds'. In flocks of **≥ 6000 ducks**, the strategies mentioned above would be effective, together with sampling all dead birds up to 10 every 2 weeks, or up to 15 dead birds every 3 weeks.

For flocks of **≥ 6000 turkeys**, the strategy of molecular testing all dead birds up to a number of 5, every week resulted effective to early detect HPAIV. Longer sampling intervals can be considered if higher numbers of dead birds are collected (up to a number of 15 every 2 weeks or up to 20 for a 3-week interval). For flocks of **< 6000 turkeys**, the only effective surveillance strategy was based on 'mortality thresholds' and it should be carried out combined with molecular testing of live birds every 2 weeks to increase the level of confidence of early detection.

After emergency vaccination is applied and **surveillance for disease freedom (E3, E4)** implemented, there is a phase where no further outbreaks are detected. With consistently negative results at both establishment and zone level and a high probability of detection (> 99%) of infected flocks within an establishment, a high level of confidence of disease freedom would be achieved.

According to Delegated Regulation (EU) No 2023/361 **surveillance in a peri-vaccination zone**, during emergency protective vaccination, should be applied to detect any possible new outbreak. A previous risk assessment on control measures for Category A disease has been here used to assess the radius of the peri-vaccination zone. Based on it, a 10-km peri-vaccination zone radius was identified as suitable to contain the spread of the disease and avoid any jump outside the area with 95% confidence. In the peri-vaccination zone, the type of surveillance depends on the vaccination status of the establishments. In preventively vaccinated farms, the surveillance strategy for early detection in emergency vaccination is to be applied. For non-vaccinated farms, passive surveillance for layer chickens and turkeys and weekly sample of available dead birds for ducks and geese applies.

Surveillance results for **early detection** and **demonstration of freedom** in preventive vaccinated populations **(P1, P3, P4)** are assessed based on simulated scenarios in the Netherlands, France and Italy. The focus is on geographical areas previously categorised as high-risk zones for HPAIV incursion. In these zones, coexistence of vaccinated and unvaccinated populations is present. The model tested active surveillance carried out in all vaccinated flocks based on monthly collection of all dead birds up to a number of 15, collected in the 48 h before sample submission, while passive surveillance is applied in unvaccinated flocks. The results indicated that the estimated probability of disease freedom (Pfree) would exceed 99% in the three evaluated high-risk regions, given that all test results are negative. The model outcomes also showed that early detection surveillance sensitivities (EDSe) in vaccinated flocks are higher (layer chickens and turkeys) or similar (ducks) to the EDSe of passive surveillance in unvaccinated flocks of the other poultry types in the regions. Scenarios with variations in sampling intervals (30, 15 and 7 days) and different proportions of vaccinated establishments (100%, 50% and 25%) were also explored, showing that EDSe increase with shorter sampling interval and decrease with smaller proportion of vaccinated establishments sampled. In contrast, these changes have a limited effect on the probability of freedom of the zone (Pfree).

Surveillance can also be used to **assess vaccination effectiveness** (VE) **(E2)**. Implementing vaccination at the regional level enables the observation of numerous vaccinated establishments, facilitating the assessment of vaccination effectiveness and its progression in time under natural exposure conditions to improve emergency preparedness. This approach also offers the opportunity to evaluate vaccination characteristics, such as the onset of protection after vaccination and the duration of protection. The data needed to estimate VE include vaccination status of an establishment, date of vaccination, HPAI infection status, date of detection. Data should be collected and grouped at the smallest administrative area. Probabilities of infection before vaccination and at different time intervals following vaccination (e.g. 7, 14 and ≥ 21 days) can be then compared. During preventive vaccination, it is expected that all establishments of a specific poultry type have been vaccinated, so no comparison can be drawn with unvaccinated ones; nevertheless, an unexpected high number of outbreaks could point at vaccination failure.

Surveillance to assess the **immune response induced by vaccination (P2)**. In the absence of virus circulation, the level of immunity at establishment level induced by vaccination can be measured using different serological assays aimed at the detection of antibodies induced by vaccination. The type of vaccine applied determines the most appropriate diagnostic test to monitor the immune response at flock level. The animals should be sampled at a precise time for the detection of a specific correlate of protection. However, the identification of a correlate of protection remains elusive. So, to assess the percentage of a vaccinated flock showing an 'effective' immune response (as an indication of 'adequate protection' and possibly of the level of herd immunity reached), a pragmatic approach was suggested. First, it should be defined the desired level of herd immunity. Then, a sampling approach has to be chosen, it could be the one adopted in the emergency vaccination plans for the control of LPAI in Italy in 2000 (currently used in France), which is based on at least 20 random samples per establishment. For chicken layers, ducks and turkeys, the level of immunity to be targeted was specified and, based on different sample sizes, it was possible to obtain different results combinations.

After cessation of vaccination, when the last vaccine has been administered and no further vaccination plans are in place, a transition is expected from vaccinated flocks to unvaccinated ones at the end of each production cycle. These unvaccinated flocks will no longer be subject to HPAI surveillance implemented after vaccination. Due to limited knowledge on protective immunity duration and on protection threshold values, determining surveillance duration post-vaccination remains inconclusive. Therefore, vaccinated flocks should be under surveillance up to their replacement, with dead birds being preferred for testing. Meanwhile, passive surveillance should be applied on unvaccinated flocks present in the vaccination area.

Surveillance in vaccinated captive birds. A surveillance system should be in place to early detect and intercept the presence of HPAIV in vaccinated kept birds such as those in zoo, ornamental birds or birds of high value. Passive surveillance is considered the only feasible option in most cases. It could be based on a frequent (e.g. weekly) observation and clinical examination of birds. In this setting, clinical signs can be immediately detected, coupled with a prompt analysis of any sick and dead birds, including, for zoo, wild birds found dead in the zoo premises. For kept ornamental birds, certain movements (i.e. those with a high risk of HPAIV spread such as for trade, exhibitions, shows, markets, etc.) may represent a risk, and testing should be carried out before it, since the premovement clinical examination alone may not be sufficient. For holding keeping captive birds above a certain number, the surveillance suggested for poultry might apply.

ToR 4

Risk mitigation measures

According to Delegated Regulation (EU) No 2020/687, during emergency vaccination authorisation for movement of animals and hatching eggs can be granted if the competent authorities deem the risk to be negligible. This risk assessment relies on a comprehensive evaluation that includes results of clinical examination, laboratory examination (if necessary) and an official veterinary visit. As per Delegated Regulation (EU) No 2023/361, for **emergency vaccination**, the vaccinated establishments are already under surveillance, so laboratory examination is ongoing. According to EFSA assessment, these emergency vaccinated establishments should be under surveillance as described in Sections 3.1.2.1 and 3.1.2.2 (Tables 10A–12B). Therefore, including the effectiveness of the combined clinical examination and surveillance established in this Opinion, the risk of movement of animals and eggs is considered negligible when both are performed within the 72 h before the movement. If the process of movement (e.g. loading) is not completed within 72 h, then testing should be repeated every 48 h until it ends. Given the already ongoing surveillance, testing could coincide with the sampling session of the surveillance in place after emergency vaccination (i.e. no additional sampling and testing should be done). Note that, differently from what stated in Delegated Regulation (EU) No 2020/687, suggesting that laboratory examinations should be conducted only if necessary, laboratory examinations have been assessed as an integral component of the risk mitigation measures to be implemented in case of emergency vaccination.

For **preventive vaccination**, according to Delegated Regulation (EU) No 2023/361, safe movements of vaccinated birds and their products are guaranteed if the vaccinated animals were negative to the active and enhanced passive surveillance implemented after preventive vaccination against HPAI (Table 1). In addition, the implementation of other mitigation measures further contributes to this guarantee (see Figure 7). In the current Opinion, the surveillance to be applied after preventive vaccination is assessed in Section 3.1.2.3 and prescribes the monthly sampling of dead birds with a number of 15 collected in a flock during 48 h before the testing to assess a zone as free from HPAIV. Therefore, in the case of preventive vaccination, where no outbreaks are present, the surveillance as described in Section 3.1.2.3, that is already in place, provides a very high certainty that the area is free from infection (see Tables 15A–17B). Accordingly, if the birds are negative to the clinical inspection done not earlier than 72 h before the movement and all establishments follow the surveillance protocol with no outbreak detection, the risk of HPAI spread via movement of such birds is considered negligible. If the establishment from where the vaccinated birds are to be moved is not under surveillance, such as in case where less than 100% of vaccinated establishments are subjected to testing (such as 50% or 25%), then vaccinated birds are clinically inspected and dead birds up to a number of 15, should be collected not earlier than 72 h before the movement. Negative test results should be provided before the movement and no clinical signs should be observed.

Sources of uncertainty

The current Opinion identifies sources of uncertainty across various assessment sections, including performance of diagnostic tests used in the context of HPAIV surveillance, and the effectiveness of surveillance strategies at the establishment level and of surveillance strategies at the area/zone level.

Conclusions and Recommendations

ToR 3

Diagnostic methods

There are a number of different diagnostic methods available that can be used according to vaccine type, strategy and surveillance objective, but lack of validation and field experience in the context of vaccinated poultry populations

of these methods may be an issue. Serology may increase confidence of virological surveillance results but, the ability to use serological tests may be compromised when vaccination is carried out with non-DIVA compatible vaccines. Samples collected from the environment could be a valid, time- and labour-saving alternative to animal samples, but there is currently a lack of validated protocols; research to extend and deepen knowledge here is needed. When implementing a vaccination programme, it is important that the most appropriate diagnostic method for surveillance is selected according to vaccine type; methods with high sensitivity are recommended when expected viral levels to be detected are low due to vaccination. In addition to routine molecular monitoring, viral isolation, genetic sequencing and phenotypic characterisation of HPAIVs eventually detected in vaccinated flocks are highly recommended to detect putative escape mutants as early as possible.

Surveillance

For the surveillance, some important **general considerations** and **conclusions** were drawn: The surveillance strategies modelled included the random sampling of the dead birds up to a number of 5, 10, 15 or 20 (increasing the sample size above this target does not increase the probability of virus detection); if fewer dead birds are present, then all the available ones should be tested, given that the probability of early detecting HPAIV is higher than testing live birds; to achieve the highest sensitivity, birds intended for testing, are those found dead in the last 48 h; the effectiveness of the above-mentioned approach is maximised by repeated sampling in time; the lack of knowledge regarding the correlate of protection and, consequently, the duration of protective immunity in vaccinated poultry, precludes any definitive conclusion on the surveillance duration following the cessation of vaccination. For this reason, vaccinated flocks should be under surveillance upon their replacement (sampling and testing of dead birds) and passive surveillance should be carried out in both unvaccinated and vaccinated flocks.

For **early detection** at **establishments in case of emergency vaccinations (E1)**, different options are proposed according to poultry species. For **chicken layers**, a number of effective options based on molecular testing of dead animals at a certain interval have been identified and should be selected according to country's specific circumstances and resources. For both flock sizes considered (**≥ 3000** and **< 3000**), the surveillance strategy identified in Delegated Regulation (EU) No 2023/361 (virological testing of 60 live birds) was assessed as not effective, as well as the serological testing and the strategies based on passive reporting and 'mortality threshold'. However, for flocks **< 3000** birds, combining the molecular testing of dead birds with molecular testing of live birds would increase the level of confidence of early detection.

For **ducks**, a number of options based on the molecular testing of dead birds at a certain interval have been identified as effective and should be selected according to country's specific circumstances and resources. Other surveillance strategies can also be carried out (molecular or serological testing of 60 live ducks and 'mortality thresholds'), but they may present more challenges (both for welfare and logistical reasons) and their effectiveness may depend on various factors (availability and accuracy of information about mortality thresholds). The only strategy assessed as not effective for ducks is the one relying solely on passive reporting.

For flocks of **≥ 6000 turkeys**, a number of effective options based on the molecular testing of dead birds at a certain interval have been identified and should be selected according to country's specific circumstances and resources. The surveillance strategy identified in Delegated Regulation (EU) No 2023/361 (virological testing of 60 live birds) was assessed as not effective, as well as the serological testing and the strategies based exclusively on passive reporting and 'mortality thresholds'. However, for flocks **< 6000 turkeys**, the only valid strategy identified as effective was the one based on 'mortality thresholds' (0.21%), that should be combined with molecular testing of live birds to increase the level of confidence of early detection.

For the **surveillance in peri-vaccination zone**, it is recommended to establish a 10-km radius zone to increase the confidence that the probability of jump spread is lower than 0.04. In relation to the type of surveillance, it is recommended to apply either early detection surveillance in vaccinated establishments and passive surveillance for layer chickens and turkeys and bucket sampling for ducks and geese in unvaccinated farms.

For **disease freedom** at establishment and zone following **emergency vaccination** (E3, E4), the testing schemes as reported above by poultry species apply, given the sampling is repeated in time.

For the **surveillance to demonstrate disease freedom** following **preventive vaccination**, in the three high-risk zones assessed, it is recommended to carry out monthly molecular testing of all dead birds up to a number of 15, in all vaccinated establishments. Passive surveillance is to be maintained in both vaccinated and unvaccinated flocks. This testing scheme is also applied to **early detect** infections following **preventive vaccination**, but if the aim is to increase the early detection surveillance sensitivities (EDSe), then it is recommended to reduce the sampling intervals. Reducing the proportion of vaccinated establishments to be tested showed a limited impact on the Pfree, but a negative impact on the EDSe. However, for each of the targeted species, a number of effective options (maintaining high level of EDSe and Pfree) have been identified, testing lower proportions of establishments (25% or 50%) at different sampling intervals. These testing schemes should be selected according to country's specific circumstances and resources.

To estimate **vaccination effectiveness (E2)**, it is required a careful planning, sufficient human resources, follow-up of all flocks and a good data collection framework.

Surveillance to assess the **level of immune response induced by vaccination (P2)** can be performed when virus circulation is absent or expected to be low. To best detect how many birds have a measurable immune response, testing within a certain time window, depending on the vaccine used, is recommended. Since it is important to have accurate estimates of the minimum proportion of protected birds required to stop transmission, further studies quantifying R_0 values for the currently circulating HPAIV are recommended. Even if there is no established correlate of protection, an assessment of the prevalence of birds showing an immune response following vaccination is assumed to provide an indication of sufficient protection (for chickens, 20 blood samples per establishment, using appropriate diagnostic test, are appropriate to determine whether vaccines are correctly used in the field).

ToR 4

Restriction and risk mitigation measures

In case of **emergency vaccination**, HPAI outbreaks are present in the area, and vaccinated establishments are already under the surveillance applied after emergency vaccination (i.e. laboratory examination is already ongoing). In addition to ensuring negligible risk as set out in Delegated Regulation (EU) No 2020/687, the laboratory examination to demonstrate freedom from HPAI, conducted according to Sections 3.1.2.1 and 3.1.2.2, is considered as part of the risk mitigation measures to be implemented in addition to the clinical inspection in the 72 h before the movement. Negative test results should be provided before the movement and no clinical signs should be observed. Testing to guarantee safe movement of the birds could coincide with the sampling session of the surveillance in place (i.e. no additional birds/samples to be tested).

In case of **preventive vaccination**, where no outbreaks are present, vaccinated establishments are already under the surveillance applied after preventive vaccination. To guarantee safe movement of vaccinated poultry or the day-old chickens and hatching eggs deriving from such poultry, negative surveillance test results, conducted according to Section 3.1.2.3, should be provided, and no clinical signs should be observed before the movement. If the vaccinated establishment is not under surveillance, such as in case where less than 100% of them are subjected to testing, then, in addition to the absence of clinical signs, also negative test results should be provided before the movement.

1 | INTRODUCTION

1.1 | Background and Terms of Reference as provided by the requestor

The background and the Terms of Reference (ToR) as provided by the requestor are reported in Section 1.1 of Part 1 of the Scientific Opinion on Vaccination of poultry against highly pathogenic avian influenza (HPAI) (EFSA AHAW Panel, 2023). This Scientific Opinion focuses on ToR 3 and ToR 4, which are reported in Appendix A.

1.2 | Interpretation of the Terms of Reference

The interpretation of ToRs 3 and 4 is reported in Sections 1.2.3 and 1.2.4 of Part 1 of the Scientific Opinion on Vaccination of poultry against highly pathogenic avian influenza (EFSA AHAW Panel, 2023).

2 | DATA AND METHODOLOGIES

The methodological approach adopted to address the ToRs 3 and 4 is described in the protocol reported in Appendix A of part 1 of the Scientific Opinion on Vaccination of poultry against HPAI (EFSA AHAW Panel, 2023). This protocol was developed upfront of the initiation of the risk assessment. In this section, a more detailed description of the specific data and methodology used for ToRs 3 and 4 is provided. A mapping of ToRs 3 and 4 as received from the European Commission and how they were addressed in the current Opinion is presented in Appendix A.

2.1 | ToR 3 – Surveillance in the vaccinated zone and/or vaccinated establishments

ToR 3 entails the assessment of different surveillance strategies (or surveillance approaches as mentioned in Table A1 APRIO elements for the assessment question and subquestions) to be implemented on poultry establishments vaccinated against HPAI. Vaccination scenarios include emergency protective and preventive vaccination and have been presented in Part 1 of the Scientific Opinion on Vaccination of poultry against HPAI (EFSA AHAW Panel, 2023). The surveillance assessed in this Opinion is the reinforced surveillance laid down in Delegated Regulation (EU) No 2023/361[1] (see Section 2.1.1); those described in the WOAH surveillance requirements (see Section 2.1.2); and alternative surveillance strategies as suggested by the EFSA Working Group (see Section 2.1.3).

Particularly, the current assessment was targeted at the surveillance objectives indicated in ToR 3 and in Delegated Regulation (EU) No 2023/361, namely:

1. Early detection of HPAIV infection in vaccinated establishments (ToRs 3.1, 3.2, Delegated Regulation (EU) No 2023/361 Article 9 and Part 5.2.b).
2. Demonstration of freedom from infection with HPAIV in vaccinated establishments and/or the vaccinated zone in order to guarantee movement of vaccinated and non-vaccinated animals and animal products within and from the vaccination zone during and after cessation of vaccination (ToR 3.1, 3.2); freedom from HPAIV in a vaccinated zone should be also demonstrated considering specifically the WOAH standards of surveillance (i.e. in all vaccinated establishments as provided in Chapter 10.4, Article 10.4.28) and possible alternatives to that (ToR 3.3).
3. Assessment of vaccination effectiveness (Delegated Regulation (EU) No 2023/361 Article 9).

The suitability and effectiveness of these surveillance strategies were assessed using mathematical models (see Sections 2.1.5 and 3.1.2) and the suitable diagnostic methods to be used according to each objective were described (Section 2.1.4) and assessed (Section 3.1.1). As stated in Opinion Part 1, backyard poultry were not considered due to data limitations. Also, previous studies in the Netherlands and France have shown that captive birds and backyard holdings likely played a limited role in disease spread (Bavinck et al., 2009; Souvestre et al., 2019).

2.1.1 | Surveillance as per Regulation (EU) 2023/361 during/after vaccination

To define surveillance strategies for the assessment, the Working Group initially provided an overview of the legal framework. In doing so, they considered Delegated Regulation (EU) No 2023/361, which sets rules for reinforced surveillance in the vaccination zones established as detailed in Art. 9 and in Annex XIII Part 2, Part 4 and Part 5.

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[1]Commission Delegated Regulation (EU) No 2023/361 of 28 November 2022 supplementing Regulation (EU) 2016/429 of the European Parliament and the Council as regards rules for the use of certain veterinary medicinal products for the purpose of prevention and control of certain listed diseases. *OJ L 52, 20.2.2023, pp. 1–42.*

2.1.1.1 | *Rules for implementation of surveillance during/after emergency protective vaccination*

According to Article 9, Delegated Regulation (EU) No 2023/361, when applying emergency protective vaccination, reinforced clinical and laboratory surveillance should be implemented in both the vaccination and peri-vaccination zones established. The objectives of such surveillance will be to assess **vaccination effectiveness** in the vaccination zone, and to **detect any possible new outbreaks** in the vaccination and peri-vaccination zones. According to Part 2, in all the vaccinated establishments, virological testing should be implemented every 2 weeks, and the sampling has to enable the detection of a minimum within-flock prevalence of HPAIV infection of 5% with a confidence level of 95% (Table 1). These measures should remain in place during the whole recovery period following emergency protective vaccination (as per Part 4). The recovery period lasts 28 days where vaccination is carried out in a zone that does not overlap with restricted zones due to HPAI outbreaks; where the vaccination zone overlaps with HPAI restricted zones, the recovery period ends when the restricted zones are lifted, if the date of lifting is later than 28 days after the last vaccine inoculation.[2] After the end of the recovery period, the rules for implementation of surveillance during/after preventive vaccination apply as long as an establishment keeps vaccinated birds.

Sampling methods of animals for laboratory examination are outlined in Annex I of Delegated Regulation (EU) No 2020/687[3] and include the following requirements:

- Animals should be chosen randomly in a number large enough to detect the disease if present;
- Samples should be collected at random in each epidemiological unit of the establishment;
- Disease profile and level of exposure risk should be taken into account;
- Minimum number of animals to sample will depend on expected prevalence, level of confidence and performance of tests used.

The *diagnostic methods* chosen should aim at maximising surveillance sensitivity and may include using laboratory examinations to assess previous exposure to the disease.

2.1.1.2 | *Rules for implementation of surveillance during/after preventive vaccination*

For preventive vaccination, both enhanced passive and active surveillance should be implemented to early **detect occurrence of infection with HPAI**. Enhanced passive surveillance foresees weekly virological testing of a representative sample of dead birds collected within 1 week; according to the Diagnostic Manual for Avian Influenza (2006/437/EC),[4] it is recommended to pool sampling of dead birds (with a maximum of 20) over the week equally subdivided from each flock[5] of the establishment[6]: up to five carcasses/samples of birds deceased on the day of sampling should be collected by flock. Active surveillance should be carried out, by monthly clinical examination (clinical examination of poultry, check of production records, check of health records of each epidemiological unit[7]), and by monthly collection of representative samples from live birds for laboratory surveillance by virological and serological testing to enable detection of a minimum within-flock prevalence of HPAIV infection of 5% with 95% confidence level (Table 1). These measures will remain in place as long as the establishments keep vaccinated animals.

Requirements for *sampling of animals for laboratory examinations* and the *diagnostic methods* for surveillance for preventive vaccination against HPAIV in poultry are the same as in emergency vaccination and are summarised in Section 2.1.1.1.

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[2]According to Annex XIII, Part 4, Delegated Regulation (EU) No 2023/361.

[3]Commission Delegated Regulation (EU) No 2020/687 of 17 December 2019 supplementing Regulation (EU) 2016/429 of the European Parliament and the Council, as regards rules for the prevention and control of certain listed diseases. OJ L 174, 3.6.2020, pp. 64–139.

[4]https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006D0437&from=EN.

[5]'Flock' means all poultry or captive birds of the same health status kept on the same premises or in the same enclosure and constituting a single epidemiological unit; in housed poultry includes all birds sharing the same airspace, Delegated Regulation (EU) No 2020/689.

[6]'Establishment' means any premises, structure, or, in the case of open-air farming, any environment or place, where animals or germinal products are kept, on a temporary or permanent basis, Regulation (EU) No 2016/429 on transmissible animal diseases ('Animal Health Law' – AHL).

[7]'Epidemiological unit' means a group of animals with the same likelihood of exposure to a disease agent, Regulation (EU) No 2016/429 on transmissible animal diseases ('Animal Health Law' – AHL).

TABLE 1 Surveillance activities according to Delegated Regulation (EU) No 2023/361 during/after implementing vaccination against HPAIV in poultry.

Type of vaccination	Surveillance category	Testing procedure	Frequency	Minimum detectable prevalence/type of information collected	Duration
Emergency protective	Reinforced laboratory	Virological	2 weeks	5% prevalence with 95% confidence level[a]	According to the duration of the recovery period
	Reinforced clinical	–	–	–	–
Preventive	Enhanced passive	Virological	Weekly	Representative sample of dead birds	As long as there are vaccinated birds in the establishment
	Active	Clinical examination	30 days	Clinical examination of poultry, check of production records, check of health records of each epidemiological unit	
		Serological or virological	30 days	5% prevalence with 95% confidence level (representative sample)[a]	

[a]To detect HPAIV with 5% design prevalence with 95% confidence level, a sample size of 60 randomly selected live birds is required to be tested.

2.1.2 | Surveillance as per WOAH

To define surveillance strategies for the assessment, the Working Group also considered the WOAH recommendations for surveillance systems, in particular Chapters 1.4 and 10.4 of the Terrestrial Animal Code on Animal Health Surveillance and on surveillance for HPAI, respectively (WOAH, online-a).

Considering the diversity of situations regarding HPAI in the world, the WOAH approach is to provide generic recommendations on the approach and parameters to be considered when designing and implementing a surveillance system for early detection and for demonstration of freedom from infection with HPAIV; those principles are also necessary to support vaccination programmes. The WOAH does not have a standard operating procedure for official recognition of a country's status for HPAI, but Members Countries have the possibility to submit self-declaration of freedom from disease, under its sole responsibility and according to the conditions listed in Article 10.4.2 (Chapter 10.4) of the Terrestrial Code (WOAH, online-a). Self-declaration should include details of the surveillance system in place to sustain the health status. For areas that need to recover their freedom status following infection, the absence of infection should be demonstrated by the surveillance programme for a minimum period of 28 days starting after cleaning and disinfection of the last affected establishment if a stamping out policy is implemented. If the stamping out policy is not implemented, the absence of infection in poultry during a period of 12 months should be demonstrated by the surveillance programme.

In zones where vaccination is carried out, surveillance for demonstration of freedom from HPAI requires that all vaccinated flocks are tested to prove the absence of viral circulation, with a frequency that is proportional to the risk in that zone. Evidence of the effectiveness of the vaccination programme is also part of the requirements for the surveillance programme.

2.1.3 | Surveillance strategies and key attributes

To effectively define and compare surveillance strategies for the assessment, the following key attributes were considered:

- **Objective of surveillance**: (i) The early detection of HPAIV infection in a vaccinated establishment and in the vaccination/peri-vaccination zone; (ii) substantiating freedom from disease in an establishment/area in order to grant derogation to movements to animals and animal products; (iii) assessing vaccination effectiveness.
- **Type of surveillance** can be either defined as 'active' or 'passive' (Hoinville et al., 2013). Passive surveillance means reporting of suspect cases raised by increased mortality, other signs of serious disease or significantly reduced production rates. The health authorities are notified by veterinarians or farmers at their discretion. It is more likely to be useful for diseases that are characterised by typical clinical signs and for which subclinical infection is rare (Doherr & Audigé, 2001). Active surveillance is the detection of the disease through activities instigated following a plan. These activities can be carried out randomly or non-randomly and they can be based on clinical, serological or virological detection of disease or infection.
- **Performance indicator** describes concrete and measurable information serving as a measurable benchmark for assessing the effectiveness of a surveillance activity concerning the objective, i.e. for early detection that would be the time from virus introduction until detection of the disease, for freedom from disease that would be the confidence of (non) detecting the disease if it was present at a specified level.

- **Sampling frequency**: It characterises the surveillance activities i.e. how often samples are collected., being the sampling or collection of data carried out on a continuous basis (data are gathered continuously), on a regular basis (data are gathered at defined time intervals), or ad hoc (undertaken occasionally, when the need arises).
- **Duration and period** of surveillance refer to the period of time and the period of the year (e.g. season), in which the surveillance activities are carried out in order to meet the objective of the strategy.
- **Poultry population to be sampled** is the animal population from which the samples are collected; it refers both to the vaccinated establishments and the birds within each establishment and/or each flock within the establishment, considering species and production type, and immunity status dynamics, and the criteria for its selection (census, random, targeted, based on the risk, etc.).
- **Design prevalence** also known as the minimum detectable prevalence or target prevalence, it is defined as a threshold prevalence value associated with a statistical risk of error, at the animal or herd level, to test the hypothesis that disease is present in a population of interest and it is used to determine the needed sample size. It is the hypothetical proportion of animals/herds that are infected given the disease was present and it is used for evaluating the sensitivity of the surveillance systems. The error represents the probability of not detecting the disease among the sampled animals/herds if it was present at the minimum value set by the design prevalence.
- **Diagnostic methods**: It refers to the most suitable method with appropriate diagnostic tools to diagnose rapidly and accurately the disease or to detect the virus circulation in a population where evidence of those conditions (i.e. clinical signs and virus shedding) is modulated by the effectiveness of vaccination implemented and the field strain circulation. An overview of the benefits and limitations of diagnostic methods for HPAI is extensively provided in Section 3.1.1.
- **Epidemiological unit**: It refers to the establishment (any premises where animals are kept in a temporary or permanent basis); an establishment can comprise one or more flocks and if one flock is infected then the whole establishment is considered infected; for the number of birds to be sampled within an establishment, it always refers to the flock (all poultry of the same health status kept in the same enclosure (house/barn)).
- **Sampling unit**: flock(s) within the establishment

All those characteristics are described in Table 2 for both situations of emergency protective vaccination and preventive vaccination.

2.1.3.1 | *Surveillance strategies*

Table 2 provides an overview of the attributes associated with the different surveillance strategies considered in the assessment. The strategies encompass those specified in Delegated Regulation (EU) No 2023/361 as well as alternative surveillance strategies proposed by the Working Group and are separated by emergency protective (E) and preventive (P) vaccination scenarios. These alternative strategies explore variations in the sampled population, including differences in sample number (reduced vs. increased) or bird categories (dead vs. live), in sampling frequency (reduced vs. increased) and diagnostic methods while keeping the surveillance's objectives as they are in Delegated Regulation (EU) No 2023/361 and WOAH standard, with exception of objective in strategy P2 (assessment of level of immune response to vaccination) that was proposed by the Working Group (WG). The strategies are compared based on a performance indicator, which depends on the objective of the surveillance. An explanation of how each performance indicator is estimated is provided in Sections 2.1.5.1 and 2.1.5.2. For the surveillance set out in Delegated Regulation (EU) No 2023/361, for emergency vaccination, as the aim is to detect 5% prevalence and the animals to be sampled, according to Regulation as reported in Annex XIII part 2, should be chosen randomly in a number large enough, it is here interpreted as the sampling of live birds with a number of 60.

TABLE 2 Attributes of the different surveillance strategies considered in the assessment described by emergency protective (E) and preventive vaccination (P) scenarios.

Surveillance strategy	Emergency protective vaccination scenario – Surveillance within the vaccination zone				Preventive vaccination scenario – Surveillance within vaccination zone			
	Strategy E1	**Strategy E2**	**Strategy E3**	**Strategy E4**	**Strategy P1**	**Strategy P2**	**Strategy P3**	**Strategy P4**
Objective of surveillance	HPAIV early detection (to be implemented also in the peri-vaccination zone)	Assessment of vaccination effectiveness	Demonstrating freedom from HPAIV in the vaccinated establishment (to authorise the movement of birds from that establishment)	Demonstrating freedom from HPAIV in the vaccinated zone	Early detection in case of HPAIV introduction	Assessment of level of immune response induced by vaccination	Demonstrating freedom from HPAIV in the vaccinated establishment (to authorise the movement of birds from that establishment)	Demonstrating freedom from HPAIV in the vaccinated area (considering that also non-vaccinated establishments might be present)
Performance indicator	Between-flock reproduction number (R_h), i.e. the average number of farms infected by one infected farm in its infectious period. The surveillance was considered to be effective for early detection if it contributed to $R_h < 1$	Vaccination effectiveness, estimated as 1 minus a measure of relative risk (RR) such as: incidence or rates of infection, prevalence ratio (attack rates, cumulative incidence), the odds ratio (OR) or the hazard ratio (HR)	Probability of freedom Conditions required: no virus-positive samples	Probability of freedom within and between-flock/establishment Required: no virus-positive flocks	Surveillance sensitivity for early detection in the vaccinated population	% of animals showing immune response following vaccination	Confidence of freedom	Confidence of freedom
Establishments to be sampled	Regulation (EU) 2023/361: all vaccinated establishments Alternative: Since this is emergency surveillance, no alternative strategy at this level is considered	Regulation (EU) 2023/361: all farms in the vaccination and peri-vaccination zones	Regulation (EU) 2023/361: all vaccinated establishments Alternative: since this is emergency surveillance, no alternative strategy at this level is considered	Regulation (EU) 2023/361: all vaccinated establishments	Regulation (EU) 2023/361: all vaccinated establishments Alternative: a sample of vaccinated establishments	All vaccinated establishments that completed the vaccination scheme	Regulation (EU) 2023/361: all vaccinated establishments	Regulation (EU) 2023/361: all vaccinated establishments Alternative: a sample of vaccinated establishments

(Continues)

TABLE 2   (Continued)

Surveillance strategy	Emergency protective vaccination scenario – Surveillance within the vaccination zone				Preventive vaccination scenario – Surveillance within vaccination zone			
	Strategy E1	**Strategy E2**	**Strategy E3**	**Strategy E4**	**Strategy P1**	**Strategy P2**	**Strategy P3**	**Strategy P4**
Birds to be sampled	Regulation (EU) 2023/361: a representative sample of live birds to detect a design prevalence of 5% with 95% CL[a] Alternative: assessing different sample sizes based on sampling dead or live birds (see details in Section 3.1.2.1)	NR	Regulation (EU) 2023/361: a representative sample of live birds to detect a design prevalence of 5% with 95% CL[a] Alternative: assessing different sample sizes based on sampling dead or live birds (see details in Section 3.1.2.2)	Regulation (EU) 2023/361: a representative sample of live birds to detect a design prevalence of 5% with 95% CL[a] Alternative: assessing different sample sizes based on sampling dead or live birds (see details in Section 3.1.2.2)	Regulation (EU) 2023/361: representative sample of dead birds and a representative sample of live birds to detect design prevalence of 5% with 95% CL[a] Alternative: Assessing different sample sizes of dead or live birds to enable both high within flock sensitivity for early detection and high level of confidence in disease freedom (see details in Section 3.1.2.3)	≥ 20 randomly selected birds per establishment (to estimate the percentage of birds developing an immune response above predefined-thresholds)	Regulation (EU) 2023/361: representative sample of dead birds and a representative sample of live birds to detect design prevalence of 5% with 95% CL[a] Alternative: Assessing different sample sizes of dead or live birds to enable both high within flock sensitivity for early detection and high level of confidence in disease freedom (see details in Section 3.1.2.3)	Regulation (EU) 2023/361: representative sample of dead birds and a representative sample of live birds to detect design prevalence of 5% with 95% CL[a] Alternative: Assessing different sample sizes of dead or live birds to enable both high within flock sensitivity for early detection and high level of confidence in disease freedom (see details in Section 3.1.2.3)

TABLE 2 (Continued)

Surveillance strategy	Emergency protective vaccination scenario – Surveillance within the vaccination zone				Preventive vaccination scenario – Surveillance within vaccination zone			
	Strategy E1	**Strategy E2**	**Strategy E3**	**Strategy E4**	**Strategy P1**	**Strategy P2**	**Strategy P3**	**Strategy P4**
Diagnostic methods	Regulation (EU) 2023/361: clinical examination and virological testing in live birds Alternative: molecular virological testing on live or dead birds (i.e. real time RT-PCR targeting M gene for screening. H5/H7 subtyping by real-time RT-PCR or sequencing methods); Virus isolation followed by H5/H7 subtyping by classical or molecular virological methods	Regulation (EU) 2023/361: clinical examination and virological testing in live birds Alternative: any virological method suitable for the detection and identification of HPAI infection in vaccinated farms	Regulation (EU) 2023/361: clinical examination and virological testing in live birds Alternative: molecular virological testing on live or dead birds (i.e. real time RT-PCR targeting M gene for screening. H5/H7 subtyping by real-time RT-PCR or sequencing methods); Virus isolation followed by H5/H7 subtyping by classical or molecular virological methods	Regulation (EU) 2023/361: clinical examination and virological testing in live birds Alternative: a combination of molecular virological (i.e. real time RT-PCRs) and serological tests such as NP-based ELISAs (if DIVA compatible vaccines are used). For NP-ELISA-positive and PCR-negative birds, HI with selected HPAI and LPAI antigens; real-time RT-qPCRs only (if inactivated vaccines are used or when the epidemiological situation makes impractical the use of serological tests)	Regulation (EU) 2023/361: clinical examination and virological testing of dead or live birds and serological testing in live birds Alternative: molecular virological testing by real-time RT-PCR targeting M gene for screening, confirmation of subtype and pathotype on positive samples at screening; Virus isolation followed by H5/H7 subtyping by classical or molecular virological methods	Alternative: serological testing in live birds (e.g. H5-HI or H5-ELISA); molecular virological if an RT-qPCR tailored to the specific vaccine product is available	Regulation (EU) 2023/361: clinical examination and virological testing of dead or live birds and serological testing in live birds Alternative: molecular virological testing in live or dead birds (i.e. real time RT-PCR targeting M gene for screening, confirmation of subtype and pathotype on positive samples at screening); Virus isolation followed by H5/H7 subtyping by classical or molecular virological methods	Regulation (EU) 2023/361: clinical examination and virological testing of dead or live birds and serological testing in live birds Alternative: a combination of molecular virological (i.e. real time RT-PCRs) and serological tests such as NP-based ELISAs (if DIVA compatible vaccines are used). For NP-ELISA-positive and PCR-negative birds, HI with selected HPAI and LPAI antigens; real-time RT-qPCRs only (if inactivated vaccines are used or when the epidemiological situation makes impractical the use of serological tests)

(Continues)

TABLE 2 (Continued)

Surveillance strategy	Emergency protective vaccination scenario – Surveillance within the vaccination zone				Preventive vaccination scenario – Surveillance within vaccination zone			
	Strategy E1	Strategy E2	Strategy E3	Strategy E4	Strategy P1	Strategy P2	Strategy P3	Strategy P4
Sampling frequency	Regulation (EU) 2023/361: every 14 days Alternative tested: 7, 14, 21 or 30 days	Regulation (EU) 2023/361: every 14 days Alternative tested: 7, 14, 21 or 30 days	Regulation (EU) 2023/361: every 14 days Alternative tested: Use of cumulative evidence from repeated sampling	Regulation (EU) 2023/361: 14 days Alternative tested: Use of cumulative evidence from repeated sampling	Regulation (EU) 2023/361: weekly virological testing of dead birds and monthly clinical examination and testing of live birds (virological or serological) Alternative tested: sampling frequency of 7, 14, 21 and 30 days	To be done once (from 2 to 4 weeks after completion of immunisation of after 7 weeks for vectored vaccines administered at 1 day of age)	Regulation (EU) 2023/361: weekly virological testing of dead birds and monthly clinical examination and testing of live birds (virological or serological) Alternative tested: sampling frequency of 7, 14, 21 and 30 days	Regulation (EU) 2023/361: weekly virological testing of dead birds and monthly clinical examination and testing of live birds (virological or serological) Alternative tested: sampling frequency of 7, 14, 21 and 30 days
Duration and period of surveillance	Regulation (EU) 2023/361: for the duration of the recovery period, after the surveillance for preventive vaccination applies Alternative: for the duration of the recovery period, after the surveillance for preventive vaccination applies (i.e. as long as vaccinated animals in the establishment have protective immunity)		Regulation (EU) 2023/361: for the duration of the recovery period, after the surveillance for preventive vaccination applies Alternative: for the duration of the recovery period, after the surveillance for preventive vaccination applies (i.e. as long as vaccinated animals in the establishment have protective immunity)	Regulation (EU) 2023/361: for the duration of the recovery period, after the surveillance for preventive vaccination applies Alternative: For the duration of the recovery period, after the surveillance for preventive vaccination applies (i.e. as long as vaccinated animals in the establishment have protective immunity)	Regulation (EU) 2023/361: as long as there are vaccinated animals in the establishments Alternatives: as long as vaccinated animals in the establishment have protective immunity (after that they should be under normal surveillance for non-vaccinated flocks)	As long as vaccination is performed	Regulation (EU) 2023/361: as long as there are vaccinated animals in the establishments Alternatives: as long as vaccinated animals in the establishment have protective immunity (after that they should be under normal surveillance for non-vaccinated flocks)	Regulation (EU) 2023/361: as long as there are vaccinated animals in the establishments Alternatives: as long as vaccinated animals in the establishment have detectable protective immunity (after that they should be under normal surveillance for non-vaccinated flocks)

Abbreviation: NR, not relevant.

[a]As the animals to be sampled, according to Delegated Regulation (EU) No 2023/361 in Annex XIII part 2, should be chosen randomly in a number large enough to detect 5% prevalence, it is here interpreted as the sampling of live birds with a number of 60; CL means confidence level.

2.1.4 | Diagnostic methods

Vaccination against HPAI in poultry requires the appropriate tailoring not only of surveillance strategies but also of the matching diagnostic methods to be applied in AI-vaccinated flocks to robustly verify or rule out the presence of AI virus circulation in such establishments. The effect of vaccination on clinical signs, on magnitude and duration of viral shedding following field virus exposure and on the serological profile of AI-vaccinated birds requires careful selection of the most appropriate assays according to the vaccination protocol implemented and the objective of surveillance as described in Table 2. In this Opinion, a description of the following different diagnostic tests that can be used as part of HPAIV surveillance is reported: classical virological methods (virus isolation), molecular virological methods (conventional RT-PCR and real-time RT-PCR (RT-qPCR)), rapid antigen detection systems and serological methods (agar gel immunoprecipitation (AGID), haemagglutination inhibition (HI) and ELISA technology). An updated list of diagnostic protocols for AIV detection and typing is maintained by the EURL and is available at the EURL website (EURL, online).

For each of the described methods, the WG conducted a qualitative assessment, according to the objective of the surveillance, whose results are reported in Section 3.1.1.

Serological data from experimental trials conducted in laboratory on ducks, turkeys and chickens performed by AI-NRLs were obtained from France (ducks), Italy (turkeys), the Netherlands (chicken layers) and Belgium (chicken layers). Those data were analysed with the aim to assess:

- Expected hemagglutination inhibition (HI) titres in vaccinated birds according to the vaccine(s) used for immunisation. Such values can be used to evaluate if immunisation has been obtained in the field.
- HI titres in experimentally vaccinated challenged birds. Irrespective of the severity of the clinical presentation and the magnitude of shedding, vaccinated infected birds, are expected to seroconvert following exposure to field virus. The magnitude of seroconversion is, however, strongly dependent on the effective replication of the virus in the host.
- Performances like sensitivity (Se) and specificity (Sp) of the ELISA assays used for the detection of antibodies against the nucleoprotein (NP) in birds vaccinated with DIVA compatible vaccines following infection.

Also, the monitoring of clinical signs and the possible use of sentinels are included in the assessment in Sections 3.1.1.5 and 3.1.1.6, respectively.

2.1.5 | Methods to assess surveillance strategies

To assess the effectiveness of the surveillance strategies in poultry after vaccination as described in Table 2, different methods were used at the animal and flock level (e.g. within establishment and between establishments). These methods were applied independently or combined, depending on whether the surveillance was conducted during emergency protective or preventive vaccination. Note that surveillance in captive birds was qualitatively assessed in Section 4.2.5.

2.1.5.1 | *Methodology to assess surveillance for early detection (E1) and demonstration of freedom (E3, E4) following emergency vaccination*

Early detection is here defined as the identification of infection with HPAIV in order to remove an infected poultry establishment before it transmits the infection on average to one or more establishments. Considering that a poultry establishment may consist of one or more flocks, in this Scientific Opinion, an establishment was considered infected if HPAIV was detected in one of its flocks and the flock was the sampling unit. We used the between-flock reproduction number (R_h) as a measure of transmission, and the surveillance was considered to be effective whenever it contributed to keeping R_h below 1 (meaning the epidemic would fade out) as performance indicator. The attributes that define surveillance effectiveness are the sample size at flock level, frequency of sampling, type of sample and diagnostic test sensitivity. For this assessment, we followed a methodology based on the approach previously described by Gonzales et al. (2014).

Transmission dynamics of HPAIV

To identify the critical surveillance parameters such as prevalence of infectious animals, the infectiousness of a flock at the time of detection and the expected efficacy of the surveillance strategy at the establishment level, it is key to understand the infection dynamics in vaccinated flocks where the protection conferred by the vaccine may not be sufficient to stop the disease transmission ($R_h > 1$).

Transmission parameters for unvaccinated and vaccinated chicken layer, duck and turkey flocks are summarised in Table 3. These data were collected as part of the systematic literature review (SLR) performed in Part 1 of the Scientific Opinion on vaccination of poultry against HPAI (EFSA AHAW Panel, 2023). There, it was assumed that in a vaccinated poultry population, 70% of the flocks were fully protected ($R < 1$) 3 weeks after vaccination, while 30% were partially protected ($R > 1$). Virus introduction into fully protected farms will result in infection of one or a few birds (minor outbreaks) and the probability of detecting such incursions is extremely low given that the number of infected birds is very small and that they will not show clear signs of infection. The probability of identifying them in a serological or virological survey (random

sample) are also extremely low given the very low number of infected/seroconverted birds relative to the sample size. In addition, such minor outbreaks do not pose a risk as the few infected birds will completely recover (no carriership of any kind) and the probability that they will infect birds on other farms is negligible due to lack of contact. Consequently, surveillance is here focused on detecting major HPAIV outbreaks in the vaccinated flocks given that the 30% of the vaccinated ones will be only partially protected; it is also assumed that the surveillance in unvaccinated flocks will not change and it will be based on detection of signs of infection via passive surveillance.

Parameters for within-flock transmission such as the transmission rate, latent and infectious periods were derived from transmission experiments performed in the Netherlands (Germeraad et al., 2023), France (Grasland et al., 2023) or identified during the previous Opinion (Ssematimba et al., 2019; Tatár-Kis et al., 2019) (Table 3).

Case fatality which is the mortality attributed to HPAI was derived from the results of SLR reported in Table 2 in Opinion Part 1 (EFSA AHAW Panel, 2023). The case fatality, in the reviewed experiments, was calculated as the number of dead birds due to HPAI over the total number of infected birds. Values for daily mortality not attributed to HPAI ('baseline mortality rate') were obtained from Gonzales and Elbers (2018), Elbers and Gonzales (2021) and Gonzales et al. (2023). These authors analysed daily mortality, during the production cycle, in a sample of not-infected flocks. During an outbreak of HPAI, the daily mortality will consist of uninfected and HPAIV-infected dead birds (Table 3). This information was used to assess the efficacy of testing dead birds. Of note, based on the SRL performed for the SO Part 1 (EFSA AHAW Panel, 2023), case fatality lower than 20% was associated with vaccines effective to stop sustained transmission (no outbreaks would take place in these flocks).

Mortality on the day of reporting suspicion parameter (Table 3) was calculated based on the daily mortalities recorded at the time of reporting suspicions of HPAI by farmers in the Netherlands and France (Unpublished data). This parameter was used as a performance indicator of passive surveillance and as reference for comparison of the infectiousness of a flock up to the time of detection when using enhanced passive surveillance or active surveillance.

Mortality thresholds for reporting suspicion (Table 3) were defined based on the studies by Gonzales and Elbers (2018), Elbers and Gonzales (2021) and Gonzales et al. (2023). They established mortality levels that could trigger farmers to report suspicions of HPAI. Whenever the observed mortality exceeds this predefined percentage, farmers are assumed to report their suspicions. Although these thresholds were defined using data from unvaccinated flocks., we assessed the efficacy of using such thresholds for early detection of vaccinated flock.

TABLE 3 Within-flock transmission parameters for unvaccinated and vaccinated partially protected bird flocks (i.e. 30% of the vaccinated flocks where $R > 1$).

Parameter	Chicken layers		Ducks		Turkeys	
	Unvaccinated[a]	Vaccinated[a]	Unvaccinated[b,c]	Vaccinated[c]	Unvaccinated[d,e]	Vaccinated[f]
Transmission rate (day^{-1})	1.13	0.47	4.02|10.8	1	3.2	0.64
Latent period (days)	1	1	1|1	1	1	
Infectious period$_{survive}$ (days)	3.2	6.8	7|8.1	2.7	–	4
Infectious period$_{die}$ (days)	3.2	4.5	4.9|–	–	4	4
Case fatality (range)	0.95–1	0.2–1	0–0.8	0–0.1	0.9–1	0.37–0.62
Daily mortality not attributed to HPAI (baseline mortality rate)	0.0002		0.0004		0.0007	0.0007
Mortality at day of reporting suspicion. Mean (range)	1.66 (0.25–5.27)%		1.8 (0.46–15.34)%		1.50%	
Proposing reporting thresholds	0.08 (indoor layers)–0.13 (outdoor)%		0.17%		0.21%[g]	0.21%[g]

[a]Germeraad et al. (2023).

[b]Tatár-Kis et al. (2019). The value at the left of '|' come from this reference.

[c]Grasland et al. (2023). Values for vaccinated are the upper confidence limits of the parameter estimates. Mean estimates resulted in R values lower than 1. These values are for mule ducks. These values are those provided at the right of '|'.

[d]Ssematimba et al. (2019).

[e]Reference laboratory EURL.

[f]These values are assumed since no data on transmission parameters in vaccinated flocks could be found.

[g]No literature was found, hence we assumed a value three times higher than the 'normal' daily mortality as a potential threshold for evaluation.

For illustration purposes, graphical representations showing the expected number of infectious birds over time during an outbreak and the expected daily mortality for unvaccinated and vaccinated flocks are shown for chicken layers, ducks and turkeys in Figures 1–3, respectively. These figures have been built using a deterministic (frequency dependent) compartmental SEIRD model (S=Susceptible, E=exposed, I=infectious, R=recovered, D=dead) (Keeling & Rohani, 2008), which includes normal mortality affecting all compartments and case fatality affecting the I compartment only (Hobbelen et al., 2020). Dynamics were built using the parameters reported in Table 3: the transmission rate, the rate at which birds

of compartment E become I (1/latent period) and the rate at which I birds either die or recover (1/infectious period). Note that for the assessment, a stochastic model was employed to offer a more detailed understanding of virus transmission dynamics (see below).
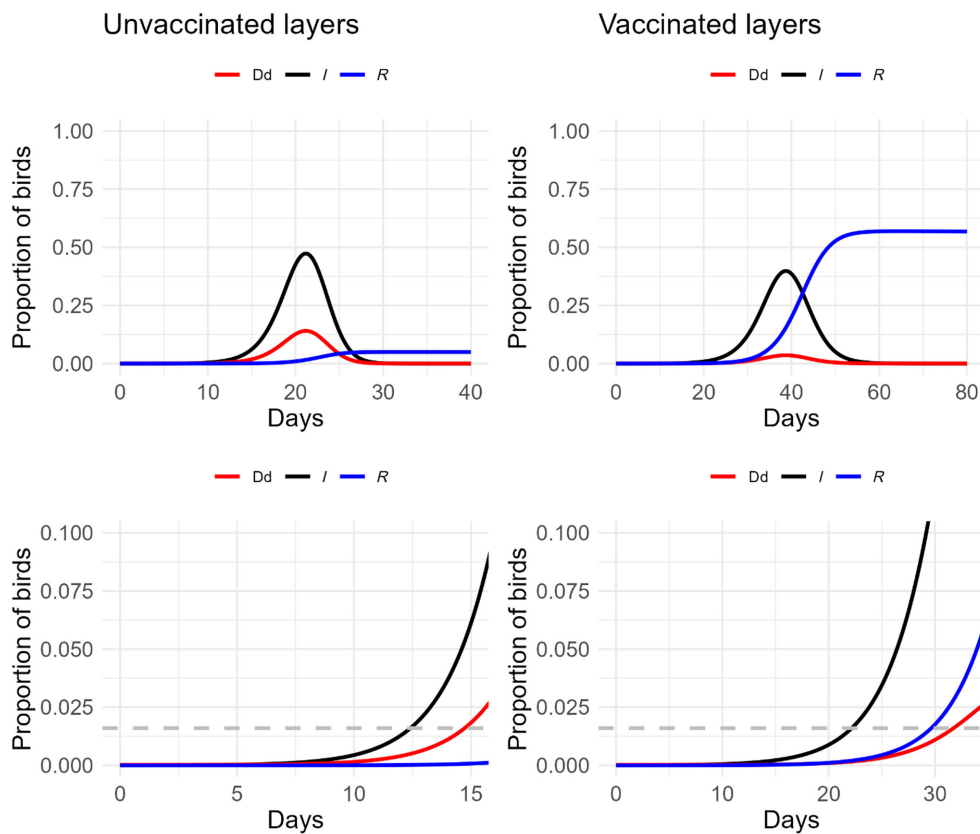
FIGURE 1 Infection dynamics (deterministic modelling) of HPAI H5N1 in unvaccinated (left panels) (reproduction number = 3.6) or vaccinated (right panels) (partially protected, vaccine does not fully stop sustained transmission, R = 2.6) **chicken layers**. Dd, daily mortality; I, Infectious birds; R = recovered-seroconverting birds. The two lower panels provide a zoomed in view of the upper panel, facilitating the visualisation of the reported suspicion mortality level (dashed line) and how this level (1.66%, Table 3) intersects the infection dynamics curves. Case fatalities assumed were 0.95 (95%) and 0.4 (40%) for unvaccinated and vaccinated flocks, respectively. The two lower panels provide a zoomed-in view of the upper panel, facilitating the visualisation of the reported suspicion mortality level (dashed line) and how this level (1.66%, Table 3) intersects the infection dynamics curves. Case fatalities assumed were 0.95 (95%) and 0.4 (40%) for unvaccinated and vaccinated flocks, respectively.
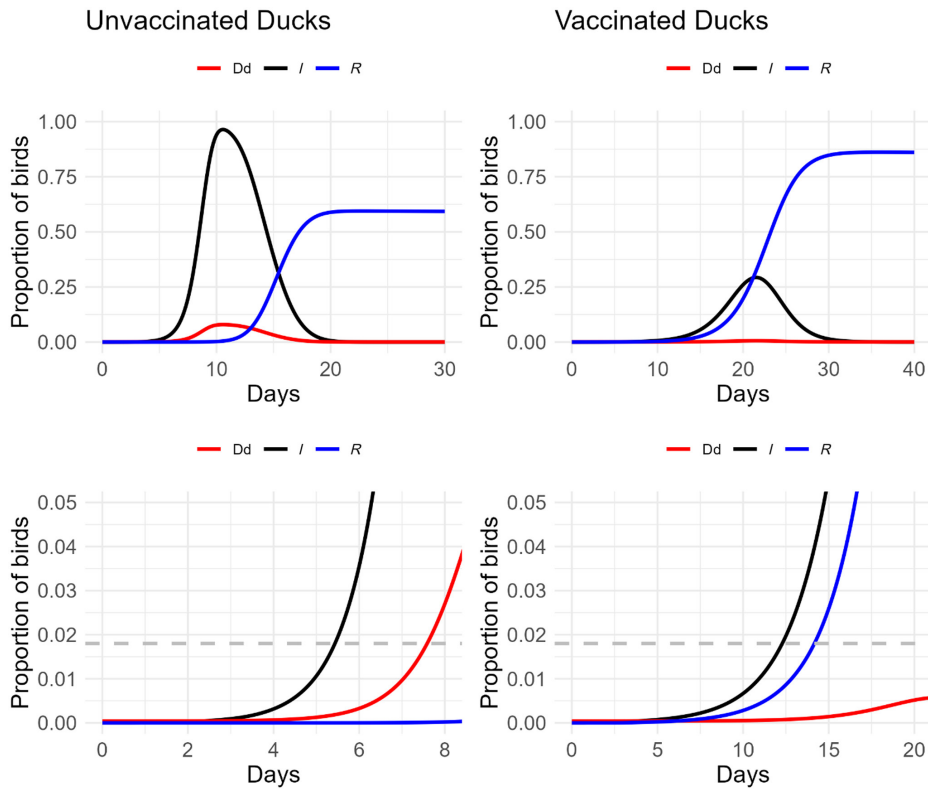
FIGURE 2   Infection dynamics (deterministic modelling) of HPAI H5N1 in unvaccinated (left panels) (reproduction number = 24) or vaccinated (right panels) (partially protected, vaccine does not stop sustained transmission, $R = 2.7$) **ducks**. Dd, daily mortality; I, Infectious birds; R, recovered-seroconverting birds. The two lower panels provide a zoomed-in view of the upper panel, facilitating the visualisation of the reported suspicion mortality level (dashed line) and how this level (1.8%, see Table 3) intersects the infection dynamics curves. Case fatalities assumed were 0.4 (40%) and 0.05 (5%) for unvaccinated and vaccinated flocks, respectively.
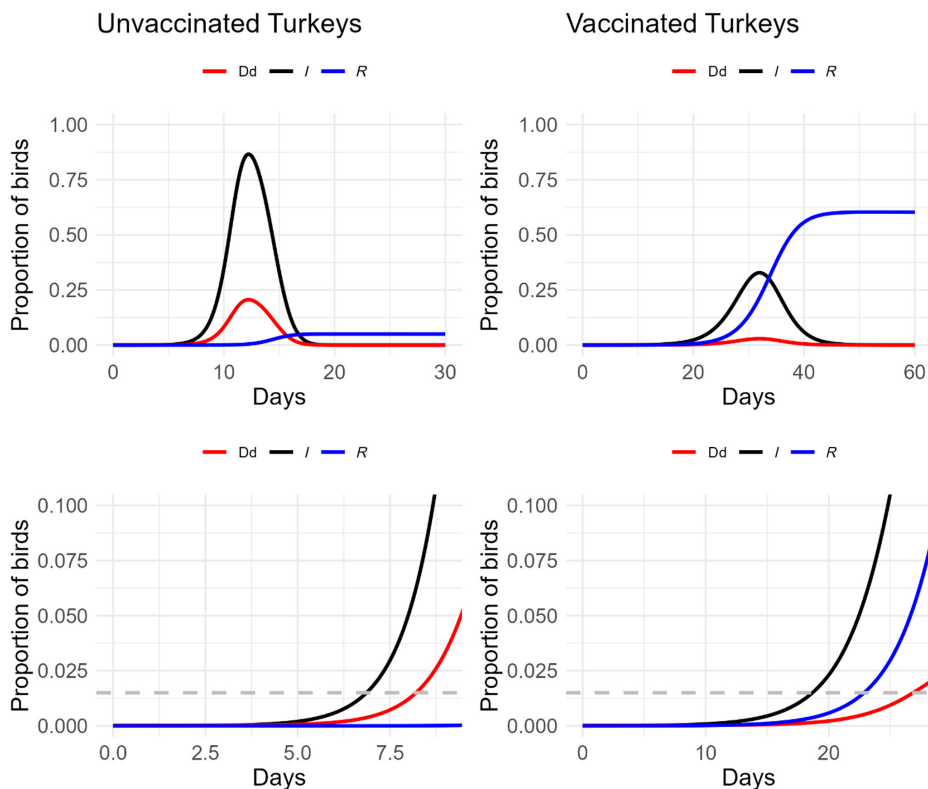
FIGURE 3   Infection dynamics (deterministic modelling) of HPAI H5N1 in unvaccinated (left panels) (reproduction number = 12.8) or vaccinated (right panels) (partially protected, vaccine does not stop sustained transmission, $R = 2.6$) **turkeys**. Dd, daily mortality; I, Infectious birds; R, recovered-seroconverting birds. The two lower panels provide a zoomed-in view of the upper panel, facilitating the visualisation of the reported suspicion mortality level (dashed line) and how this level (1.5%, see Table 3) intersects the infection dynamics curves. Case fatalities assumed were 0.95 (95%) and 0.4 (40%) for unvaccinated and vaccinated flocks, respectively.

Of note are the differences in the duration of the epidemic, the number of infectious birds (I) in time and the observed daily mortality when comparing unvaccinated and partially protected establishments in Figures 1–3. Also, note the differences between the three poultry species assessed and the impact of vaccination on reducing case fatality and therefore the proportion of daily dead birds in these vaccinated flocks. For example, the very low case fatality assumed in vaccinated ducks leads to low proportions of daily dead birds. However, a consistent increase in daily mortality (above the 'baseline mortality') can still be observed later than the day 11 of the epidemic. In simple numbers, if a flock consisted of 6000 ducks, at day 12 there would be around five dead ducks with at least two out of those five being dead due to HPAIV infection.

Definition of infectiousness of a flock and between-flock transmission

Reporting and control of HPAI is compulsory in the EU, and HPAI outbreaks in unvaccinated flocks are generally detected by passive surveillance before the outbreaks reach their completion (no infectious or susceptible birds left in the flock) (Figures 1–3). We assumed that a flock is infectious (i.e. the establishment is therefore infected) and can infect other establishments from the time the flock becomes infected and the first infected birds become infectious (generally 1 day post infection) until the daily mortality rate within the flock reaches the observed average mortality rates of the reported and confirmed outbreaks. At this stage, it is assumed that the flock would be detected and removed (Table 3). We defined infectiousness by function $A(t)$, and assumed that infectiousness is dependent on the prevalence of infectious (shedding virus) birds in time, so that the higher this prevalence the higher the infectiousness. Therefore, $A(t)$ was estimated as (Gonzales et al., 2014):

$$A(t) = \int_0^t I(\tau)d\tau,$$ (1)

where $I(\tau)$ is the prevalence of infectious birds at time t and the expected overall infectiousness was obtained by integrating over t from the time of introduction of infection ($t = 0$) to the time of detection by passive surveillance ($t = \mathrm{ps}$). This overall infectiousness multiplied by a constant c, which contains information on the between-flock contact rate and the probability of transmission, defines the expected between-flock reproduction number R_h:

$$R_h = cA(\mathrm{ps}).$$ (2)

Because the transmission dynamics in vaccinated flocks are different from the one in unvaccinated flocks and mortality levels are likely to be lower, thereby compromising detection by passive surveillance, we did not estimate infectiousness for vaccinated flocks, and we assumed instead that an infected vaccinated flock would remain undetected by passive surveillance until it reaches a level of infectiousness similar to the one we estimated for an unvaccinated flock. In other words, we used the estimated $A(\mathrm{ps})$ for unvaccinated flocks as a reference to assess the efficacy of implementing active or enhanced passive surveillance (see below).

Active or enhanced passive surveillance and its effect on transmission

Even when surveillance is performed on a regular basis, there remains a probability that an infected flock escapes detection. This can occur, for example, when the prevalence of infected birds at the time of sampling is still lower than the predefined design prevalence. In such cases, there is a potential risk of transmission to flocks in other establishments. The probability of escaping detection (P_{esc}) at time t is defined as

$$P_{\mathrm{esc}}(t) = \prod_{\tau=0}^{\tau=t} \exp\left(-\frac{p(\tau)n\mathrm{Se}}{\Delta}\right),$$ (3)

where $p(\tau)$ is the prevalence of infected birds, which depending on the sampled population could be dead birds, infectious live birds or recovered-seroconverting live birds. Se is the diagnostic test sensitivity expressed in values between 0 and 1 (e.g. qPCR for swab samples from dead or infectious live chickens or ELISA for serum samples to detect seroconverting live birds) and n is the number of birds sampled (sample size). Finally, Δ is the sampling interval (in days) and refers to the repeated sampling performed on a flock at a determined time interval with the probability of escaping detection at time τ with $p(\tau)$ being conditional on the probability of having escaped detection at an earlier sampling time with $p(\tau - 1)$ within the sampling interval. A key parameter determining the probability of escaping detection is the prevalence of infected birds at the time of sampling $p(t)$. However, in reality, it is not known when a flock could become infected. Therefore, this model considers that the introduction of infection takes place at a random moment between two adjacent sampling events. Following this consideration, the expected prevalence of infected birds can be calculated by averaging the prevalence of infected birds at the moment of sampling $p(\tau)$ over all possible introduction moments within Δ (Gonzales et al., 2012; Graat et al., 2001). In this analysis, we assumed perfect specificity, which means that any positive result, particularly if detection is based on a serological test, would need to be followed up to confirm or exclude infection.

Next, we estimated the expected infectiousness of a flock that escapes detection ($A_s(t)$). This infectiousness is a function of the fraction of infectious birds escaping detection at time (t) of sampling as

$$A_s(t) = \int_0^t I(\tau)P_{\mathrm{esc}}(\tau)d\tau.$$ (4)

The expected infectiousness of a flock that escaped detection was then obtained by integrating over $t \in [0, \mathrm{ps}]$. If surveillance is effective, we expect $A_s(\mathrm{ps})$ to be lower than $A(\mathrm{ps})$ and this reduction in infectiousness would result in a proportional reduction in R_h, hence similar to Equation (2), the expected between-flock reproduction number given surveillance (R_s) (Gonzales et al., 2012) can be calculated as

$$R_s = cA_s(\mathrm{ps}).\tag{5}$$

From Equation (2), we obtained $c = \frac{R_h}{A(\mathrm{ps})}$ and by substituting c into Equation (5) we got:

$$R_s = \frac{A_s(\mathrm{ps})}{A(\mathrm{ps})}R_h.\tag{6}$$

When $R_s < 1$ a flock would be detected on average before it transmits infection to another flock in one or more other establishments ('early detection'). Under this requirement, large epidemics would not be expected, due to early detection and removal.

A key parameter to consider in the model is R_h. We have only estimated R_h for unvaccinated poultry populations in some European countries (EFSA AHAW Panel, 2023) and there are no specific estimates of R_h in populations where there is a combination of vaccinated-protected poultry flocks, vaccinated-partially protected flocks and unvaccinated flocks. Because of that, we had to assume a hypothetical value. In poultry-dense populations like those included in the Opinion Part 1, one could expect that R_h exceeds 1, but is lower than the R_h estimated in unvaccinated populations.

Model implementation

Using the parameters reported in Table 3, a stochastic compartmental SEIRD model (Hobbelen et al., 2020) was built using the library SimInf (Widgren et al., 2019) in the statistical software R (R Core Team, 2018). We refer the reader to Widgren et al. (2019) for a detailed overview of stochastic compartmental models within this package. A total of 1000 simulations of HPAI outbreaks (for each poultry species) in vaccinated flocks were performed. Building on these simulations, the above-mentioned surveillance model was implemented to quantify $A(t)$ and $A_s(t)$. These values were then related to a hypothetical R_h to calculate R_s as the reference measure to compare different surveillance strategies, encompassing different sample types, sizes and frequencies at the flock level. The methodological steps were as follows:

– We selected outbreak simulations in which a significant number of infected birds (I) occurred, i.e. those with a peak of I exceeding five birds (regardless of the assessed flock size). We considered these outbreaks 'major outbreaks'.
– For each of these major outbreaks, we estimated $A(t)$ and $A_s(t)$. The latter depends on the surveillance strategy assessed.
– Surveillance strategies assessed were a combination of the type of sample, the sample size, sampling frequency and the diagnostic test used.
– Different sampling strategies were considered, involving the random selection of birds and sample sizes. Specifically, all dead birds up to a number of 5, 10 and 15 or more dead birds were randomly sampled and tested. If there were fewer dead birds than the required sample size (e.g. 5) on a given day (or a combination of 2 days), all available dead birds are sampled and tested. Note that in the model one additional day was assumed between this sampling/testing day and the diagnostic results. In addition, scenarios where live birds are randomly selected for sampling were also assessed. We assessed a sample size of 60 birds (as suggested in the EU Regulation), but also tested lower ($n = 30$) or higher sample sizes ($n = 90$ or 120). Note that sampling of birds refers to sampling birds in a flock, considering that an establishment can have more than one flock, this sample sizes would have to be taken for each flock within the establishment.
– Each considered sample size was assessed in combination with different sampling intervals: every 7, 14 and 30 days as recommended in the EU Regulation. In addition, we assessed longer sampling intervals. A combination of sample size and sampling interval was considered suitable only if the probability to escape detection reached levels lower than 0.01 (Figure 4) for more than 95% of the outbreak simulations. This means that at least 99% of outbreaks are detected in ≥ 95% of the simulations, given that surveillance strategy.
– To assess the effectiveness of surveillance for early detection, we defined the following hypothetical R_h values (see model description) in unvaccinated flocks ranging from 1.4 to 1.8, which are the mean R_h estimates obtained, from the transmission kernel analyses done for the Opinion Part 1 using data from epidemics in France, Italy and the Netherlands (EFSA AHAW Panel, 2023).
– We then derived the following performance indicators:

  ○ Reduction of infectiousness $A_s(t)$ and R_s, given detection.
  ○ Median time from introduction to detection of an outbreak, which was derived as the time when the probability of escape at the farm level was 0.5 (Figure 4).
  ○ In addition, the prevalence of infectious and recovered (seroconverting) birds at the time of detection was also reported.
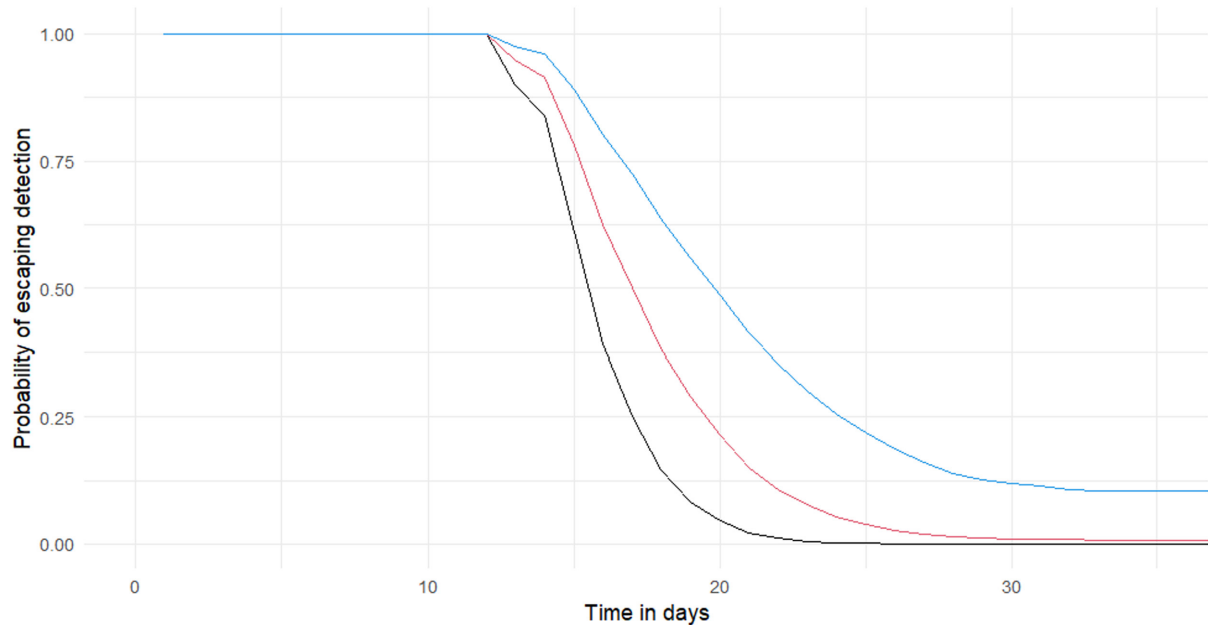
FIGURE 4 Probability of an infected vaccinated flock (one outbreak simulation is shown) escaping detection given three surveillance strategies based on sampling five dead birds at an interval of 7 days (black line), 14 days (red line) and 30 days (blue line). In this flock the probability of escaping detection when sampling at intervals of 14 and 30 days does not decrease below the required level of detection 0.01 (≥ 99% of probability of detection). This requirement could be reached by increasing the number of birds sampled. The x-axis is the time in days from the start of outbreak in the flock.

2.1.5.2 | *Methodology to assess surveillance for early detection (P1) and demonstration of freedom (P3, P4) following preventive vaccination*

Scenario tree models were used to estimate the sensitivity (Se) of the surveillance systems to demonstrate freedom from disease and to provide inputs for assessing early detection of HPAI, the latest only in a preventive vaccination scenario. A scenario tree model is a structured approach to describe the process of disease detection of a surveillance system or a component of that. A surveillance system component is a surveillance activity that, in itself, can contribute evidence to disease freedom; it has the capacity to detect disease if/when it occurs. This includes both general and targeted surveillance activities; both active and passive. A scenario tree graphically traces all pathways by which a single unit can be detected as infected by the system; it defines the conditional probabilities associated with each step in the pathway and it is useful for calculating surveillance components' sensitivity and the sensitivity of the surveillance system as a whole (Martin, Cameron, Barfod, et al., 2007; Martin, Cameron, & Greiner, 2007). This analysis provides an estimation of the probability that the surveillance would be able to detect an infection or a disease in the unit of interest (an animal, a sample, a pooled sample, a group of animals, a region), given that the condition is present in the population at a level specified by the design prevalence. Surveillance component sensitivity (CSe, see Glossary) is the average probability that a surveillance system component will return a positive surveillance outcome, given that the disease is present in the population at a level equal to or greater than the specified design prevalence.

The logical and practical structure of the surveillance strategy applied can be outlined by a scenario tree, in which the process of disease detection is quantitatively described by including all factors affecting the probability that a surveillance unit will be infected and all those affecting the probability that will be detected. In each branch of the tree, which usually identifies a surveillance component, proportions and probabilities are assigned to a node, one for each stage of the diagnostic process. We can identify different types of nodes: category nodes, which define groups into which units or group of units fall, risk nodes (the population into subsets with different risks of being infected at the design prevalence), infection nodes (infection status of a unit), detection nodes (any event, action or test which contributes to an infected unit's detection in the surveillance process). An example of the scenario tree model used for this assessment is provided in Figure 5. This tree illustrates the category of the population which is vaccinated and represents the target of surveillance (which is the focus of the assessment), commercial chicken layers in this example. The tree includes multiple risk nodes. The first risk node is associated with the relative risk (RR) of virus incursion in the zone, which describes the probability of virus incursion in a given geographical area relative to the probability of the same event in the other geographical areas (RRLow, RRMod, RRHigh). The proportion of poultry present in this incursion zone is also attributed to this risk node (Prpoultry). The second risk node refers to the RR of virus incursion in poultry, which describes the probability of virus incursion in a given poultry type, relative to the probability of the same event in the other poultry types (RRLayers&breeders, RRBroilers, RRTurkeys, RRDucks). The proportion of poultry type being present in this incursion zone is attributed to this risk node (Prlayers, Prbroilers, Prturkeys, Prducks). The tree also includes probability nodes, that are the probability that a vaccinated establishment is protected (P_{vp}) and the probability that sustained transmission is established following incursion (P_{inf}). P_{inf} was calculated based on a design prevalence of 0.01 multiplied by the probability of having major outbreaks (for simplicity, in

unvaccinated flocks, this probability is assumed to be 1 and for vaccinated flocks this probability is $1 - 1/R_v$, where R_v is the within-flock transmission parameter).

As ultimate outcome of the tree there is the probability that an outbreak is detected (P_{pos}) given the Se of testing at flock level (F_{se}). Note that one or multiple flocks could be present within an establishment, and that the detection of one infected flock is enough to consider the whole establishment infected (see Table 4 for an explanation of these elements).
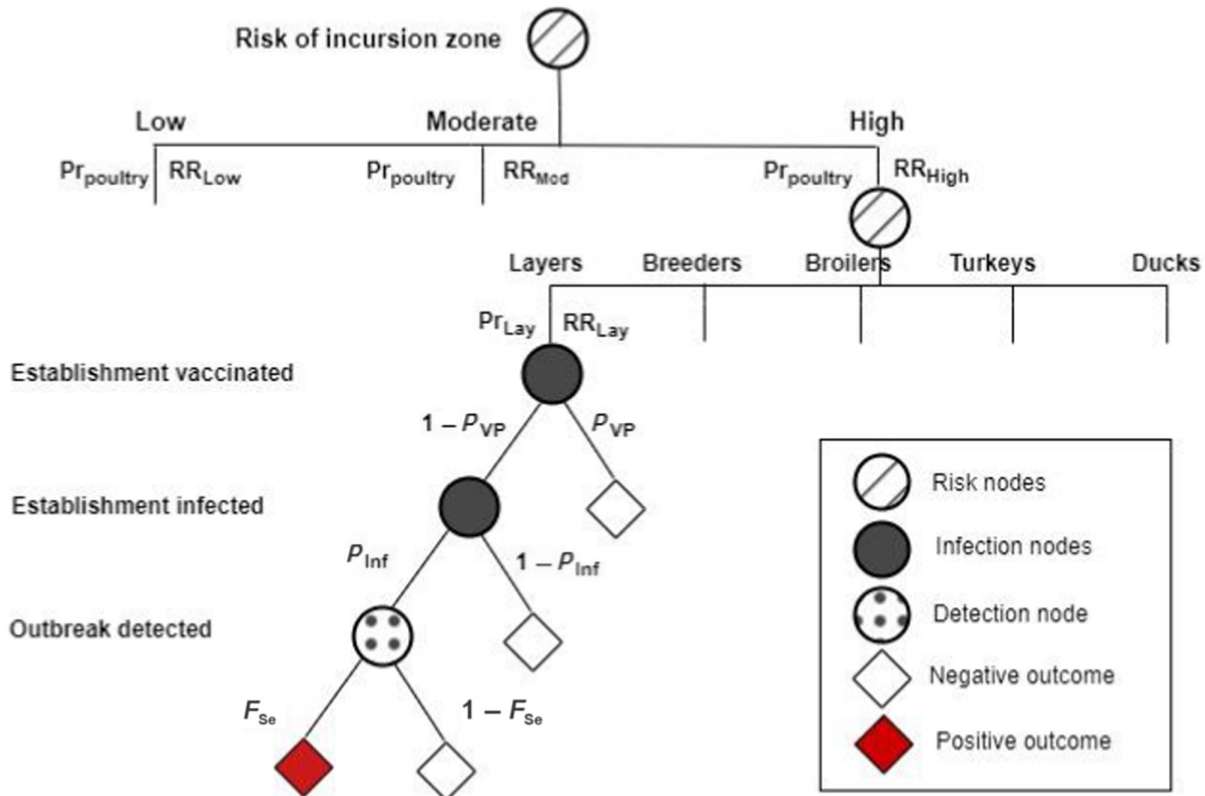
FIGURE 5 Illustrative diagram of a scenario tree model describing the surveillance process applied to the poultry population vaccinated. For this illustration, chicken layers represent the target for preventive vaccination in a given country and the unit where surveillance is applied is the establishment. A similar scenario can be derived for any other poultry sector/species targeted for vaccination (e.g. ducks in France or turkeys in Italy) and at other unit level (e.g. flock, zone). F_{Se}, sensitivity of testing at establishment level; Lay, layers; P_{Inf}, probability of infection; Pr, proportion; P_{VP}, probability that a vaccinated establishment is protected; RR, relative risk.

The risk nodes contain information on the proportion of the population within each node and the RR attributed to that. The RR describes the probability of virus incursion in one group relative to the probability of the same event in the other group. These two are combined into adjusted RR (AR for the zone: ARLow, ARMod, ARHigh; AR for poultry: ARLayers&breeders, ARBroilers, ARTurkeys, ARDucks), which ensures that the average design prevalence is constant across the population (for more details, see Martin, Cameron, & Greiner, 2007).

The probability of detection of a truly infected flock/establishment (outbreak) was given by:

$$p_{pos} = AR_{zone} \times AR_{pt} \times (1 - P_{vp}) \times P_{inf} \times F_{Se}. \tag{7}$$

For unvaccinated flocks, $P_{vp} = 0$. The testing strategy at the establishment (flock) level and corresponding F_{Se} is derived from the early detection model described in the previous section. Then, the sensitivity of a surveillance component (CSe), which represents the probability that at least one infected flock (establishment) is detected and thus refers to a specific part of the overall surveillance system, was given by:

$$CSe_i = 1 - (1 - P_{pos})^n. \tag{8}$$

With i identifying each specific component of the system and n being the number of establishments sampled within the component. If all farms are sampled, then the exact method to calculate CSe was used as described by Martin, Cameron, Barfod, et al. (2007) and Martin, Cameron, and Greiner (2007). Note that, if an establishment has more than one flock, each flock needs to be sampled following the defined surveillance strategy. One infected flock is sufficient to consider the whole establishment infected. The WOAH standards state that all vaccinated establishments need to be sampled. Hence, n in our reference surveillance scenario includes the total number of vaccinated establishments within the target surveillance area/zone. Additionally, we assessed scenarios based on random sampling of a representative number of establishments.

TABLE 4 Parameter values included in the model and the source from which these values were obtained.

Parameter	Description	Values	Reference
Relative Risk of virus incursion in the geographical zone	The relative risk of virus incursion in the zone describes the probability of virus incursion in one given geographical area, relative to the probability of the same event in other geographical areas (RRLow, RRMod, RRHigh). These relative risks were estimated by analysing data of primary introductions of HPAI in poultry in the Netherlands and were used for modelling surveillance in France and Italy as well	Mean (95% CI)[a] RRLow = 1 (reference) RRModerate = 2.98 (1.80–4.78) RRHigh = 6.62 (4.40–9.90)	This Opinion. Estimates were derived from data in Gonzales et al. (2023)
Relative risk of virus incursion in poultry species	The relative risk of virus incursion in poultry describes the probability of virus incursion in one given poultry species, relative to the probability of the same event in the other poultry species (RRLayers&Breeders, RRbroilers, Rrturkeys, Rrducks). The relative risks estimated for the Netherlands were used for modelling surveillance in France and Italy as well	Mean (95% CI)[a] RRLayers&breeders = 1 (reference) RRBroilers = 0.3 (0.1–0.7) RRTurkeys = 2.8 (1.1–6.5) RRDucks = 3.7 (1.8–7.6)	This Opinion. Estimates were derived from data in Gonzales et al. (2022)
Proportion of poultry population (Pr)	The proportion of specific poultry type establishments (e.g. turkeys) within the area/zone targeted for surveillance	Country specific	Opinion Part 1 (EFSA AHAW Panel, 2023)
Probability protected (P_v)	The proportion of establishments (flocks within the establishments) effectively protected following vaccination	0.7 (0.5–0.8)[a]	Opinion Part 1 (EFSA AHAW Panel, 2023)
Probability of infection (P_{inf})	This is the probability of having a major outbreak following virus incursion adjusted by the hypothetical design prevalence. $P_{inf} = DP \times (1 - 1/R_v)$, where R_v is the within-flock transmission parameter. For unvaccinated flocks, the probability of a major outbreaks is assumed to be 1	R_v values[a] for Chickens: 2.1 (1.03–4.0) Ducks: 2.7 (1.3–4.0) Turkeys: 2.6 (1.3–4.0) Design Prevalence (DP) = 0.01	Germeraad et al. (2023) Grasland et al. (2023). Table 3
Se sampling at flock level (F_{Se})	Combination of sample size and sampling interval derived using the methodology for early detection described in Section 2.1.5.1	Se values[a] [Median (lower – higher)][b] when sampling 15 birds every 30 days were: Chickens: 97 (92–99) Ducks: 96 (89–98) Turkeys 96 (87–97)	This Opinion

[a]For all these parameters, a pert distribution was used in the stochastic simulations.

[b]These values are the percentage of outbreak simulation detected (Sensitivity) for probabilities of escaping early detection of < 0.05 (median). Lower and higher values used for the stochastic simulations around this parameter (using a pert distribution) were derived by using the escaping probabilities < 0.01 (lower value) and < 0.1 (upper value).

Then, to assess the early detection surveillance sensitivity of each SSC (EDSe$_i$), that is the probability that the surveillance activity is able to correctly detect, among the poultry establishments, the epidemiological unit(s) affected by a new incursion within a specific time frame, we followed the method proposed by Cameron et al. (2020):

$$EDSe_i = Cp_i \times Ct_i \times CSe_i, \qquad (9)$$

where Cp$_i$ corresponds to the proportion of the population, in component i, targeted for surveillance, with Ct$_i$ being the temporal coverage, indicating the conditional probability that an establishment will be tested within a predefined time frame, e.g. the time corresponding to median time from introduction to detection of an outbreak by passive surveillance divided by the given sampling interval. Time frame values for unvaccinated species and vaccinated species were extracted from results provided in Tables 15A–17B. CSe$_i$ corresponds to the sensitivity of the surveillance component i (Equation. 8).

After the EDSe for each surveillance component had been calculated, the total surveillance system sensitivity (TotalSe, see Glossary) could be summarised as:

$$TotalSe = 1 - \prod_i \left(1 - EDSe_i\right). \qquad (10)$$

With i being the number of component's EDSe.

Finally, the probability that the population is free from HPAI (Pfree), given that surveillance did not detect any infected establishment and assuming perfect specificity, as stated in the previous section, was based on Martin, Cameron, Barfod, et al. (2007) and Martin, Cameron, and Greiner (2007) and given by:

$$\text{Pfree} = \frac{1 - \text{priorInf}}{1 - \text{priorInf} \times \text{EDSe}}, \tag{11}$$

where priorInf is the prior probability that the poultry population at the establishment level would be infected (hypothetical prior prevalence in the population, see Glossary). To determine this prior probability, we used the hypothetical design prevalence of 1% times the risk (probability) for infection during the autumn–winter months relative to that in the spring–summer months. This relative risk was considered to be 5 (Gonzales et al., unpublished data). Consequently, priorInf was assumed to be 5%.

In Equation (11), we used either EDSe or TotalSe depending on the vaccination status of poultry establishments for which the probability of freedom was estimated. We used EDSe if we focused on the vaccinated establishment and TotalSe if we focused on all the establishments (vaccinated and unvaccinated) subjected to surveillance in all five components of the scenario tree.

2.1.5.3 | *Methodology to assess vaccination effectiveness and level of immune response induced by vaccination (surveillance strategies E2, P2)*

The surveillance strategies to assess vaccination effectiveness and the level of immunity response, E2 and P2, respectively, were addressed descriptively and the assessment performed is reported in Sections 3.1.2.4 and 3.1.2.5.

Vaccine effectiveness (VE) (E2) was considered as the ability of a vaccine to protect animals from a disease or an infection under field conditions. It differs from **vaccine efficacy**, which is measured under controlled conditions in laboratory settings typically through challenge experiments. The effectiveness of vaccination in real scenarios is likely to vary across countries and vaccination campaigns and can be assessed by observing the occurrence of HPAI outbreaks in a country where vaccination is implemented.

VE can be estimated as 1 minus a measure of RR in the vaccinated population compared with the unvaccinated population. These RR measures depending on the type of data collected and method of analysis could be for example: the RR, based on incidence or rates of infection, prevalence ratio (attack rates, cumulative incidence), the odds ratio (OR) or the hazard ratio (HR).

Level of immune response induced by vaccination (P2). Given the lack of knowledge on identifying a correlate of protection, e.g. a given HI titre, which can be used to monitor the level of immunity reached within a flock (EFSA AHAW Panel, 2023), possible pragmatic approaches to estimate the level of immune response induced by vaccination were given and general considerations, e.g. on when to apply the sampling, were discussed.

2.2 | ToR 4 – Risk mitigation measures

In ToR 4, the assessment focused on risk mitigation measures to be implemented in vaccinated poultry aiming at preventing the spread of HPAIV by moving poultry and their products following emergency and preventive vaccination. Firstly, the measures laid down in the Delegated Regulation (EU) No 2023/361 and in the general and specific conditions of Delegated Regulation (EU) No 2020/687[8] for derogation were assessed (ToR 4.2). To enhance clarity, in Figures 6 and 7, these risk mitigation measures provided in the current legislation are described as two distinct groups: testing measures and other measures. It is important to highlight that these two categories of measures are not alternatives, but they complement each other. Secondly, alternative risk mitigation measures were defined by the Working Group, for both emergency and preventive vaccination scenarios, as requested in ToR 4.2.

2.2.1 | Risk mitigation measures as per delegated regulation (EU) No 2023/361 after vaccination

Delegated Regulation (EU) No 2023/361 envisage different risk mitigation measures to be implemented following vaccination in poultry, captive birds and wild birds.

According to Part 3 in Delegated Regulation (EU) No 2023/361, when implementing **emergency protective vaccination**, vaccinated animals and their products will be subject to the prohibition of movements within and outside the vaccination zone. The competent authority may allow the movement of animals if they are subject to compulsory killing or if their movement has been authorised. Movements of vaccinated poultry/captive birds/their product can be authorised if all the conditions for moving them from protection and surveillance zones provided in Delegated Regulation (EU) No 2020/687[9] (Article 28, 29, 30, 31(1), 33, 34 and 37) are fulfilled (Figure 6). At the end of the recovery period (i.e. 28 after completion of emergency protective vaccination or when lifting the restricted zones), all the measures for granting a

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[8]As per Articles 28, 29, 30, 31 (1), 33, 34 and 37.
[9]As per Articles 28, 29, 30, 31 (1), 33, 34 and 37.

derogation for moving that are provided for preventive vaccination applies (Figure 7), including the reinforced surveillance to be implemented for preventive vaccination (see Table 1).

According to Points 3 to 4 of Part 5 in Delegated Regulation (EU) No 2023/361, when implementing **preventive vaccination**, the movement of animals and their products from the establishment of vaccination is prohibited. The competent authority may allow the movement of animals and their products if they are not included in the list of animals and products subject to the prohibition of movements or if they comply with relevant conditions and their movement has been authorised. The conditions for granting a derogation for movement are described in Figure 7.
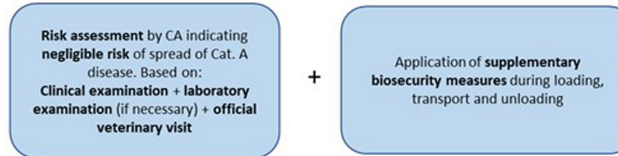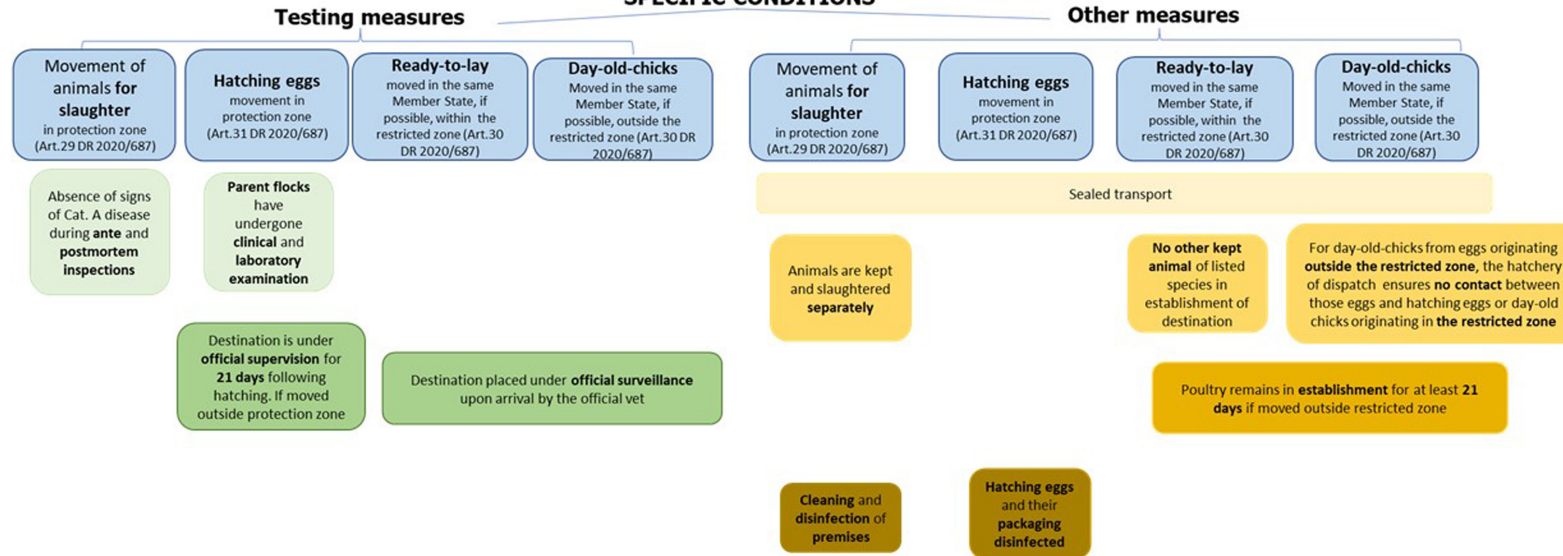
FIGURE 6 Provisions as in Delegated Regulation (EU) No 2020/687 to grant authorisation for movement of poultry/captive birds/their product within and from emergency vaccinated zones.
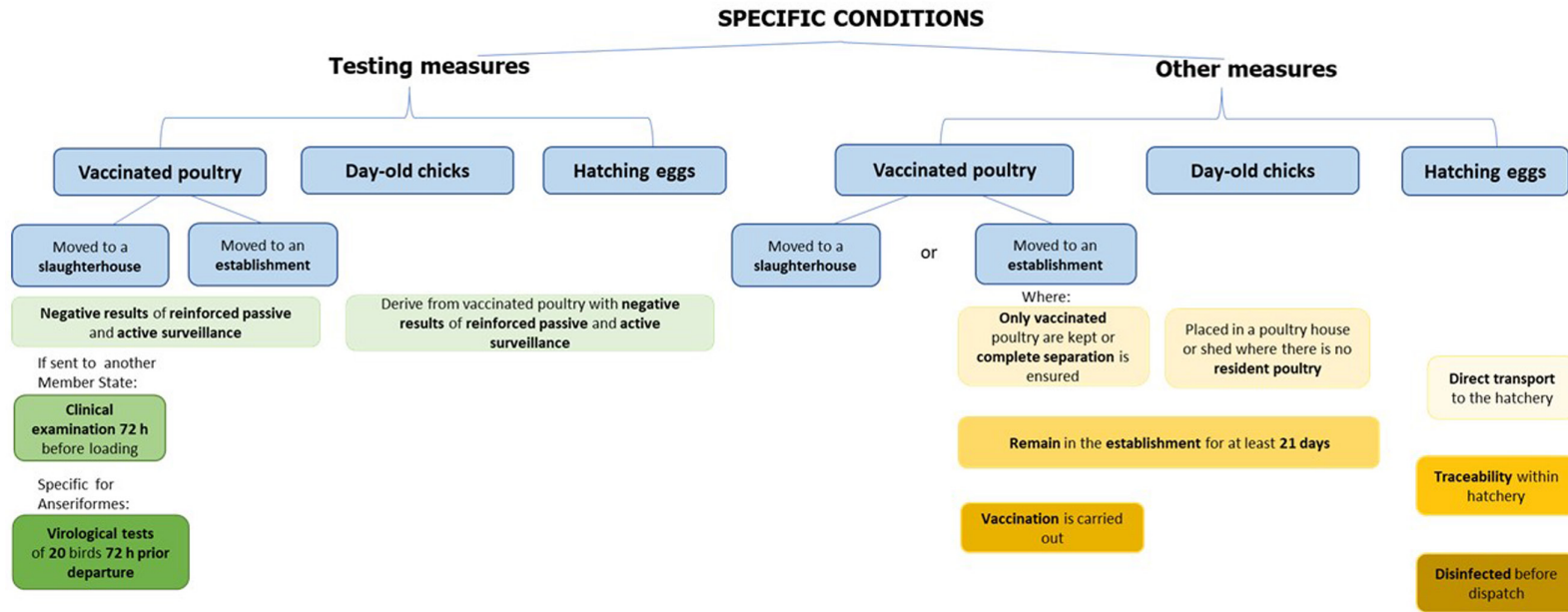
FIGURE 7 Provisions as in Delegated Regulation (EU) No 2023/361 to grant authorisation for movement of poultry/captive birds/their product within and from preventive vaccinated zones.

2.2.2 | Definition of risk mitigation measures scenarios

In addition to the mitigation measures described in Section 2.2.1, the Working Group identified and assessed the provision of demonstrating freedom from HPAIV as an alternative approach to enable safety movement of poultry and their products within and outside vaccinated zones and from a vaccinated establishment. The methodology used to demonstrate freedom from HPAIV was the scenario tree model as it is described in Section 2.1.5.2.

2.3 | Uncertainty analysis

The uncertainty analysis was performed following the procedure detailed in the EFSA guidance on uncertainty analysis in scientific assessments (EFSA Scientific Committee, 2018). The sources of uncertainty associated with the data available and methodology used are listed and discussed in Section 3.3, but their impact on the assessment was not quantified. For the assessment of the effectiveness of the surveillance strategies at the establishment level (except 'passive reporting' and 'mortality threshold'), the percentage of simulated outbreaks in which the desired detection probability threshold (> 99%) was achieved was used as a proxy for the certainty regarding the effectiveness of a particular surveillance strategy, so that only strategies in which > 95% certainty was achieved were considered effective. In the case of surveillance strategies based on 'passive reporting' and 'mortality threshold', the certainty on their effectiveness was quantified using the 97.5% confidence intervals for the between-flock transmission (R_s) obtained using the transmission models, so that these strategies were considered effective for HPAIV early detection if the 95% upper confidence limit was below 1 (i.e. their use stopped sustained between-flock transmission).

3 | ASSESSMENT

3.1 | ToR 3: Surveillance

3.1.1 | Diagnostic methods

Ideally, efficient vaccination results in sterile immunity, i.e. the vaccinated animals can no longer become infected upon virus exposure and therefore can neither fall ill nor replicate and excrete the virus. In reality, this state is usually not achieved on a population base. Thus, individual infections in a vaccinated flock that also lead to virus excretion by vaccinated animals cannot be fully excluded. However, the threshold to become infected should be higher and subsequent virus excretion should be significantly lower in amplitude and shorter in time than that of the unvaccinated animals. As a result, smaller amounts of virus at a lower prevalence compared with unvaccinated exposed flocks are available for detection. In order to detect these, the methods with the highest sensitivity should be used in the active surveillance of vaccinated flocks. When assessing various diagnostic methods in this respect, their analytical and diagnostic sensitivity and specificity must be considered along with other factors which can modify their performance characteristics: sample matrices (increased risk of the presence of PCR inhibitors in certain matrices e.g. faeces), enrichment effects (e.g. through skilful selection of target subjects – dead/diseased animals have a higher probability of being virus-positive) or dilution effects (pooling of samples). Vaccinated animals falling sick or dying due to HPAIV infection in vaccinated flocks, in contrast, in most cases are expected to produce amplitudes of virus similar to unvaccinated birds.

3.1.1.1 | *Virological methods*

Surveillance activities can rely on virological examination of samples collected in vaccinated flocks to obtain timely information about the active circulation of field viruses inside the flock. Vaccination can narrow the window of viral shedding of birds and lower viral replication titres; thus, the ability of virological tests to detect active viral circulation in flocks may diminish. Before molecular methods became widely available in veterinary laboratories, detection of AIV from clinical samples was performed by inoculation of embryonated specific pathogen free (SPF) chicken eggs (ECE). However, nowadays, inoculation of ECE is generally used only on PCR-positive samples as a confirmatory assay or to obtain viral isolates for phenotypic characterisation, since several factors can negatively affect viral isolation success and therefore the sensitivity of the method.

Virus isolation

The preferred method of growing influenza A viruses is by the inoculation of SPF ECE, or specific antibody-negative (SAN) eggs. The use of ECE in some EU Member States may be considered an animal experiment that is subject to authorisation by an ethics committee. Clarified supernatant preparations of clinical samples are inoculated in the chorioallantoic sac of three to five embryonated SPF or SAN ECE of 9–11 days of incubation. The eggs are then incubated at 37°C for 2–5 days and, if deemed necessary, a second passage can be performed. The chances of virus replication are determined by the infectivity retained in the sample. This, in turn, is influenced by characteristics of the sample and the way in which it is transported

and stored to preserve the infectivity of the virus. Virus isolation is not recommended for screening during vaccination-assisted surveillance due to the long duration of the test (embryos are monitored up to 5 days following inoculation by candling and a second passage of again up to 5 days may be necessary to prove the absence of replicating virus). In addition, the high number of samples to test generates high costs for the SPF eggs and binds staff for a considerable time. In the surveillance activities to be implemented in vaccination zones, considering these limitations, the use of virus isolation can only be considered as an alternative to molecular methods in exceptional circumstances limited in time (e.g. lack of reagents for molecular biology methods). However, following outbreak confirmation in vaccinated populations, it would be important to attempt viral isolation to allow further phenotypic characterisation that requires the availability of the live HPAI virus.

3.1.1.2 | *Molecular methods*

Conventional RT-PCR and Real-time RT-PCR (RT-qPCR)

Molecular techniques are now used preferentially for the diagnosis of HPAIV infection directly from clinical specimens of birds. The current definition of HPAIV according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals Chapter 3.3.4 – Avian influenza (including infection with high pathogenicity avian influenza viruses) (WOAH, online-b), includes the molecular identification of virulence factors in the endoproteolytic cleavage site of the haemagglutinin (HA) protein of AIV of subtypes H5 and H7 and therefore enables the use of molecular techniques in the diagnosis of HPAI. Although conventional RT-PCR techniques could result in faster detection and subtype identification (at least of H5 and H7) at lower costs compared with virus isolation, and also provide a PCR amplicon product ready for nucleotide sequencing to define the pathotype, real-time amplification techniques have largely replaced conventional RT-PCR techniques. Real-time quantitative RT-PCR (RT-qPCR) offers increased turn-around time, added cross contamination prevention and is generally applicable to high-throughput analyses at all steps of the diagnostic algorithm, i.e. generic IAV detection, subtype specification and pathotype characterisation. Thus, these types of tests are now routinely used to process samples collected during surveillance activities in the EU. Clinical samples positive in tests specific for H5 or H7 must be further investigated for the identification of molecular signatures typical of HPAI or LPAI viruses. Due to its superior analytical sensitivity, pooling clinical samples is possible with RT-qPCR which further reduces costs. Pool size represents an important measure to reduce costs and allows the processing of larger numbers of samples, if carefully applied. Yet, pooling intrinsically decreases the analytical sensitivity of detection as samples are diluted. When pooling 5–10 samples this effect is usually negligible (Fereidouni et al., 2012; Ladman et al., 2012) but, nevertheless, arithmetically may lead to false-negative results for single samples with viral loads close to the limit of detection. Guidelines based on laboratory evidence on pooling recommendations in different species are available on the EURL website (EURL, online). Laboratories processing samples in pools should validate their internal procedures aiming to maximise efficiency without significantly affecting diagnostic sensitivity of the methods.

For screening purposes, generic M gene targeting RT-qPCR methods (e.g. SOP VIR018 available on the EURL website (EURL, online)) are preferred due to higher diagnostic sensitivity (99%–100%) and specificity (99%–100%) compared with other targets. Such methods can be used on clinical samples collected from live or dead birds for early detection or monitoring freedom from infection in vaccinated or unvaccinated poultry flocks. If vaccination reduces virus shedding effectively, the probability of any PCR to detect a positive sample will decrease. Also, the interpretation of subtype-specific RT-qPCRs in flocks vaccinated with modified live/vectored H5 or H7 vaccines could be affected: Organs/injection sites where active replication of the vaccine is taking place (e.g. spleen) would yield positive results to such assays even in the absence of field virus. Further investigations on recommended diagnostic methods to be applied in animals vaccinated with live vectored vaccines are currently ongoing at EURL. Specific RT-qPCRs tailored to the vector vaccine used may be required to distinguish residual vector vaccine inserts from HPAI field virus. The residual genetic material in birds vaccinated with inactivated vaccines is not to be expected unless injection sites are sampled and examined. Data available from vaccination trials performed in the EU against recent strains belonging to 2.3.4.4b clade indicate that samples collected from the respiratory tract, such as oropharyngeal/tracheal swabs, should be favoured in surveillance activities over cloacal swabs. The use of only oral swabs would be justified by higher and longer shedding compared with the ones observed in cloacal swabs obtained from chickens and turkeys. This would help save resources, labour and time for sampling in active surveillance programmes according to Delegated Regulation (EU) No 2023/361. Any positive result in clinical samples should be interpreted as potential indication of an AI infection and prompt immediate identification of the respective AIV sub- and pathotype by appropriate methods and time for sampling in active surveillance programmes according to Delegated Regulation (EU) No 2023/361.

The same assays used for surveillance in animals could also be applied to analyse sample matrices different from clinical specimens such as environmental samples collected for surveillance purposes or for verification of cleansing efficacy (e.g. swabs from surfaces, drinking water, air samples, etc.). Delegated Regulation (EU) No 2023/361 imposes the condition to use exclusively clinical sample material in vaccination surveillance programmes. However, the extent of sampling and investigating is considerable and might itself form an obstacle to the implementation of vaccination. Thus, the use of further informative matrices of the environment in poultry holdings could be a measure to mitigate those obstacles. Appropriate systematic validation studies of these matrixes and appropriate exogenous inhibition controls for environmental samples

are still missing. Also, based on farm infrastructure and poultry species, the usefulness of different environmental samples can vary considerably (Grasland et al., 2023).

Molecular methods can also be used for monitoring vaccination coverage in birds vaccinated with live vectored vaccines. For example, vector replication and therefore expression of the immunising antigen can be monitored by qPCR assays specifically designed to detect vaccine-specific genomic sections. For HVT, vector replication, and therefore vaccine uptake, can also be assessed in non-invasive samples such as feather pulp. However, the intermittent presence of vector-associated genomic material in these types of samples is a major limitation for the accurate estimation of vaccination coverage in vaccinated poultry flocks (Cortes et al., 2011).

Next-generation sequencing (NGS) technologies

Novel DNA sequencing techniques, commonly referred to as 'next-generation' sequencing (NGS), are characterised by high speed and throughput allowing the production of an enormous volume of data with many applications in research and diagnostic settings. Several commercially available high-throughput sequencing platforms exist that vary by way of their sequencing principle, sequencing speed, expense and read length. The amount of total nucleic acids belonging to viral particles in clinical samples is often too low for sequencing and may require amplifying of viral nucleic acid using targeted or non-targeted approaches. The aim of these sequencing approaches is to (i) sequence all eight IAV gene segments, (ii) verify the IAV H gene subtype as reported by existing RT-PCR assays, (iii) determine the IAV N subtype and (iv) generate sequence data for analysis of the IAV hemagglutinin cleavage site, for pathotype assessment. In optimal scenarios and using real-time devices, this information can be obtained in less than 24 h, which is in many cases a longer turn-around time than RT-qPCR-based approaches. NGS technologies do not see a wide application for surveillance purposes mainly because of costs and time for analysis, while they are of high value in the further characterisation of detected AI viruses. Analysis of sequencing data can provide valuable information to monitor viral evolution and possible sources of introduction. Moreover, several studies have identified genetic markers associated with specific phenotypic behaviours that can be identified and used to infer the properties of the virus. In the context of HPAI vaccination, monitoring of viral genetic characteristics under the selective pressure exerted by immunity towards HPAI in vaccinated flocks allows the detection of mutant viruses and informs on the need for vaccine updates and the risks to public health.

Other amplification techniques

Several alternative amplification strategies have been developed and published including loop-mediated isothermal amplification (LAMP), strand displacement amplification (SDA) or Crispr amplification techniques. Some applications have been linked to point-of-care testing also. However, validation data for use in vaccinated poultry populations are scarce, and commercial products are not readily available at this moment. The potential of these methods has not been yet fully explored.

3.1.1.3 | Rapid antigen detection systems

Commercially available generic antigen detection kits are mostly based on enzymatic immunoassays or on immunochromatography (lateral flow devices) and generally use a monoclonal antibody against the nucleoprotein or other highly conserved regions of the structural proteins of AIVs. Some of these tests have been designed for the detection of influenza A viruses in poultry and should be able to detect any influenza A virus independent of its subtype. The main advantage of these tests is the rapid confirmation of the presence of an influenza A virus in a few (< 20) minutes. They do not require special laboratory equipment and therefore can be used directly on poultry premises without the need of highly trained personnel. However, they suffer from a substantial lack of sensitivity compared with RT-qPCR, which can produce false-negative results. Given the intended and expected reduction of viral replication in exposed vaccinated birds and the reported lack of sensitivity, it is highly unlikely that such tests would be useful for active surveillance purposes in vaccinated flocks. However, birds that succumb to HPAIV infection in infected vaccinated flocks might still produce sufficient virus loads to be detected by rapid antigen assays (i.e. bucket sampling). Nevertheless, at the current stage, there is not enough information on the diagnostic sensitivity of these tests in field conditions to recommend their use in surveillance activities aimed at the identification of outbreaks or for pre-movement virological testing of vaccinated flocks. For similar reasons, any positive result obtained with such assays must be further investigated by validated methods characterised by higher specificity to confirm the results.

3.1.1.4 | Serological methods

Upon infection with HPAIV in birds, antibodies are mounted by the animals as part of the immunological response. The antibodies produced are generally directed towards all the viral proteins expressed although variabilities exist in terms of the relative quantities of antibodies produced. Neutralising antibodies, particularly those directed towards HA, are able to abrogate the infectivity of the virus. Different vaccine technologies vary in their ability to stimulate preferentially a humoral or cell-mediated immune response. For example, adjuvanted whole virus inactivated vaccines generally induce high levels of antibodies but with a poor stimulation of the cell-mediated immune response arm. On the opposite side,

replication-competent vectored vaccines are generally capable of more robust stimulation of cell-mediated immunity but might be slow to induce higher levels of circulating antibodies. The type of vaccine applied in a population can therefore affect the character and magnitude of the specific immune response, which in turn may affect its detectability by serological methods. Most of the available tests used for routine diagnostics aim at the detection of antibodies, while, at the time of writing, there are no scalable methods to assess cell-mediated immunity.

Assessment of immunity might be useful for different purposes:

1. Screening for vaccine coverage is done to assess the percentage of poultry that has mounted a measurable immune response upon vaccination. Depending on the diagnostic antigen used, the testing could be further stratified to extend the questioning to the protective efficacy of the mounted immune response against specific viruses that are in current circulation. However, defining thresholds of protection on basis of antibody titres has proven difficult (see Opinion Part 1).
2. Retrospective indirect screening for virus incursions into vaccinated flocks. Here, serological assays are part of the active surveillance in flocks that have received DIVA vaccination. The general drawback compared with the virological methodology is the delay in time until antibodies are mounted (usually 7–14 days post infection).

Agar gel immunoprecipitation (AGID)

All influenza A viruses share antigenically similar nucleoproteins and matrix antigens. For this reason, AGID tests are able to detect the presence or absence of antibodies against any influenza A virus in a subtype-independent manner. Although AGID is relatively cheap and suitable for settings with limited resources, it is increasingly being replaced by species-independent ELISA, which can provide greater sensitivity and shorter turn-around times. Limitations of this method include the restriction of its use to species which develop precipitating antibodies following infection (i.e. not suitable for use in Anseriformes). Moreover, the result of AGID is qualitative only, and targets virus proteins not involved in mounting protective immunity. For all the above reasons, AGID would be of limited value for surveillance purposes in vaccinated flocks.

Haemagglutination inhibition (HI)

The HI test is used to identify AIV subtype-specific antibodies such as H5 and H7 or other H subtypes. HI antibodies are a surrogate of neutralising antibodies and therefore often are highly strain-specific. Variability within the same subtype can be extensive, so there is a considerable risk of obtaining false-negative results in case a mismatched antigen is used. Therefore, to ensure high diagnostic sensitivity, it is always necessary to use the most appropriate antigen for each purpose. For example, to evaluate vaccination coverage, it should be fine to use the vaccine antigen or antigens antigenically very similar to the vaccine strain. However, in order to assess protection efficacy against the circulating field virus, an HI test must be carried out with this antigen or with a closely related one. HI titres may be regarded as being positive if there is inhibition at a serum dilution of 1:16 or more against four haemagglutinating units (HAU) of antigen, according to international standards.

Comparing different vaccine challenge studies (Mo et al., 2023), the target values for antibody titres to the challenge virus were identified: a value of ≥ 1:16 should be evaluated as a minimum titre that may be sufficient for survival and reduction of virus excretion of exposed poultry. In a field setting with physiological stress or immunosuppressive factors, survival and virus excretion may be accelerated and therefore the minimum positive titre in relation to protective efficacy should not be overinterpreted; it does not necessarily imply, e.g. that immunised birds with that titre will be protected against challenge or that birds with lower titres will be susceptible to challenge.

When compared with inactivated vaccines, HVT-vectored vaccines may be protective even if these threshold titres are not reached. This might be due to cellular immunity preferentially induced by vectored vaccines. Similarly, RNA vaccines are also known to elicit protective immunity through dendritic cell activation followed by stimulation of primary and memory T-cell responses and may induce protection even if HI titres remain below 1:16. Since a full evaluation of both arms of immunity, i.e. B- and T-cell-derived, is not possible at present, it is in any case recommended to use an antigen most closely related to the circulating virus in order to depict meaningful titres in vaccinated groups for an approximate evaluation of the supposed protective immunity in a vaccinated flock.

Onset of detectable immunity, that can be used to assess vaccination coverage in vaccinated poultry populations, is expected between 2 and 4 weeks following vaccination, in most cases. In birds (i.e. turkeys and chickens) vaccinated with HVT-vectored vaccines only, at 1 day of age, the development of a sufficient amount of antibodies to be detected by currently available serological tests could be delayed (i.e. 6–8 weeks following vaccination).

When longitudinal sero-surveillance is carried out using HI techniques, a 2–3 $\log_2$ increase or more in HI titres, in the absence of a new vaccine booster, could be considered a sign of introduction of a field AIV with the same HA subtype as the vaccine in a vaccinated holding after reaching the plateau phase, and should prompt additional virological investigations aimed at the detection and typing of the AIV responsible of the incursion. However, under field conditions, the use of such thresholds require stable and homogenous antibody levels at flock size for a sufficient period of time and sampling intervals need to be short enough to avoid that natural waning of immunity would mask a HPAIV incursion. HI tests therefore should be mainly used to assess vaccine coverage and not for routine surveillance activities in vaccinated flocks. However,

in monitoring flocks to assess freedom from HPAI (E4 and P4 in Table 2), HI tests using a test antigen derived from circulating HPAIV is preferred and yields a more accurate interpretation of sera that tested positive in NP-ELISA method.

ELISA technology

Commercial ELISA kits have replaced AGID assays as a more sensitive and versatile alternative for the detection of influenza A group reactive antibodies in sera. Kits with an indirect and competitive/blocking format have been developed and validated and are routinely used in poultry surveillance activities. Indirect ELISA kits, where the primary antigen-specific antibody is recognised by a secondary conjugated antibody, requires validation in multiple species as the nature of the secondary antibody limits their use to the determined species. Competitive formats instead, allow their use in a species-independent manner. Similar to AGID, most of assays use the nucleoprotein as a target antigen. These assays are qualified for use in flocks vaccinated with DIVA vaccines with NP as a negative marker.

Alternatively, subtype-specific H5 or H7 kits are available, but these kits must be carefully validated to ensure that they identify with sufficiently high sensitivity and specificity the specific seroconversion caused by the circulating strain.

Specific kits for H5 or H7, as well as the generic anti-NP kits if whole inactivated vaccines are used, can also be used for the evaluation of vaccine coverage. In general, the assays are used to produce a qualitative result. Although equations are offered with some assays to define a titre-like result, these should be used with caution; usually, examination of sera by HI cannot be replaced if specific answers on protection efficacy are sought.

ELISAs aimed at the detection of antibodies against different proteins (e.g. NA, NS1, etc.) have been developed as alternatives, although currently there are no commercially available products with a validation dossier.

The following Table 5 presents an overview of some summarised characteristics of diagnostic methods currently available for surveillance purposes.

TABLE 5　Overview of some characteristics of diagnostic methods currently available for surveillance purposes.

Diagnostic test	Target	Follow-up	Sensitivity	Specificity	Time window for detection (days post-infection)	Surveillance purposes	Time to results[a]	Susceptibility to viral mutations affecting the method	Labour intensity	Costs	Positive result interpretation
Genome detection											
Conventional RT-PCR	H5/H7	Sequencing of the PCR product for confirmation and HP/LP differentiation	++	+++	From 2 to 5–7	Confirmation of clinical cases; population freedom from infection	4–6 h	+	++	+	Presence of the targeted genetic material of H5/H7
RT-qPCR	M gene	End point	+++	+++	from 1–2 to 7–10	Confirmation of clinical cases; population freedom from infection; individual animal freedom from infection before movement	3 h	+	+	++	Presence of the targeted genetic material of AIV
	HA cleavage site	End point	++	+++	from 1–2 to 7–10	Confirmation of viral pathotype[b]	3 h	+++	+	++	Presence of the targeted genetic material of HPAI/LPAI
	H5/H7	End point	+++	+++	from 1–2 to 7–10	Confirmation of HA subtype	3 h	++	+	+	Presence of the targeted genetic material of H5/H7
	NA (1–9)	End point	+++	+++	from 1–2 to 7–10	Confirmation of NA subtype	3 h	++	+	++	Presence of the targeted genetic material of NA(X)
	Vaccine vector (e.g. HVT-H5)	End point	+	+++	Since day 21–28 post-vaccination	Vaccination coverage	3 h	+	+	++	Presence of the targeted vector-specific genetic material
Antigen detection											
Antigen-ELISA	NP	End point	+	++	3–6	Confirmation of clinical cases	< 1 h	+	+	+++	AIV
Lateral flow devices	NP	End point	+	++	3–6	Confirmation of clinical cases	< 1 h	+	(+)	++	AIV

(Continues)

TABLE 5   (Continued)

Diagnostic test	Target	Follow-up	Sensitivity	Specificity	Time window for detection (days post-infection)	Surveillance purposes	Time to results[a]	Susceptibility to viral mutations affecting the method	Labour intensity	Costs	Positive result interpretation
Detection of infectious virus											
Embryonated chicken eggs	Whole virus	Typing by HI or RT-qPCR	+	+++	2–8	Confirmation of clinical cases	2–14 days	−	+++	++	Infectious virus presence
Cell cultures	Whole virus	Typing by HI or RT-qPCR	+	+++	2–6	Confirmation of clinical cases	2–10 days	−	++	+	Infectious virus presence
Antibody detection											
ELISA	NP	Typing by HI	+++	++	> 7–10 days to 3 months	Immune status in individual animals or populations; Prevalence of infection – DIVA surveillance	3 h	(+)	+	++	Presence of antibodies against the NP of AIVs
ELISA	H5/H7	End point	++	+++	> 10–14 days to 3 months	Immune status in individual animals or populations post-vaccination (Vaccination coverage)	3 h	+	+	++	Presence of antibodies against the HA of H5/H7 (including vaccine-derived immunity)
AGID[c]	Whole virus	Typing by HI	+	++	> 14 days to 3 months	Immune status in individual animals or populations	2 days	(+)	++	+	Presence of antibodies against AIVs
HI	HA	Titration	++	+++	> 14 days to 3 months	Immune status in individual animals or populations post-vaccination (vaccine coverage) using vaccine homologous antigen; detection of field HPAIv exposure using field antigen	4 h	+	+++	+	Presence of antibodies against the HA of AIVs (subtype-specific)
V-NT	(HA)[d]	Titration	+++	+++	> 14 days to 3 months	Immune status in individual animals	3 days	+	+++	++	H5/H7

Abbreviation: I, Scaling equivalents; +++, high; ++, moderate; +, low; (+), very low.

[a]Meant as time from the arrival of the sample in the lab to the availability of the results (i.e. time for sampling and transport are not included).

[b]Use of RT-qPCR for pathotype identification should be used when epidemiological scenario is well known (e.g. circulation of 2.3.4.4b).

[c]Galliform poultry only.

[d]Most of neutralising epitopes of the AIV are located on the HA protein. Antibodies against the NA protein can also have neutralising ability.

Serological data from challenge experiments in vaccinated chickens, turkeys and ducks

Haemagglutination inhibition (HI)

Homologous HI titres represent values obtained with an antigen which is identical to the vaccine strain or which has a very short antigenic distance to the vaccine antigen (see Section 2.1.3.2 in Opinion Part 1 for estimation of the antigenic distance). Heterologous HI titres represent values obtained with any other test antigen with a larger antigenic distance. For the purpose of the analysis presented below if different antigens were tested by laboratories to analyse the same set of sera only the best performing one has been considered.

Chicken layers vaccinated with recombinant vectored vaccines showed robust immune response 42 days after vaccination at 1 day of age with mean homologous HI titres around 6–7 $\log_2$ before exposure to challenge virus and when tested against an antigen antigenically similar to the H5 HA expressed by the vector (Table 6). When tested with the heterologous challenge virus, sera of vaccinated animals show little reactivity with the majority of titres being < 4 $\log_2$ (Table 6). Sera obtained from vaccinated birds after challenge showed increased heterologous HI titres against the challenge virus antigen, while lower increase in HI titres was evident against the vaccine virus antigen (Table 6). Similar results have been obtained with nucleic acid-based vaccines (i.e. RNA and DNA-based vaccines) in birds vaccinated at 1 day or 2 weeks of age, although variable prechallenge HI titres have been observed which could compromise sensitivity of surveillance activities aimed at the evaluation of level of immune response in vaccinated flocks (Table 6).

In vaccinated **turkeys**, immune responses clearly detectable by HI are reached at approximately 7 weeks of age irrespective of the different immunisation schemes and vaccines used. Higher HI titres can be obtained using antigens homologous to the vaccines used, and therefore should be preferred for assessing immunisation of birds (Table 7). Wide variability in immune response elicited has been observed, with higher antibody titres obtained in groups immunised with vaccination schemes that included an heterologous vaccine as boost (Table 7). Seroconversion (evaluated as the Delta-Log of HI titres in sera collected before or after challenge in vaccinated and infected birds) measured with the challenge antigen (field 2.3.4.4b strain) is generally a more reliable indicator of past infection with Delta values ranging from 5 to 7 $\log_2$ compared to antigen homologous to the vaccine (Table 7).

Ducks vaccinated (at 10 and 28 days) with a subunit vaccine showed higher HI titres compared to ducks vaccinated (at 1 and 28 days) with an RNA-based vaccine in sera collected before challenge (i.e. 49 and 42 days, respectively) when tested with an antigen homologous to the vaccines (Table 8). Seroconversion (evaluated as the HI Delta-Log) was more evident in samples collected from convalescent birds, using the challenge strain as the antigen in HI assays (Table 8).

TABLE 6 Antibody titres in **chicken layers** vaccinated (pre-challenge), and vaccinated and challenged (post-challenge) based on HI test with **homologous** and **heterologous** antigens.

	Vaccine administered					
	ExactVac – Vaxliant ENABLE adjuvant (14, 28)[a] (median and 95% CI)[b]		RESPONS AI H5 (1, 28)[a] (median and 95% CI)[b]		Vectormune AI (1)[a] (median and 95% CI)[c]	
HI titres	Homologous	Heterologous	Homologous	Heterologous	Homologous	Heterologous
'pre-challenge'	6.00 (2.72;10.55)[d] 3.00 (2.17;4.00)[e]	6.00 (6.00–7.00)[d]	6.00 (2.22;6.77)[d]	3.00 (0.00;3.77)[d]	7.75 (6.24;9.26)[h]	0.00 (0.00;1)[h]
'delta log post-challenge'	0.00 (−4.27;5.50)[f] 6.50 (2.17;7.82)[g]	0.00 (−1.00;2.55)[f]	0.00 (−1.75;4.10)[f]	1.5 (−0.77;3.00)[f]	0.50 (−1.26;2.26)[i]	2.00 (0.00;7.52)[i]

	HVT-IBD-AIV-H5 haemagglutinin 'COBRA' (1)[a] (median and 95% CI)[c]		Nobilis Influenza H5N2 (8)[a] (median and 95% CI)[c]		ExactVac – Vaxliant ENABLE adjuvant (14)[a] (median and 95% CI)[c]	
HI titres	Homologous	Heterologous	Homologous	Heterologous	Homologous	Heterologous
'pre-challenge'	6.00 (1.19;9.55)[h]	0.00 (0.00;2.52)[h]	0.00 (0;1)[h]	0[h]	4.75 (0.0;7.5)[h]	0[h]
'delta log post-challenge'	0.25 (−0.5;7.1)[i]	2.00 (−0.26;6.76)[i]	6.00 (0.00;10.4)[i]	5.75 (0.00;10.91)[i]	−0.25 (−2.36;4.00)[i]	5.50 (0.00;7.85)[i]

[a]Age in days at time of vaccination.

[b]Data source: Belgium – NRL for AI.

[c]Data source: The Netherlands – NRL for AI.

[d,e]Days at testing: d = 41 days, e = 174 days.

[f,g]Delta-Log between HI titres in sera collected before (41 or 174 days of age) and after (56 or 189 days of age) the challenge in vaccinated animals f = 56–41 days, g = 189–174 days. Challenge performed at 42 or 175 days of age.

[h]Days at testing: 42 days.

[i]Delta-Log between HI titres in sera collected before (42 days of age) and after (77 days of age) the challenge in vaccinated animals. Challenge performed at 56 days of age.

TABLE 7   Antibody titres in **turkeys** vaccinated (pre-challenge), and vaccinated and challenged (post-challenge) based on HI test with **homologous** and **heterologous** antigens.

	Vaccine(s) administered									
	Volvac B.E.S.T. AI+ND (1,28)[a] (median and 95% CI)		Vectormune AI (1)[a] (median and 95% CI)		HVT-IBD-AIV-H5 haemagglutinin 'COBRA' (1)[a] (median and 95% CI)		ExactVac – Vaxliant ENABLE adjuvant (1)[a] ExactVac – Vaxliant ENABLE adjuvant (28)[a] (median and 95% CI)		Vectormune AI (1)[a]+ExactVac – Vaxliant ENABLE adjuvant (28)[a] (median and 95% CI)	
HI titres	Homologous	Heterologous	Homologous	Heterologous	Homologous	Heterologous	Homologous	Heterologous	Homologous	Heterologous
'pre-challenges'	5.00 (3.17;7.00)[b] 4.50 (3.00;6.82)[c]	1.00 (1.00;2.00)[b,c]	4.50 (3.17;6.82)[c]	2.00 (1.00;3.82)[c]	na	3.00 (1.17;4.00)[c]	1.50 (1.00;5.47)[b] 2.00 (1.00;5.47)[c]	1.00 (1.00;4.30)[b] 1.00 (1.00;3.47)[c]	3.50 (2.00;4.00)[b] 5.00 (3.00;5.00)[c]	1.00 (1.00;1.82)[b] 1.50 (1.00;2.82)[c]
'delta log post-challenges'	5.00 (5.00;5.95)[d] 6.00 (5.05;6.00)[e]	5.00 (4.05;7.85)[d,e]	3.00 (3.00;5.00)[d]	5.00 (2.3;6.0)[e]	na	8.00 (7.00;9.65)[e]	5.50 (4.07;6.92)[d] 5.00 (4.05;5.95)[e]	5.00 (–)[d] 5.50 (5.02;5.97)[e]	4.50 (3.00;7.65)[d] 3.50 (1.17;6.65)[e]	5.50 (1.35;8.82)[d] 5.00 (1.35;7.82)[e]

	Vectormune AI (1)[a]+Volvac B.E.S.T. AI+ND (28)[a] (median and 95% CI)		HVT-IBD-AIV-H5 haemagglutinin 'COBRA' (1)[a]+Volvac B.E.S.T. AI+ND (36)[a] (median and 95% CI)		HVT-IBD-AIV-H5 haemagglutinin 'COBRA' (1)[a]+ExactVac – Vaxliant ENABLE adjuvant (36)[a] (median and 95% CI)		Vectormune AI (1)[a]+Vaxigen Flu H5N8 (36)[a] (median and 95% CI)		HVT-IBD-AIV-H5 haemagglutinin 'COBRA' (1)[a]+Vaxigen Flu H5N8 (36)[a] (median and 95% CI)	
HI titres	Homologous	Heterologous	Homologous	Heterologous	Homologous	Heterologous	Homologous	Heterologous	Homologous	Heterologous
'pre-challenges'	7.00 (5.35;8.82)[b] 7.00 (5.17;8.82)[c]	1.50 (1.00;3.00)[b] 2.00 (1.00;3.82)[c]	8.00 (7.17;10.65)[c]	4.00 (2.17;5.00)[b] 5.00 (4.00;6.82)[c]	na	2.00 (2.00;3.82)[b] 3.00 (2.00;4.00)[c]	4.00 (3.00;5.00)[b] 6.00 (4.35;7.00)[c]	2.00 (–)[b] 6.00 (4.35;7.00)[c]	5.50 (4.00;6.00)[c]	2.50 (2.00;4.82)[b] 5.00 (4.00;6.00)[c]
'delta log post-challenges'	3.00 (2.00;4.82)[d] 3.00 (3.00;4.00)[e]	7.00 (3.52;7.00)[d] 6.00 (3.35;7.00)[e]	2.00 (1.17;4.00)[e]	5.50 (4.17;7.65)[d] 3.50 (3.00;6.00)[e]	na	7.00 (5.15;7.85)[d] 6.00 (5.00;7.00)[e]	4.50 (4.00;6.00)[d] 3.00 (2.00;3.00)[e]	6.00 (4.35;7.00)[d] 4.50 (3.17–6.82)[e]	4.00 (3.00;5.82)[e]	6.00 (4.17;7.82)[d] 4.00 (3.00;5.82)[e]

Data source: Italy – EURL for AI.

[a]Age in days at time of vaccination.

[b,c]Days at testing: b = 43 days, c = 50 days.

[d,e]Delta-Log between HI titres of sera collected before (42 or 50 days of age) and after (64 days of age) the challenge in vaccinated animals d = 64–43 days, e = 64–50 days. For all groups, challenge was performed at 50 days of age.

TABLE 8 Antibody titres in **ducks** vaccinated (pre-challenge), and vaccinated and challenged (post-challenge) based on HI test with **homologous** and **heterologous** antigens. Groups vaccinated with Duck H5-SRV vaccine® were challenged at 46 or 75 days of age. Groups vaccinated with Volvac B.E.S.T. AI+ND were challenged at 53 or 81 days of age.

	Vaccine administered			
	Duck H5-SRV vaccine® (1, 28)[a]		Volvac B.E.S.T. AI+ND (10, 28)[a]	
	(median and 95% CI)		(median and 95% CI)	
HI titres	**Homologous**	**Heterologous**	**Homologous**	**Heterologous**
'pre-challenge'	1.00 (–)[b,c]	1.00 (1.00;2.77)[b] 1.00 (–)[c]	7.00 (5.00;7.00)[d]	1.00 (–)[d,e] 5.00 (1.52;5.00)[e]
'delta log post-challenge'	4.00 (3;5)[f] 6.0 (3.45;7.00)[g]	5.00 (2.22;6.00)[f] 6.5 (4.45;7.00)[g]	1.50 (0.22;3.77)[h] 5.00 (4.00;8.47)[i]	3.50 (2.22;4.77)[h] 4.00 (3.00;4.82)[i]

Data source: France - NRL for AI.

[a]Age in days at time of vaccination.

[b,c,e]Days at testing: b = 42 days, c = 71 days, d = 49 days, e = 77 days.

[f,g,h,i]Delta-Log between HI titres of sera collected before (42, 49, 71 or 75 days of age) and after (60, 67, 89, 95 days of age) the challenge in vaccinated animals f = 60–42 days, g = 89–71 days, h = 67–49 days, i = 95–77 days. Groups vaccinated with Duck H5-SRV vaccine® were challenged at 46 or 75 days of age. Groups vaccinated with Volvac B.E.S.T. AI+ND were challenged at 53 or 75 days of age.

ELISA

NP-based ELISA in either an indirect or competitive format will be of importance in surveillance activities aimed at the detection of past AI-infection in flocks vaccinated with DIVA compatible vaccines. Regardless of the species tested, an indirect ELISA assay showed higher Se and Sp when compared to a competitive ELISA assay. Antibodies against the NP were elicited by HPAI replication in 100% of the infected birds, 14 days after infection in chickens and turkeys and 10 days after infection in mule ducks (Table 9). Earlier time points after exposure to the virus have been assessed for turkeys and ducks (Table 9), but showed lower Se and Sp values.

Competitive NP-ELISA assays evaluated under experimental conditions showed lower Se and Sp values than indirect ELISAs at all tested time points following infection in both chickens and ducks (Table 9). No values for turkeys were available for this type of ELISA at the time of writing.

TABLE 9 Sensitivity (Se) and specificity (Sp) of the indirect and competitive NP-ELISA at 4, 7, 10, 14 and 21 days post infection (DPI) in **chicken layers**, **turkeys**, **ducks** and **mule ducks** vaccinated with a DIVA compatible vaccine.

| | Poultry species | | | | | | | |
| | Chicken layers | | Turkeys | | Ducks | | Mule ducks | |
DPI	**Indirect NP-ELISA (exact 95% CI)**	**Competitive NP-ELISA (exact 95% CI)**	**Indirect NP-ELISA (exact 95% CI)**	**Competitive NP-ELISA (exact 95% CI)**	**Indirect NP-ELISA (exact 95% CI)**	**Competitive NP-ELISA (exact 95% CI)**	**Indirect NP-ELISA (exact 95% CI)**	**Competitive NP-ELISA (exact 95% CI)**
4	na	na	na	na	na	Se = 10% (0.25%–44.5%) Sp = 100% (71%–100%)	Se = 28.95% (15.42%–45.9%) Sp = 100% (90.7%–100%)	na
7	na	na	Se = 60% (41%–77%) Sp = 100% (63%–100%)	na	na	Se = 60% (26.2%–87.8%) Sp = 100% (71%–100%)	Se = 97.36% (86.2%–99.9%) Sp = 100% (90.7%–100%)	na
10	na	na	na	na	na	Se = 70% (34.7%–93.3%) Sp = 100% (71%–100%)	Se = 100% (90.5%–100%) Sp = 100% (90.7%–100%)	na
14	Se = 100% (59%–100%)[a] Sp = 92.3% (64%–99.8%)[a]	Se = 75% (53.2%–90.2%)[a] Sp = 79.5% (64.7%–90.2%)[a]	Se = 100% (88%–100%) Sp = 100% (63%–100%)	na	na	Se = 70% (34.7%–93.3%) Sp = 100% (71%–100%)	Se = 100% (90.5%–100%) Sp = 100% (90.7%–100%)	na
21	na	Se = 85% (62.10%–96.7%)[b] Sp = 100% (88.43%–100%)[b]	na	na	na	na	na	na

Data source: NRL for AI in Belgium, France, Italy, the Netherlands.

Abbreviation: na, not available.

[a]Data from Belgium.

[b]Data from the Netherlands.

3.1.1.5 | *Monitoring of clinical signs and production parameters*

The absence of mortality and/or overt clinical disease is the basic effect of HPAI vaccination, although variations in vaccine efficacy/effectiveness and the characteristics of the circulating viruses may cause different degrees of clinical protection (Table 3). As shown in the previous Opinion, absence of clinical signs and an increase in mortality in vaccinated flocks are poor indicators of the absence of viral circulation (EFSA AHAW Panel, 2023). Similarly, more refined parameters, which have been proven effective as indicators of ongoing disease in unvaccinated poultry flocks, such as reduced weight gain, reduced consumption of water or feed, drop in egg production, etc. bear a low predictive value of HPAIV incursions in vaccinated flocks. Clinical examination is therefore not a reliable indicator in surveillance activities aimed at early identification of HPAIV incursions in vaccinated flocks both in emergency and in preventive vaccination scenarios.

However, it must be considered that under realistic field conditions, as it has been extensively demonstrated, a certain percentage of vaccinated animals may fail to reach expected protective immunity levels. This might be due to a plethora of factors including vaccination failures or problems associated with the individual vaccinated bird such as the presence of immunosuppressive agents, nutritional diseases, genetic characteristics, etc. Therefore, there may be a certain percentage of birds in vaccinated flocks, which would show clinical signs of the disease and there may be even an increase in mortality associated with the HPAIV infection (Huynh et al., 2019; Tarigan et al., 2018). These 'fallen' animals deserve special attention in further surveillance investigations with the use of sensitive laboratory diagnostic methods (i.e. 'bucket sampling'). Similarly, if an escape-mutant arises or is introduced from outside this would be expected to spread despite vaccination, and clinical signs and increased mortality are the sequelae.

In peri-vaccination zones or in unvaccinated flocks within a vaccination zone, passive 'syndrome' surveillance based on clinical signs and production parameters would still represent a valuable method for the early detection of HPAI outbreaks.

3.1.1.6 | *Use of unvaccinated sentinel birds*

The use of unvaccinated sentinels in vaccinated flocks to determine if flocks have been exposed to infections has been a common strategy of disease detection (Bouma et al., 2008). In theory, sentinels could provide a sensitive measure of exposure to infection with HPAIV of a vaccinated flock, since the birds are completely susceptible to infection and would show clinical signs and mortality typical of HPAI. Sentinels can then be routinely tested for HPAIV infection with appropriate serological or virological methods (Suarez, 2005). However, several practical problems have been found to hamper the use of sentinels in the surveillance strategy in HPAI-vaccinated flocks and areas, and therefore, the sentinel bird approach has been abandoned in countries with long-standing vaccination experience such as Indonesia and Hongkong (Harder et al., 2023):

1. Without good compliance of farmers and a proper management system, the presence of unvaccinated sentinel birds in the flock may in fact function as the 'gap in the door of protection' since if exposed to the virus they can get infected and spread HPAIV in the flock and to other farms.
2. It is logistically challenging to identify sentinel animals in large flocks if sentinels are of the same species (conspecific) as the vaccinated part of the flock. Conspecific sentinels would enable an appropriate diffusion in the vaccinated flock. If they are from a different species (e.g. sentinel chickens in a duck flock) problems arise with compatibility of the different species leading to a separation of sentinels and vaccinees rather than observing them mixing in the flock.
3. In certain species, lateral transmission of replication-competent live-vectored HPAI vaccines would eventually immunise unvaccinated sentinels (e.g. turkeys vaccinated with a live HVT-vectored vaccine).

In addition, where LPAI viruses are circulating in the vaccination area, infection in sentinels will compromise the interpretation of virological and serological monitoring results.

The current scenarios evaluated as part of this Scientific Opinion therefore do not envisage the use of sentinels due to the above-mentioned problems and due to the increased risk of flock infection associated with the use of fully susceptible unvaccinated sentinels.

3.1.1.7 | *Use of environmental samples for surveillance*

The current sampling schemes laid down in Delegated Regulation (EU) No 2023/361 Annex XIII require surveillance of vaccinated flocks to exclude a prevalence of HPAIV infection in the flock of 5% with 95% confidence. This amounts to at least 60 samples for the average commercial holding when oropharyngeal and cloacal are taken as prescribed. Such samples are to be obtained every 2 weeks (emergency protective vaccination) or every 4 weeks (preventive vaccination) until the last vaccinated bird leaves the holding. In practice, several problems arise with these prescriptions:

- Catching the birds repeatedly troubles the flock and may create animal welfare problems.
- In weighty, long-lived poultry such as fattening turkeys or geese parents the catching and restraining of animals up to 20 kg body weight requires several stable hands and means a heavy physical workload.
- Obtaining the samples by an official veterinarian will be costly and time and human resources are often limited.
- Transporting of 60 samples per round to a laboratory requires a cool chain and appropriate logistics.
- RNA extraction and RT-qPCR analysis of samples is costly, even if pools of up to 10 samples could be formed.

Therefore, alternative sampling schemes and sample matrices are sought that would contribute to reducing workload during sampling, reduce costs for analysis and reduces stress and strains for the individual animals. However, such alternatives must have been validated to prove similar efficacy (i.e. detecting a design prevalence of 5/95) like the 60-swab scheme.

Recent investigations of environmental samples from AIV infected flocks have documented that several matrices are promising with respect to detection of viral genomes by RT-qPCR. In particular, water and biofilm samples from drinkers in turkey flocks (Muñoz-Aguayo et al., 2019), dust samples from walls and air samples (Filaire et al., 2022) have been analysed. Data showed that virus detection in such environmental samples was as sensitive as in conventional swab samples from animals. However, these investigations were all carried out in unvaccinated infected flocks. Systematic validation should clarify whether these promising data can be transposed to vaccinated flocks, since HPAI virus excretion in infected vaccinated animals is shortened in time and reduced in load. This may reduce virus loads in environmental sample matrices to a stronger extent than in infected non-vaccinated herds.

3.1.2 | Surveillance in vaccinated populations

Building upon the context of the previous Opinion (ToRs 1 and 2) we focused here on evaluating the effectiveness of surveillance in the three productive poultry groups targeted for vaccination, namely chicken layers (the Netherlands), ducks (France) and turkeys (Italy), according to the surveillance strategies as indicated in Table 2.

3.1.2.1 | *Surveillance for early detection at the establishment level for emergency vaccination (strategy E1)*

In the context of surveillance for early detection, $R_h < 1$ was chosen as indicating effectiveness. The reason was that it takes time to vaccinate flocks, so in the initial stages of an epidemic, despite the start of vaccination, R_h remains above 1 for a certain time lag and between-farm transmission may occur as shown in the previous Opinion (EFSA AHAW Panel, 2023). Surveillance is key to detecting infected flocks and the removal of those will contribute together with vaccination to reduce the number of susceptible animals/establishments and consequently to bring faster R_h below 1. Because of the methodology used in this assessment R_h is replaced by R_s, but the interpretation of R_s is equivalent to R_h.

The results of the assessment on surveillance strategies for early detection of HPAIV for emergency vaccination are shown in Tables 10A–12B, grouped by poultry species (chicken layers, ducks and turkeys) and for different surveillance strategies: passive reporting (i.e. the level of mortality at which farmers tend to report their suspicions calculated based on mortality data and reported suspicion dates, see Table 3), mortality thresholds (i.e. inferred level of mortality beyond which farmers have to report their suspicions) based on the studies by Gonzales and Elbers (2018), Elbers and Gonzales (2021) and Gonzales et al. (2023) (see Table 3), and active surveillance based on sampling dead or live birds to be tested by qPCR, or live birds to be tested by serology.

Different combinations of sampling intervals and samples sizes are presented as results; only those surveillance strategies whose combination of sampling interval and sample sizes resulted in a probability to escape detection below 0.01 at the flock level for more than 95% of the simulated within-flock outbreaks are considered effective to early detect HPAIV. The reason is that undetected outbreaks led to an underestimation of R_h and of the time between introduction and detection. Note that surveillance strategies for testing dead birds included randomly sampling all up to a number of 5, 10 and 15 dead birds. If fewer dead birds were available than these required sample sizes, all available dead birds were tested, which is why a minimum sample size was not provided.

Surveillance in chicken layers

Table 10A reports results for flocks of 20,000 chicken layers. However, the model outcomes remained consistent for flock sizes of 6000 and 60,000 birds; simulations for these flock sizes produced similar results as those presented in Table 10A. The results reported (Table 10A) indicate that sampling live birds for PCR testing or serology did not result in detecting 99% of the outbreaks in ≥ 95% of the simulations. Therefore, the strategy of 'sampling live birds ($n = 60$)' every 14 days as recommended by the EU Regulation, when emergency vaccination is applied (scenario E1) would not be effective for early detection. The proposed strategy of weekly sampling all dead birds up to a number of five achieved early detection at a median (95% confidence intervals (CI)) time of 20 (14–31) days post-introduction and a reduction of R_s to 0.13 (95% CI: 0.10–0.16). The median (95% CI) number of dead birds likely to be found at the time of sampling and detection of an infected flock was 6 (4–10). With longer sampling intervals, such as every 14 or 21 days, all dead birds up to a number of 10 or 15, respectively, need to be sampled to enable that > 99% of the outbreaks would be detected in 95% of the simulations, with median times of detection of 21 and 22 days post-introduction, respectively. The median number of dead birds likely to be found at the time of sampling and detection of an infected flock would be 8 (4–12) and 8 (6–14), respectively. Additionally, transmission would remain limited ($R_s < 1$). Consequently, testing dead birds is more effective for early detection than testing live birds and also fewer resources are needed. Furthermore, longer sampling intervals compared with the weekly sampling can be considered if more dead birds are collected (up to a number of 15 for a 3-week interval) (Table 10A).

The strategies based on 'passive reporting' or on 'mortality thresholds' would not be effective for early detection, with median detection days of 31 (25–43) and 28 (22–41), respectively, and $R_{h/s} > 1$.

TABLE 10A Efficacy of different combination of sampling types, sample size, sampling frequency and diagnostic test for early detection (defined as reduction of $R_s < 1$) of vaccinated-infected flocks, strategy E1. These simulations were done for a flock of **20,000[a] chicken layers** and assuming a case fatality equal to 0.2, a qPCR Se = 0.99 and a Serology (ELISA) Se = 0.9. Results are reported only for effective surveillance strategies, i.e. where the proportion of outbreak simulations with the probabilities of escaping detection below 1% is above 95%; otherwise, these are left empty.

Sample type (diagnostic test)	Sample size	Sampling interval (days)	Percentage of outbreak simulations with the probabilities of escaping detection below 1%[b]	Detection time as days post introduction (median (2.5–97.5 CI))	Prevalence (%) infectious birds (median (2.5–97.5 CI))	Prevalence (%) recovered birds (median (2.5–97.5 CI))	R_h/R_s (reproduction number) (median (2.5–97.5 CI))
Passive reporting (reference)				31 (25–43)	3.93 (3.44–4.5)	2.16 (1.86–2.46)	1.4
Mortality threshold (0.13%)				28 (22–39)	2.35 (2.01–2.75)	1.26 (1.06–1.49)	1.09 (1.04–1.1)
Dead birds (qPCR)	≤ 5	7	99%	20 (14–31)	0.34 (0.25–0.43)	0.18 (0.11–0.24)	0.13 (0.1–0.16)
		14	90%				
		21	51%				
		30	0%				
	≤ 10	7	99%	18 (13–30)	0.26 (0.19–0.34)	0.14 (0.08–0.19)	0.1 (0.08–0.13)
		14	98%	21 (15–33)	0.44 (0.35–0.56)	0.23 (0.15–0.31)	0.17 (0.15–0.2)
		21	94%				
		30	84%				
	≤ 15	7	99%	18 (13–30)	0.26 (0.19–0.33)	0.13 (0.08–0.19)	0.1 (0.08–0.13)
		14	99%	20 (15–32)	0.41 (0.32–0.52)	0.21 (0.15–0.29)	0.16 (0.14–0.19)
		21	97%	22 (16–34)	0.56 (0.45–0.71)	0.3 (0.21–0.39)	0.22 (0.19–0.26)
		30	92%				
Live birds (qPCR)	60	14	72%				
		30	30%				
	120	14	89%				
		30	69%				
Live birds (serology)	60	14	47%				
		30	9%				

[a]Flock sizes of 6000 and 60,000 chicken layers were also assessed. No differences in the results regarding reduction of $R_s < 1$ were observed.

[b]An individual infected flock is considered detected when the probability of escaping detection < 0.01 (probability of detection > 0.99).

The results were different when a flock size of 2500 birds was used to represent small flocks. For these flocks, none of the strategies evaluated were effective, since the requirement to detect 99% of the outbreaks in 95% of the simulations was not met (Table 10B); in these flock size, sampling all birds found dead up to a number of 10 birds weekly gave detection of 99% of the outbreaks in 90% of the simulations. For flocks ≥ 3000 and < 6000 weekly sampling of all up to a number of 10 or 15 dead birds resulted in an effective early detection in ≥ 95% of the simulations (Data not shown).

TABLE 10B Efficacy of different combination of sampling types, sample size, sampling frequency and diagnostic test for early detection (defined as reduction of $R_s < 1$) of vaccinated-infected flocks, strategy E1. These simulations were done for a flock of **2500 chicken layers** and assuming a case fatality equal to 0.2, a qPCR Se = 0.99 and a Serology (ELISA) Se = 0.9. Results are reported only for effective surveillance strategies, i.e. where the proportion of outbreak simulations with the probabilities of escaping detection below 1% is above 95%; otherwise, these are left empty.

Sample type (diagnostic test)	Sample size	Sampling interval (days)	Percentage of outbreak simulations with the probabilities of escaping detection below 1%[a]	Detection time as days post introduction (median (2.5–97.5 CI))	Prevalence (%) infectious birds (median (2.5–97.5 CI))	Prevalence (%) recovered birds (median (2.5–97.5 CI))	R_h/R_s (reproduction number) (median (2.5–97.5 CI))
Passive reporting (reference)				21 (16–33)	4.09 (3.01–5.35)	2.19 (1.59–2.91)	1.4
Mortality threshold (0.13%)				15 (10–26)	1.01 (0.6–1.56)	0.48 (0.24–0.81)	1.27 (0.44–1.41)
Dead birds (qPCR)	≤ 5	7	87%				
		14	50%				
		21	2%				
		30	0%				
	≤ 10	7	90%				
		14	79%				
		21	59%				
		30	18%				
	≤ 15	7	90%				
		14	80%				
		21	69%				
		30	47%				
Live birds (qPCR)	60	14	69%				
		30	25%				
	120	14	85%				
		30	67%				
Live birds (serology)	60	14	42%				
		30	7%				

[a]An individual infected flock is considered detected when the probability of escaping detection < 0.01 (probability of detection > 0.99).

Surveillance in ducks

In flocks of 6000 ducks or larger, the results reported in Table 11A indicate that the strategy based on 'mortality thresholds' achieved the earliest detection, at a median of 17 (13–26) days post-introduction, with a $R_s < 1$. Note that the strategy based on 'passive reporting' is ineffective for early detection, with a median detection day of 23 (19–32) and $R_h > 1$. Sampling dead birds at weekly intervals showed in more than 95% of the simulations that they would be effective for early detection, with a detection time of 14–15 days and $R_s < 1$ irrespective of collecting all up to a number of 5, 10 or 15 dead birds. Similarly, biweekly sampling of all up to a number of 10, or every 3 weeks up to 15 dead birds would be effective for early detection. The median number of dead birds likely to be found at the time of sampling and detection of an infected flock would be 4 (2–9) when sampling weekly, 6 (4–10) when sampling every 2 weeks and 8 (5–12) when sampling every 3 weeks.

The strategy of 'sampling live birds (n = 60)' every 14 days as recommended by the EU Regulation for emergency vaccination (E1), or testing 60 birds serologically, would be effective for early detection with a median detection time of 17 days (13–25) and $R_s < 1$. This is also in contrast to chicken layers (both small and larger flocks), likely because of the higher transmission rate in duck flocks. Of note, sampling live ducks implies challenges in terms of welfare consequences as the animals undergo catching and handling, as well as in term of logistics and resources needed as more samples would be collected and tested compared with those required if dead birds are sampled.

TABLE 11A Efficacy of different sample types, sample size, sampling frequency and diagnostic test combination for early detection (defined as reduction of $R_s < 1$) of vaccinated-infected flocks, strategy E1. These simulations were done for a flock **≥ 6000 ducks**[a] and assuming a case fatality equal to 0.05 and qPCR sensitivity of 0.99 and ELISA sensitivity of 0.90. Results are reported only for effectiveness surveillance strategies, i.e. those where the proportion of outbreak simulations with the probabilities of escaping detection below 1% is above 95% otherwise these are left empty.

Sample type (diagnostic test)	Sample size	Sampling interval (days)	Percentage of outbreak simulations with the probabilities of escaping detection below 1%[b]	Detection time as days post-introduction (median (2.5–97.5 CI))	Prevalence (%) infectious birds (median (2.5–97.5 CI))	Prevalence (%) recovered birds (median (2.5–97.5 CI))	R_h/R_s (reproduction number) (median (2.5–97.5 CI))
Passive reporting (reference)				23 (19–32)	20.5 (18.9–22.3)	33.31 (29.26–37.26)	1.8
Mortality threshold (0.17%)				17 (13–26)	5.84 (4.83–7.22)	6.13 (4.9–7.28)	0.62 (0.49–0.63)
Dead birds (qPCR)	≤ 5	7	98%	15 (11–24)	3.09 (2.51–3.86)	3.09 (2.33–3.88)	0.21 (0.18–0.26)
		14	70%				
		21	0[b]				
		30	0%				
	≤ 10	7	99%	14 (11–23)	2.35 (1.87–3.02)	2.35 (1.74–3)	0.17 (0.14–0.19)
		14	97%	16 (12–25)	4.29 (3.48–5.19)	4.37 (3.34–5.38)	0.3 (0.26–0.34)
		21	89%				
		30	36%				
	≤ 15	7	99%	14 (10–23)	2.33 (1.86–2.96)	2.3 (1.72–2.99)	0.16 (0.14–0.19)
		14	98%	16 (12–24)	3.93 (3.18–4.8)	3.99 (2.97–4.94)	0.28 (0.24–0.32)
		21	96%	17 (13–26)	5.5 (4.54–6.68)	5.75 (4.43–6.97)	0.39 (0.35–0.44)
		30	89%				
Live birds (qPCR)	60	14	97%	17 (13–25)	4.95 (4.03–5.98)	5.05 (3.9–6.34)	0.35 (0.32–0.39)
		30	44%				
	90	14	98%	15 (12–24)	3.34 (2.64–4.11)	3.33 (2.49–4.17)	0.23 (0.21–0.26)
		30	93%				
Live birds (serology)	60	14	97%	17 (13–25)	5.16 (4.25–6.28)	5.34 (4.17–6.62)	0.36 (0.29–0.44)
		30	93%				

[a]Flock size of 15,000 ducks was also assessed. No differences in the results were observed.

[b]An individual infected flock is considered detected when the probability of escaping detection < 0.01 (probability of detection > 0.99). Results for scenarios where this probability is 0 are results for flocks where the probability of escaping detection was > 0.01 and hence not considered an effective surveillance scenario for early detection.

In flocks of 2500 ducks, the results reported in Table 11B indicate that the strategy based on 'mortality thresholds' achieved the earliest detection, at a median of 10 (7–18) days post-introduction, with an $R_s < 1$. Sampling dead birds at weekly intervals showed in more than 95% of the simulations that they would be effective for early detection, with a detection time of 14–15 days and $R_s < 1$ irrespective of collecting all up to a number of 5, 10 or 15 dead birds. Different from the simulations made for flocks with ≥ 6000 birds, sampling intervals longer than 7 days would not be effective (< 95% of the simulations detected > 99% of the outbreaks). The median number of dead ducks likely to be found at the time of sampling and detection of an infected flock would be around 3 (2–6) when sampling up to five dead birds, or 3 (2–5) when sampling up to 10 (sampling up to 15 birds does not improve further the efficacy). This is in contrast to surveillance in small flocks of chicken layers, where weekly sampling of dead birds could only reach detection of 90% of the simulations. This contrast is likely to be due to the faster transmission rate expected in ducks than in chickens.

TABLE 11B Efficacy of different sample types, sample size, sampling frequency and diagnostic test combination for early detection (defined as reduction of $R_s < 1$) of vaccinated infected flocks, strategy E1. These simulations were done for a flock of **2500 ducks**[a] and assuming a case fatality equal to 0.05 and qPCR sensitivity of 0.99 and ELISA sensitivity of 0.90. Results are reported only for effectiveness surveillance strategies, i.e. those where the proportion of outbreak simulations with the probabilities of escaping detection below 1% is above 95% otherwise these are left empty.

Sample type (diagnostic test)	Sample size	Sampling interval (days)	Percentage of outbreak simulations with the probabilities of escaping detection below 1%[b]	Detection time as days post introduction (median (2.5–97.5 CI))	Prevalence (%) infectious birds (median (2.5–97.5 CI))	Prevalence (%) recovered birds (median (2.5–97.5 CI))	R_h/R_s (reproduction number) (median (2.5–97.5 CI))
Passive reporting (reference)				21 (17–30)	20.76 (18.37–23.21)	34.5 (29.9–38.74)	1.8
Mortality threshold (0.17%)				10 (7–18)	1.29 (0.77–1.97)	1.21 (0.72–1.73)	0.63 (0.1–0.76)
Dead birds (qPCR)	≤ 5	7	96%	15 (11–24)	5.83 (4.64–7.25)	6.13 (4.4–7.56)	0.4 (0.34–0.48)
		14	33%				
		21	0%				
		30	0%				
	≤ 10	7	97%	14 (11–24)	5.42 (4.37–6.81)	5.63 (4.12–7.11)	0.37 (0.31–0.44)
		14	92%				
		21	12%				
		30	0%				
	≤ 15	7	97%	14 (11–24)	5.42 (4.37–6.81)	5.63 (4.12–7.11)	0.37 (0.31–0.44)
		14	92%				
		21	20%				
		30	0%				
Live birds (qPCR)	60	14	97%	14 (11–23)	4.91 (3.98–6.16)	5.04 (3.67–6.72)	0.34 (0.31–0.37)
		30	48%				
	90	14	98%	13 (9–22)	3.33 (2.51–4.34)	3.3 (2.34–4.38)	0.23 (0.2–0.25)
		30	93%				
Live birds (Serology)	60	14	97%	14 (11–24)	5.22 (4.2–6.61)	5.36 (3.9–7.02)	0.36 (0.27–0.45)
		30	93%				

[a]This was the median size for flocks smaller than 4000 in France.

[b]An individual infected flock is considered detected when the probability of escaping detection < 0.01 (probability of detection > 0.99).

Surveillance in turkeys

Table 12A reports results for flocks of 18,000 turkeys. However, the model outcomes remained consistent for flock sizes of 6000 and 60,000 birds; simulations for these flock sizes produced similar results as those presented in Table 12A. The results reported in Table 12A indicate that sampling all dead birds up to a number of five every week is effective to early detect HPAIV. The earliest detection is achieved using all up to a number of 15 dead birds at weekly intervals, with a median of 15 (10–27) days post-introduction and with the lowest R_s at 0.11 (0.09–0.13). The median number of dead birds likely to be found at the time of sampling and detection of an infected flock would be 14 (7–22). Less frequent sampling intervals, such as every 14 days with a sample size of all up to a number of 15 dead birds and every 21 days with a sample size of up to a number of 20 birds, remains effective for early detection and limited transmission ($R_s < 1$). The median number of dead birds likely to be found at the time of sampling and detection of an infected flock would be 16 (11–23) when sampling bi-weekly and 18 (11–25) when sampling every 3 weeks.

The strategies involving 'sampling live birds' (qPCR or serology) ($n = 60$ or 90) would not be effective, including the one recommended by the EU Regulation for emergency vaccination (E1), e.g. 60 live birds every 14 days.

The strategies based on 'passive reporting' or on 'mortality thresholds' would not be effective for early detection, with median detection days of 27 (21–38) and 24 (19–34), respectively, and $R_s > 1$.

TABLE 12A Efficacy of different sample types, sample size, sampling frequency and diagnostic test combination for early detection (defined as reduction of $R_s < 1$) of vaccinated partially protected infected flocks, strategy E1. These simulations were done for a flock of **18,000 turkeys**[a] and assuming a case fatality equal to 0.2. A qPCR sensitivity of 0.99 and an ELISA sensitivity of 0.9. Results are reported only for effective surveillance strategies, i.e. those where the proportion of outbreak simulations with the probabilities of escaping detection below 1% is above 95% otherwise these are left empty.

Sample type (diagnostic test)	Sample size	Sampling interval (days)	Percentage of outbreak simulations with the probabilities of escaping detection below 1%[b]	Detection time as days post introduction (median (2.5–97.5 CI))	Prevalence (%) infectious birds (median (2.5–97.5 CI))	Prevalence (%) recovered birds (median (2.5–97.5 CI))	R_h/R_s (reproduction number) (median (2.5–97.5 CI))
Passive reporting (reference)				27 (21–38)	4.1 (3.51–4.79)	3.31 (2.83–3.83)	1.6
Mortality threshold (0.21%)				24 (19–34)	2.5 (2.07–2.96)	1.97 (1.64–2.34)	1.42 (1.68–1.04)
Dead birds (qPCR)	≤5	7	95%	19 (14–31)	0.66 (0.53–0.81)	0.52 (0.38–0.66)	0.28 (0.23–0.34)
		14	66%				
		21	5%				
		30	0[b]				
	≤10	7	99%	16 (11–28)	0.33 (0.25–0.44)	0.25 (0.16–0.36)	0.14 (0.11–0.18)
		14	94%				
		21	77%				
		30	59%				
	≤15	7	99%	15 (10–27)	0.26 (0.18–0.35)	0.2 (0.12–0.29)	0.11 (0.09–0.14)
		14	98%	18 (12–29)	0.46 (0.35–0.6)	0.35 (0.25–0.48)	0.2 (0.17–0.24)
		21	94%				
		30	87%				
	≤20	7	99%	15 (10–27)	0.25 (0.17–0.33)	0.18 (0.12–0.27)	0.1 (0.08–0.13)
		14	98%	17 (12–29)	0.42 (0.31–0.53)	0.31 (0.22–0.43)	0.18 (0.15–0.21)
		21	96%	19 (13–30)	0.59 (0.48–0.75)	0.45 (0.33–0.6)	0.26 (0.22–0.3)
		30	93%				
Live birds (qPCR)	60	14	70%				
		30	21%				
	90	14	82%				
		30	55%				
Live birds (serology)	60	14	61%				
		30	22%				

[a]Flock sizes of 6000 and 60,000 turkeys were also assessed. No differences in the results were observed.

[b]An individual infected flock is considered detected when the probability of escaping detection < 0.01 (probability of detection > 0.99).

In flocks < 6000 neither testing dead birds, nor testing live birds would be effective for early detection. Different to chicken layers but similar to small duck flocks, using a daily mortality threshold to raise suspicions and issuing guidelines for sampling and detection could result in an effective early detection (Table 12B).

TABLE 12B Efficacy of different sample types, sample size, sampling frequency and diagnostic test combination for early detection (defined as reduction of $R_s < 1$) of vaccinated partially protected infected flocks, strategy E1. These simulations were done for a flock of **2500 turkeys** and assuming a case fatality equal to 0.2. A qPCR sensitivity of 0.99 and an ELISA sensitivity of 0.9. Results are reported only for effective surveillance strategies, i.e. those where the proportion of outbreak simulations with the probabilities of escaping detection below 1% is above 95% otherwise these are left empty.

Sample type (diagnostic test)	Sample size	Sampling interval (days)	Percentage of outbreak simulations with the probabilities of escaping detection below 1%[a]	Detection time as days post-introduction (median (2.5–97.5 CI))	Prevalence (%) infectious birds (median (2.5–97.5 CI))	Prevalence (%) recovered birds (median (2.5–97.5 CI))	R_h/R_s (reproduction number) (median (2.5–97.5 CI))
Passive reporting (reference)				19 (13–29)	4.24 (3.18–5.56)	3.37 (2.52–4.29)	1.6 (1.6–1.6)
Mortality threshold (0.21%)				5 (3–9)	0.16 (0.04–0.28)	0.04 (0–0.15)	0.89 (0.06–0.98)
Dead birds (qPCR)	≤ 5	7	11%				
		14	11%				
		21	9%				
		30	0%				
	≤ 10	7	11%				
		14	11%				
		21	11%				
		30	10%				
	≤ 15	7	11%				
		14	11%				
		21	11%				
		30	11%				
	≤ 20	7	11%				
		14	11%				
		21	11%				
		30	11%				
Live birds (qPCR)	60	14	64%				
		30	12%				
	90	14	77%				
		30	43%				
Live birds (serology)	60	14	44%				
		30	6%				

[a]An individual infected flock is considered detected when the probability of escaping detection < 0.01 (probability of detection > 0.99).

Surveillance in peri-vaccination zone (only for emergency protective vaccination)

According to Article 9 1(c)(ii) Delegated Regulation (EU) No 2023/361, HPAIV surveillance should be applied in a peri-vaccination zone established for emergency vaccination to detect any possible new outbreaks. The risk assessment carried out by EFSA in the opinion on control measures for Category A diseases (EFSA AHAW Panel, 2021) (pp. 35–38) has been used to assess the **radius of the peri-vaccination zone**. Given that the 3-km recommended radius for an emergency vaccination zone (EFSA AHAW Panel, 2023) corresponds to the 3-km radius of a classical 'protection zone' following HPAIV outbreaks in poultry, the peri-vaccination zone always overlaps with the 'surveillance zone' that, according to Delegated Regulation (EU) No 2020/687 should have a 10-km minimum radius.

In the EFSA Opinion on Category A diseases, a simple kernel based on three parameters was used on unvaccinated population to estimate the radius of a surveillance zone based on the probability of HPAIV transmission beyond given distances (jump spread), as reported in Table 13 for scenarios of the Netherlands (epidemic in 2003) and Italy (epidemic 1999). In particular, if the aim of the peri-vaccination zone is to contain the spread of the disease avoiding any jump outside the area with 95% confidence, a zone radius of at least 10 km would be needed (probability of jump spread equal to 0.04, meaning probability of containment equal to 0.96, so 96%). For emergency vaccination, the establishments within the 3-km protection zone would be vaccinated and therefore should have a reduced risk of HPAIV transmission compared with that observed in the absence of vaccination; therefore, emergency vaccination applied in the 3-km radius around the outbreak

increases the confidence that the probability of transmission beyond 10 km is lower than 0.04. Given this consideration, a 10-km peri-vaccination zone radius can be considered as a worst-case scenario estimate.

TABLE 13 Probability of transmission of highly pathogenic avian influenza virus beyond different distances in the scenario of the Netherlands (epidemic in 2003) and Italy (epidemic 1999) as estimated in EFSA AHAW Panel (2023).

	Distance (km)						
	3	**5**	**10**	**15**	**20**	**25**	**50**
Netherlands, 2003 epidemic	0.52	0.22	0.04	0.01	0.007	0.004	< 0.001
Italy, 1999 epidemic	0.33	0.15	0.04	0.02	0.01	0.006	0.001

In relation to the **type of surveillance** to detect any new outbreaks in the peri-vaccination zone, the options depend on the vaccination status of the establishments within that area. If the establishments are vaccinated (e.g. when preventive vaccination was applied before the outbreak), the scenarios presented in Tables 10A–12B would also apply here.

If establishments in the peri-vaccination zone are not vaccinated, the recommendations provided in the EFSA Opinion on control measures for Category A diseases (EFSA AHAW Panel, 2021) are applicable, i.e. passive surveillance would be applied for gallinaceous poultry species (chickens and turkeys) and bucket sampling for Anseriformes (ducks and geese). The reason for the latter is due to the risk that some HPAIV strains could cause limited mortality (case fatality) in these species. Therefore, we have assessed scenarios where we assumed a low case fatality of 0.2 (20%) (Table 14) in unvaccinated duck flocks. Weekly sampling of all available dead birds (preferably dead within the last 48 h), as recommended by the EFSA opinion on Category A diseases, would be effective to early detect the infection.

TABLE 14 Efficacy of different sample size, sampling frequency and diagnostic test combination for early detection (defined as reduction of $R_s < 1$) of unvaccinated infected flocks, strategy E1. These simulations were done for a flock of **2500 ducks**[a] and assuming a case fatality equal to 0.2, a qPCR sensitivity of 0.99 and ELISA sensitivity of 0.90.

Sample type (diagnostic test)	Sample size	Sampling interval (days)	Percentage of outbreak simulations with the probabilities of escaping detection below 1%[b]	Detection time as days post-introduction (median (2.5–97.5 CI))	Prevalence (%) infectious birds (median (2.5–97.5 CI))	Prevalence (%) recovered birds (median (2.5–97.5 CI))	R_h/R_s (reproduction number) (median (2.5–97.5 CI))
Passive reporting (reference)				6 (5–8)	59.81 (45.26–68.1)	7.66 (4.4–11.5)	1.8
Mortality threshold (0.13%)				4 (3–5)	5.41 (2.85–11.43)	0.44 (0.12–1.07)	0.2 (0.1–0.33)
Dead birds (qPCR)	≤ 15	7	96%	3 (2–5)	2.33 (1.04–4.5)	0.16 (0–0.48)	0.08 (0.06–0.12)
		14	95%	3 (3–5)	3.41 (1.68–6.9)	0.24 (0.04–0.67)	0.13 (0.1–0.18)
		21					
		30					

[a]This was the median size for flocks smaller than 4000 in France.

[b]An individual infected flock is considered detected when the probability of escaping detection < 0.01 (probability of detection > 0.99).

3.1.2.2 | Surveillance for demonstrating disease freedom following emergency vaccination (strategy E3, E4)

During the process of controlling an epidemic where emergency vaccination is applied and surveillance is implemented, following the strategies identified and described in Tables 10A–12B, there is a moment in time when no more outbreaks are detected. This will result in only negative results at both establishment (flock(s) within an establishment) and zone levels. In some cases (and for some establishments), multiple samplings would be done in time and all would be negative. Given these repeated negative results in time and the high probability of detection (> 99%) of infected flocks within an establishment, one could expect a high level of confidence in freedom. If the estimation of the probability of freedom is required, then Equation (10) (Section 2.1.5.2) could be used.

3.1.2.3 | Surveillance for early detection and demonstrating freedom in preventive vaccination populations (strategy P1, P3, P4)

In this section, results are presented based on simulated scenarios in the Netherlands, France and Italy, focusing specifically on areas previously identified as high-risk zones for the incursion of HPAIV (see Opinion Part 1). We assessed scenarios for demonstrating freedom from disease within these high-risk zones, where specific poultry species are vaccinated (chicken layers in the Netherlands, ducks in France and turkeys in Italy), leading to the co-existence of vaccinated and unvaccinated

poultry populations. This situation could occur if, for instance, only certain poultry species are vaccinated during a campaign of preventive vaccination as described in Opinion part 1 (EFSA AHAW Panel, 2023). In this high-risk zone, it is assumed that active surveillance is conducted in all vaccinated flocks by collecting every 30 days in a 48-h period all dead birds up to a number of 15 to be tested by qPCR, while passive surveillance is applied in unvaccinated flocks (Tables 15A, 16A and 17A). As mentioned in Table 4, this testing scheme corresponded to a median of 97% of outbreak simulations being detected (Sensitivity) in chicken layers, 96% in ducks and 96% (CL: 87–97) in turkeys. Note that passive surveillance is by default applied also on vaccinated flocks; therefore, if unusual increased mortality is observed between two consecutive sampling events, additional testing should be considered to exclude the presence of HPAIV.

In addition, we explored scenarios with variations in sampling intervals (30, 15 and 7 days) and proportions of vaccinated flocks (100%, 50% and 25%) (Tables 15B, 16B and 17B). For definitions of the measures used in the tables, see Glossary.

As indicated in the methods, the probability of freedom from the disease is here provided and, considering that this probability is highly dependent on the prior chosen for the prevalence of the disease in a certain area (Equation 11 and see Glossary), we also provided the early detection sensitivity (EDSe, i.e. the probability that at least one infected establishment is detected by the surveillance component in the selected time frame for a given design prevalence) and the surveillance system sensitivity (TotalSe), which combines the sensitivity of all surveillance components in the Tables below.

Results show that by testing all dead birds up to a number of 15 collected in a flock during 48 h by qPCR every 30 days in all vaccinated flocks and by applying passive surveillance in unvaccinated flocks, the estimated probability of disease freedom (Pfree) would exceed 99% in the three high-risk regions assessed, if all results show negative. The overall sensitivity of active and passive surveillance in all the poultry (Surveillance system sensitivity) was 95% in the high-risk regions of the Netherlands and 99% in the high-risk regions of France and Italy. The probability of detecting at least one infected vaccinated establishment (EDSe) in the time frame of 30 days was 92% for vaccinated chickens in the Netherlands, 74% for vaccinated ducks in France and 93% for vaccinated turkeys in Italy. This indicates that the early detection sensitivity in vaccinated flocks is higher (chicken layers and turkeys) or similar (ducks) to the early detection sensitivity of passive surveillance in unvaccinated flocks of other poultry types in the regions (Tables 15A, 16A and 17A)

Results show that the early detection surveillance sensitivities (EDSe) vary with different sampling frequencies and proportions of vaccinated establishments sampled (Tables 15B, 16B and 17B). For example, reducing the proportion of tested turkey flocks from 100% to 50% reduces the EDSe from an average of 93% to 49% (Table 17B). Similarly, reducing it to 25% further reduces the early detection surveillance sensitivity to 25% (Table 17B). Conversely, the early detection surveillance sensitivities are increased by reducing the sampling intervals. For example, reducing the sampling intervals from 30 to 7 days in the turkey flocks that are tested increases the early detection sensitivity from an average of 93% to 100% (Table 17B).

However, reducing the proportion of the establishments tested or reducing the sampling intervals had a more limited effect on the probability of freedom (Pfree), given all tests were negative. This is likely due to the low hypothetical prior probability of infection (priorInf) chosen (5%) and the inclusion of passive surveillance in non-vaccinated poultry types. For example, sampling every 30 days and reducing the proportion of vaccinated turkey flocks tested to 25% resulted in a probability of freedom of 96% (compared with 99% if 100% of vaccinated turkey flocks are tested).

The monthly clinical examination of poultry, check of production records, check of health records of each epidemiological unit, as set out in Delegated Regulation (EU) No 2023/361 for surveillance in preventive vaccination, when applied in addition to the surveillance scheme here proposed, further contributes at increasing the estimated confidence for early detection and disease freedom.

TABLE 15A Early detection sensitivity and probability of freedom from disease in a high-risk zone for both introduction and transmission in the Netherlands. Here, the active surveillance is performed in vaccinated **chicken layer** flocks (testing all dead birds up to a number of 15 and up to 30 days) and passive surveillance in unvaccinated flocks.

Farm type	Number of establishments in the high-risk zone	Time frame (days)[a]	Sensitivity of surveillance component (CSe)[b]	Early detection sensitivity (EDSe)[c]	Surveillance system sensitivity (TotalSe)[d]	Probability of freedom (Pfree)[e]
Unvaccinated breeder flocks	1	16 (14–23)	0.02 (0.01–0.03)	0.02 (0.01–0.03)	0.95 (0.70–0.99)	0.997 (0.985–0.999)
Unvaccinated broiler flocks	33	16 (14–23)	0.20 (0.08–0.34)	0.19 (0.07–0.32)		
Unvaccinated duck flocks	2	6 (5–8)	0.16 (0.07–0.28)	0.15 (0.07–0.25)		
Vaccinated Layer flocks	**242**	31 (25–43)	0.93 (0.56–0.99)	0.92 (0.56–0.99)		
Unvaccinated turkey flocks	1	7 (6–9)	0.07 (0.03–0.13)	0.06 (0.03–0.12)		

[a]Time period between virus introduction and detection by passive surveillance.

[b]Cse as in Equation (8).

[c]EDSe as in Equation (9). For passive surveillance of unvaccinated flocks, the cumulative EDSe is calculated (assuming no outbreaks was reported) up to the end of the sampling interval.

[d]TotalSe as in Equation (10).

[e]Pfree as in Equation (11).

TABLE 15B Early detection sensitivity and probability of freedom from disease (if all sampled establishments are negative) when considering different sampling frequency and proportion of vaccinated **chicken layer** establishments tested.

Sampling interval	Proportion of vaccinated chicken layer establishment sampled (%)	Number of establishments	Early detection sensitivity (EDSe)[a]	Probability of freedom (Pfree)
30	100	242	0.92 (0.56–0.99)	0.996 (0.977–0.999)
30	50	121	0.46 (0.28–0.50)	0.973 (0.963–0.974)
30	25	61	0.23 (0.14–0.25)	0.961 (0.957–0.962)
14	100	242	0.986 (0.80–0.99)	0.999 (0.989–1)
14	50	121	0.71 (0.48–0.75)	0.985 (0.973–0.987)
14	25	61	0.41 (0.26–0.44)	0.970 (0.962–0.971)
7	100	242	1	1
7	50	121	0.99 (0.99–1)	1
7	25	61	0.99 (0.99–0.99)	0.999 (0.999–1)

[a]For sampling intervals shorter than the time frame, the cumulative EDSe up to the end of the time frame (expected detection by passive surveillance) is calculated.

TABLE 16A Early detection sensitivity and probability of freedom from disease in a high-risk zone for both introduction and transmission in France. Here, active surveillance is performed in vaccinated **duck** flocks (testing all dead birds up to 15 and every 30 days) and passive surveillance in unvaccinated establishments.

Farm type	Number of establishments in the high-risk zone	Time frame (days)[a]	Sensitivity of surveillance component (CSe)[b]	Early detection sensitivity (EDSe)[c]	Surveillance system sensitivity (TotalSe)[d]	Probability of freedom (Pfree)[e]
Unvaccinated breeder flocks	625	16 (14–23)	0.99 (0.99–1)	0.77 (0.74–0.81)	0.99 (0.99–1)	0.999 (0.999–1)
Unvaccinated broiler flocks	3194	16 (14–23)	1	0.77 (0.74–0.81)		
Vaccinated duck flocks	**1907**	21 (17–30)	1	0.74 (0.60–0.90)		
Unvaccinated layer flocks	1000	16 (14–23)	1	0.77 (0.74–0.81)		
Unvaccinated turkey flocks	154	7 (6–9)	0.99 (0.99–1)	0.68 (0.67–0.69)		

[a]Time period between virus introduction and detection by passive surveillance.

[b]Cse as in Equation (8).

[c]EDSe as in Equation (9). For passive surveillance of unvaccinated flocks, the cumulative EDSe is calculated (assuming no outbreaks was reported) up to the end of the sampling interval.

[d]TotalSe as in Equation (10).

[e]Pfree as in Equation (11).

TABLE 16B Early detection sensitivity and probability of freedom from disease (if all sampled establishments are negative) when considering different sampling frequency and proportion of vaccinated **duck** establishments tested.

Sampling interval	Proportion of vaccinated duck establishment sampled (%)	Number of establishments	Early detection sensitivity (EDSe)[a]	Probability of freedom (Pfree)
30	100	1907	0.74 (0.60–0.90)	0.999 (0.998–0.999)
30	50	954	0.37 (0.30–0.45)	0.968 (0.964–0.972)
30	25	478	0.18 (0.15–0.22)	0.959 (0.957–0.961)
14	100	1907	1	1
14	50	954	0.75	0.987 (0.987–0.999)
14	25	478	0.44	0.971 (0.970–0.999)
7	100	1907	1	1
7	50	954	0.95	0.997
7	25	478	0.71	0.985

[a]For sampling intervals shorter than the time frame, the cumulative EDSe up to the end of the time frame (expected detection by passive surveillance) is calculated.

TABLE 17A Early detection surveillance sensitivity, and probability of freedom from disease in a high-risk zone for both introduction and transmission in Italy. Here, active surveillance is performed in vaccinated **turkey** flocks (testing all dead birds up to a maximum of 15 every 30 days) and passive surveillance in unvaccinated establishments.

Farm type	Number of establishments in the high-risk zone	Time frame[a] (days)	Sensitivity of surveillance component (CSe)[b]	Early detection sensitivity (EDSe)[c]	Surveillance system sensitivity (TotalSe)[d]	Posterior probability free (Pfree)[e]
Unvaccinated breeder flocks	120	16 (14–23)	0.89 (0.68–0.99)	0.71 (0.58–0.71)	0.99 (0.99–1.00)	0.999 (0.999–1.00)
Unvaccinated broiler flocks	501	16 (14–23)	0.93 (0.36–0.99)	0.72 (0.34–0.80)		
Unvaccinated duck flocks	10	6 (5–8)	0.35 (0.13–0.79)	0.30 (0.12–0.58)		
Unvaccinated layer flocks	300	16 (14–23)	0.99 (0.98–1)	0.77 (0.74–0.81)		
Vaccinated turkey flocks	**385**	27 (21–38)	0.99 (0.97–1)	0.93 (0.83–1)		

[a]Time period between virus introduction and detection by passive surveillance.
[b]Cse as in Equation (8).
[c]EDSe as in Equation (9). For passive surveillance of unvaccinated flocks, the cumulative EDSe is calculated (assuming no outbreaks was reported) up to the end of the sampling interval.
[d]TotalSe as in Equation (10).
[e]Pfree as in Equation (11).

TABLE 17B Early detection sensitivity and probability of freedom from disease (if all sampled establishments are negative) when considering different sampling frequency and proportion of vaccinated **turkey** establishments tested.

Sampling frequency	Proportion of vaccinated turkey establishment sampled (%)	Number of establishment	Early detection sensitivity (EDSe)[a]	Probability free (Pfree)
30	100	385	0.93 (0.83–1)	0.988 (0.986–0.990)
30	50	193	0.49 (0.46–0.50)	0.974 (0.973–0.975)
30	25	97	0.25 (0.24–0.25)	0.962 (0.961–0.962)
14	100	385	0.99 (0.99–1)	0.999 (0.999–1)
14	50	193	0.75 (0.74–0.75)	0.987 (0.986–0.987)
14	25	97	0.44 (0.43–0.44)	0.971 (0.970–0.971)
7	100	385	1	1
7	50	193	1	1
7	25	97	0.99 (0.99–1)	0.999 (0.999–1)

[a]For sampling intervals shorter than the time frame, the cumulative EDSe up to the end of the time frame (expected detection by passive surveillance) is calculated.

3.1.2.4 | *Surveillance to assess vaccination effectiveness (E2)*

Estimation of vaccine effectiveness – data needed and possible analysis

Surveillance can also be used to assess vaccination effectiveness (VE), e.g. the ability of vaccination to prevent outbreaks upon exposure. The regional implementation of vaccination allows the observation of many vaccinated establishments to assess vaccination effectiveness following natural exposure and it may also be possible to assess vaccination characteristics like the onset of protection upon vaccination (e.g. following emergency vaccination) or the duration of protection. In addition, the effectiveness of different vaccines or vaccination schemes can be compared. However, some challenges can be foreseen: (i) Natural virus circulation has to occur in the population, (ii) an effective surveillance system has to be in place to early detect the virus, (iii) the probability of exposure to the pathogen is not necessarily equal across the territory or across an epidemic (i.e. among study groups). To estimate the effectiveness of vaccination, the probability of exposure of vaccinated and non-vaccinated flocks to the field virus should be similar on average (EFSA, 2018; Klement et al., 2020). Given that vaccination is expected to be applied in those regions most at risk, it would be difficult to have all those conditions in place.

During an epidemic, where emergency vaccination is applied, it will be of interest to assess VE at different time points following vaccination. During emergencies, it is of interest to assess the onset of protection and progression of VE in time post-vaccination, e.g. assessment of VE at 7, 14, ≥ 21 days following vaccination would be desirable. This information would help, for example, improve emergency preparedness by using these VE parameters to inform simulation models such as those used for the assessment of ToRs 1 and 2 in the Opinion Part 1 (EFSA AHAW Panel, 2023).

To estimate the vaccination effectiveness based on relative risk in the vaccinated population compared with the unvaccinated population, the data needed are the vaccination status of an establishment (Y/N), the date of vaccination, the HPAI infection status (Y/N) and the respective date of detection. Based on this date of detection, inferences on the date of infection could be made (e.g. suspected date of virus introduction).

It is recommended to collect and group the data at the smallest administrative area (e.g. municipality). This is to ensure (as much as possible) that unvaccinated/vaccinated establishments within the geographical region have a similar probability of exposure. In addition, during emergencies, these establishments are likely to be vaccinated practically at the same time. During epidemics, it could be challenging to separate vaccinated from unvaccinated groups geographically. Instead, separation can be made temporally, by comparing the probabilities (or Hazard) of infection before vaccination and at different time points following vaccination (e.g. 7, 14 and > 21 days). Depending on the analytic method, either regression or survival models, RR (Poisson regression), OR (logistic regression) or HR (Cox proportional hazard) could be calculated and VE values could be derived.

In summary, assessing VE following emergency vaccination would help to improve emergency preparedness, to make inferences of the vaccine used and to assess the efficacy of the implemented control strategies and related allocation of resources, and together define more effective interventions for future epidemics.

During preventive vaccination, all establishments of a specific poultry type are likely to be vaccinated, so no comparison with unvaccinated ones is possible. Nevertheless, the occurrence of outbreaks in vaccinated establishments points to vaccination failure. Given the assumption that vaccination is effective in 70% of the establishments (EFSA AHAW Panel, 2023), some outbreaks may be expected if HPAIV exposure from wild birds occurs. Nevertheless, this should not result in extensive between farm transmission (EFSA AHAW Panel, 2023). To detect this as soon as possible, it is important to sequence viruses from the outbreaks to estimate the likelihood of between farms spread in contrast to separate introductions from the wild compartment.

3.1.2.5 | *Surveillance to assess the level of immune response induced by vaccination (P2)*

In the absence of virus circulation, vaccination effectiveness cannot be assessed, but the level of immunity at the establishment level induced by vaccination can be measured using seropositivity, or any other correlate of protection, following the administration of the vaccine. Therefore, in addition to monitoring the level of immunity, there needs to be confirmation that there is no virus circulation in the vaccinated flock, following the surveillance approach discussed in Section 3.1.2.3.

The type of vaccine applied can affect the character (humoral or cell-mediated) and magnitude of immune response, this may in turn determine the most appropriate diagnostic test to monitor the immunity response at the flock level, as thoroughly discussed in Section 3.1.1. To do this, the animals should be sampled at a precise time within the time window for detection of birds with a specific correlate of protection (e.g. 28–35 days post vaccination for HVT-H5 vectored vaccine, not earlier than 21 days post-vaccination or latest booster for inactivated vaccines). However, identifying a correlate of protection, e.g. a given HI titre, which can be used to monitor the level of immunity reached within a flock (referred to as 'herd immunity') remains elusive (EFSA AHAW Panel, 2023). Hence, we suggest a pragmatic approach to assess the percentage (prevalence) of a vaccinated flock showing an 'effective' immune response (as defined by the diagnostic test to be used) as an indication of 'adequate vaccination' and assume that this could also provide an indication of the level of herd immunity reached in the vaccinated flock. First, the desired level of herd immunity (minimum prevalence of birds with a positive vaccine induced immune response) should be defined. This herd immunity level is equal to $1-1/R_0$. Median (range) of experimental and field estimates of R_0 for HPAI H5 viruses (CVI and APHA, 2017; EFSA AHAW Panel, 2023) are 3 (2–6) for chickens and 20 (16–88) for ducks. For turkeys, only one estimation was available, this estimate was 13 (95% Confidence Intervals: 9–17) (Ssematimba et al., 2019). Given these estimates, the desired level of immunity would be ≥ 95 (94–99)% for domestic ducks ($R_0 = 24$), ≥ 92 (89–94)% for turkeys ($R_0 = 12.8$) and ≥ 73 (50–83)% for chicken layers ($R_0 = 3.6$).

A sampling approach could be the one previously adopted in the emergency vaccination plans, set out in Commission Decision 2000/721/EC,[10] applied for the control of LPAI in Italy in 2000 and currently being used to monitor vaccination in France. The approach is based on taking and assessing at least 20 random samples per establishment. In Table 18 sample sizes, prevalence estimates based on the number of positive (for immune response) birds (the 'sample prevalence') and the one-sided 95% lower confidence limit (LCL) of the prevalence are provided. The latter gives an indication of the confidence that the 'true' prevalence of immune response in the establishment is at the required level. For example, if we were monitoring vaccinated chicken layers, the level of immunity we could target is ≥ 73%. If 20 samples were taken and 15 are positive, the sample prevalence is 75% but we cannot be 95% confident that the 'true' prevalence in the establishment is ≥ 73%, since the 95% LCL of the prevalence is 58%. If, however, 18 samples were positive, then one can be 95% confident that the prevalence in the establishment is ≥ 73%, since the 95% LCL of the true prevalence is 74% (Table 18). Similar interpretations can be made for different sample sizes and results combinations, for chickens, ducks or turkeys.

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[10]Commission Decision 2000/721/EC of 7 November 2000 on introducing vaccination to supplement the measures to control avian influenza in Italy and on specific movement control measures. OJ L 291, 18.11.2000, pp.33–36.

TABLE 18 Sample sizes, to determine the prevalence of birds showing a positive immune response induced by vaccination and assess whether this prevalence is at the required level.

Poultry species	Required prevalence of immune responders	Sample size	Number positives	Sample prevalence	95% lower confidence limit[a]
Chickens	≥ 73 (50–83)%	**20**	15	75%	58%
Turkeys	≥ 92 (89–94)%		16	80%	62%
Ducks	≥ 95 (94–99)%		17	85%	69%
			18	90%	74%
			19	95%	80%
			20	100%	88%
		30	25	83%	70%
			26	87%	73%
			27	90%	77%
			28	93%	82%
			29	96%	86%
			30	100%	92%
		40	35	87%	76%
			36	90%	79%
			37	92%	83%
			38	95%	86%
			39	97%	89%
			40	100%	94%
		50	45	90%	81%
			46	92%	83%
			47	94%	86%
			48	96%	89%
			49	98%	92%
			50	100%	95%

[a]These are one-sided Wilson exact 95% confidence limits.

3.1.2.6 | *Surveillance after cessation of vaccination (duration and type)*

After cessation of vaccination, i.e. after the last vaccine has been administered and there are no further plans to vaccinate the birds, it is expected that vaccinated flocks will be gradually replaced by unvaccinated flocks at the end of each production cycle. This gradual replacement will end to a time point where all the flocks in the area will be unvaccinated and, therefore, no longer subject to HPAI surveillance to be implemented after vaccination. As assessed in the Opinion Part 1 (EFSA AHAW Panel, 2023), currently, there is no knowledge on the length of protective immunity in vaccinated birds, particularly for long-living birds (e.g. breeders) and on threshold values for correlate of protection. Therefore, an assessment of the duration of the surveillance after cessation of vaccination based on the demonstration that the vaccinated birds are still protected at a certain time point cannot be conclusive. Vaccinated flocks should be under surveillance depending on the context of vaccination (emergency/preventive) up to their replacement, with dead birds being preferred for testing. Meanwhile, passive surveillance should be applied on unvaccinated flocks present in the vaccination area.

3.1.2.7 | *Surveillance in vaccinated captive birds*

According to Regulation (EU) No 2016/429[11] on transmissible animal diseases ('Animal Health Law' – AHL), captive birds[12] are defined as any birds other than poultry[13] that are kept in captivity for any reason other than poultry, including those that are kept for shows, races, exhibitions, competitions, breeding or selling.

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[11]Regulation (EU) No 2016/429 of the European Parliament and of the Council of 9 March 2016 on transmissible animal diseases and amending and repealing certain acts in the area of animal health ('Animal Health Law'). OJ L 84, 31.3.2016, p. 1.

[12]According to Regulation (EU) No 2016/429, **captive birds** means any birds other than poultry that are kept in captivity for any reason other than those referred to in point (9), including those that are kept for shows, races, exhibitions, competitions, breeding or selling.

[13]According to Regulation (EU) No 2016/429, poultry means birds that are reared or kept in captivity for: (a) the production of: (i) meat; (ii) eggs for consumption; (iii) other products; (b) restocking supplies of game birds; (c) the purpose of breeding of birds used for the types of production referred to in points (a) and (b).

Vaccinated birds in EU-approved confined establishments

Vaccinated birds in EU-approved confined[14] establishments such as zoos, wildlife park or rehabilitation centres, are exempted from the surveillance as set out by Delegated Regulation (EU) No 2023/361 (see Part 5, point 2.3) (e.g. serological or virological testing). This is likely due to the negligible risk of HPAIV transmission from zoos to poultry holdings because of their confinement. Also, active surveillance is impractical, because periodically catching, swabbing and testing live wild birds of high value are difficult. Thus, passive surveillance is deemed more feasible and practical. Regular clinical examinations (e.g. weekly) by zoo personnel and zoo veterinarians should be conducted to early detect and intercept the presence of HPAIV in vaccinated zoo birds. In this setting, any clinical signs can be promptly detected, coupled with prompt analyses of any sick and dead birds, including wild birds found dead on the zoo premises. Moreover, environmental samples collected from each cage regularly (e.g. monthly) from e.g. cage structures, drinkers, feeders, faeces, feathers might improve the sensitivity of the surveillance system. If vaccinated birds are to be moved to another confined establishments, they should be subjected to clinical examination and molecular virological testing with negative results within 72 h before movement. Of note, there is limited knowledge in relation to the dynamic of HPAI infection of vaccinated zoo bird species.

Establishments with vaccinated ornamental birds

For establishments housing vaccinated ornamental birds, the surveillance approach may be tailored to their size and trade activities. In limited size establishments, passive surveillance similar to that proposed for confined establishments could be appropriate. However, in establishments with more extensive trade activities, surveillance strategies as proposed for poultry in Section 3.1.2.1 might be more suitable. Pre-movement clinical examination and molecular virological testing with negative results within 72 h before movement are recommended, considering the potential risks associated with movements of such birds to other establishments or to events (e.g. for trade, markets, exhibitions, shows, etc.) with a gathering of HPAI-sensitive animals. Of note, there is limited knowledge in relation to the dynamic of HPAI infection of vaccinated ornamental birds.

3.2 | ToR 4: Restrictions and risk mitigation measures

3.2.1 | Emergency vaccination

According to Delegated Regulation (EU) No 2020/687, during emergency vaccination authorisation can be granted for the movement of animals and eggs if the risk is considered negligible by the competent authority based on a risk assessment including results of clinical examination, laboratory examination (if necessary) and an official veterinary visit.

It should be considered that, according to Delegated Regulation (EU) No 2023/361, the establishments that have been vaccinated under emergency are already under surveillance, so laboratory examination is ongoing. According to the EFSA assessment, the surveillance to be applied in these establishments is reported in Sections 3.1.2.1 and 3.1.2.2. to early detect HPAI and to demonstrate freedom from HPAI (Tables 10A–12B). Therefore, including the effectiveness of the combined emergency vaccination and surveillance established in this Opinion, the risk of movement of animals and eggs is considered negligible in the following situations described in Delegated Regulation (see Figure 6). Note that, laboratory examinations, differently from what is stated in Delegated Regulation (EU) No 2020/687 where it should be done only if necessary, are here considered as part of the risk mitigation measures to be implemented.

For movement to the slaughterhouse, vaccinated **poultry** is clinically inspected and dead birds for molecular testing should be collected not earlier than 72 h before the movement. Negative test results should be provided before the movement and no clinical signs should be observed. Sampling should focus on the birds that have died on the day of clinical inspection or/ and the day before. This combination of inspection and virological checks results in a negligible risk and, consequently, from the perspective of HPAIV risk, there is no need to slaughter vaccinated animals separately from non-vaccinated animals.

For the movement of **ready to lay** poultry, vaccinated birds are clinically inspected and dead birds for testing should be collected not earlier than 72 h before the movement. Negative test results should be provided before the movement and no clinical signs should be observed. If the recipient establishment is located in the vaccinated zone, the appropriate testing scheme provided in Tables 10A–12B should be followed. If the recipient establishment is located outside the vaccinated zone, the establishment should be under official supervision for at least 21 days, during which the appropriate testing protocol provided in Tables 10A–12B should be followed. In addition, no other animals of listed species should be present in such an establishment.

For the movement of **hatching eggs**, vaccinated poultry in the breeding flock should be clinically inspected and dead birds for testing should be collected no earlier than 72 h before the movement. Negative test results should be provided before the movement and no clinical signs should be observed. Eggs should not come in contact with eggs or day-old chickens from outside the restriction zone.

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[14]Confined establishments are defined according to Regulation (EU) No 2016/429 Art. 4 as any permanent, geographically limited establishment, created on a voluntary basis and approved for the purpose of movements, where the animals are: (a) kept or bred for the purposes of exhibitions, education, the conservation of species or research; (b) confined and separated from the surrounding environment; and (c) subject to animal health surveillance and biosecurity measures.

For the movement of **day-old chickens**, it should be confirmed that the breeding flock of origin has been free from HPAIV following the appropriate testing protocol provided in Tables 10A–12B. The establishments of destination should be under official supervision for 21 days.

Placing the establishments of destination of day-old-chickens, ready to lay poultry and hatcheries under official surveillance should facilitate proper implementation of biosecurity measures and assure early detection of the disease, thus reducing the potential risk of spread of the infection.

If the process of movement (e.g. loading) is not completed within 72 h, then testing should be repeated every 48 h until the process is finished.

Given the already ongoing surveillance, in all the situation described above, testing could coincide with the sampling session of the surveillance in place after emergency vaccination.

For movement of chicken layers from establishments < 3000 birds, given the reduced confidence of early detection in this flock size of the surveillance applied (Section 3.1.2.1), molecular testing of live birds (as recommended in Delegated Regulation (EU) No 2023/361) should be considered before movement in addition to clinical inspection and molecular virological testing of dead birds (all up to a number of 10 dead birds weekly as indicated in Section 3.1.2.1).

For movement of turkeys from establishments < 6000 birds, in addition to the surveillance strategy based on mortality threshold (see Section 3.1.2.1), it is recommended to combine clinical inspection and molecular testing of live (as recommended in Delegated Regulation (EU) No 2023/361) and dead birds before movements. It should be noted that those flock sizes are likely to contribute less to HPAIV transmission than larger flocks at a regional level.

3.2.2 | Preventive vaccination

According to Delegated Regulation (EU) No 2023/361, safe movements of vaccinated birds and their products are guaranteed if the vaccinated poultry or the day-old chickens and hatching eggs deriving from such poultry were negative to the active and enhanced passive surveillance implemented after preventive vaccination against HPAI (Table 1) and also other mitigation measures are implemented (see Figure 7). In the current Opinion, the surveillance to be applied after preventive vaccination is assessed in Section 3.1.2.3 and prescribes the monthly sampling of all dead birds up to a number of 15 collected in a flock 48 h before the testing to assess a zone as free from HPAIV.

Therefore, for preventive vaccination, where no outbreaks are present, the surveillance as described in Section 3.1.2.3 that is already in place provides a very high certainty that the area is free from infection (see Tables 15A–17B). Accordingly, if no outbreaks are detected and all establishments follow the surveillance protocol, the risk of movement is considered negligible in the following situations.

For movement to the slaughterhouse, vaccinated **poultry** should be clinically inspected not earlier than 72 h before the movement. Negative surveillance test results should be provided, and, before the movement, no clinical signs should be observed. In addition, at the slaughterhouse ante mortem inspection should be taking place. For Anseriformes, the sampling scheme to be applied under surveillance is the one provided in Tables 16A and 16B.

For the movement of **ready to lay** poultry, vaccinated poultry should be clinically inspected not earlier than 72 h before the movement. Negative surveillance test results should be provided and, before the movement, no clinical signs should be observed. If the recipient establishment is located in the vaccinated zone, the appropriate testing scheme provided in Tables 15A–17B should be followed. If the recipient establishment is located outside the vaccinated zone, the farm should be under official supervision for at least 21 days, during which the appropriate testing protocol provided in Tables 15A–17B should be followed. In addition, no other animals of listed species should be present in such an establishment.

For the movement of **hatching eggs** to a hatchery, the breeding establishment should follow the appropriate surveillance protocol given in Tables 15A–17B.

For the movement of **day-old chickens**, it should be clear that the breeding flock of origin has been free from HPAIV following the appropriate testing protocol provided in Tables 15A–17B. The establishment of the destination is under official supervision for 21 days.

If the establishment from where the vaccinated birds are to be moved is not under surveillance, for example when fewer than 100% of vaccinated establishments are subjected to testing (such as 50% or 25%), then vaccinated poultry or day-old chickens and hatching eggs deriving from such poultry are clinically inspected and dead birds for testing, all up to a number of 15, should be collected not earlier than 72 h before the movement. Negative test results should be provided before the movement and no clinical signs should be observed.

All other measures described for preventive vaccination, e.g. disinfection, traceability, are considered useful and suitable to prevent HPAIV spread via the movement of vaccinated birds or their products.

3.3 | Sources of uncertainty

The source of uncertainty found in the current assessment are here listed. In relation to the assessment on diagnostic tests used in the context of HPAIV surveillance, there is a lack of information on several aspects including: the impact of vaccination on the performance of molecular tests under field conditions given the expected decrease in viral loads/shedding; the suitability of different environmental samples for surveillance in vaccinated flocks; the usefulness of amplification-based techniques other than PCR-based methods (e.g. LAMP, SDA, CRISPR) for detection of HPAIV; the performance of rapid

antigen detection systems on dead birds for monitoring of HPAIV circulation in vaccinated flocks; on the duration of the immunity elicited by vaccination; and on the correlate of protection which are described in Section 3.1.1.

Regarding the sources of uncertainty for the assessment of the effectiveness of the surveillance strategies at the establishment level, these are mainly related to the within-flock and between-flock transmission models chosen, which assumed values for transmission and clinical parameters such as the transmission rates, duration of latent and infectious periods, case fatality rate and baseline mortality and a given range of values for the between-flock reproduction number R_h based on previously published estimates when available or based on expert opinion (mainly for vaccinated turkeys) which may not be applicable to certain chicken layer, duck and turkey production systems in Europe. Also, there is uncertainty on species different from chicken layers, ducks and turkeys, given that data available for this assessment only relate to those species.

Similarly, for the assessment of surveillance strategies at area/zone level, there is uncertainty related to the parameters used as inputs for the scenario tree modelling, such as the relative risks of virus incursion in a zone/poultry species, which could differ among Member States, the proportion of establishments effectively protected following vaccination and the probability of infection in vaccinated flocks, which were informed by previously published studies which may not be representative of all the scenarios/populations covered by this assessment.

4 | CONCLUSIONS AND RECOMMENDATIONS

4.1 | ToR 3: Diagnostic methods

- A broad spectrum of diagnostics methods is available for selection according to vaccine type, vaccination strategy and surveillance objective.

  - **Recommendation**: The vaccination plan should already pre-select the most appropriate diagnostic assays based on vaccine type, vaccination strategy and surveillance objective. Recommended diagnostic tests are provided in Tables 2 and 5.

- There is a lack of field experience and validation data for diagnostic methods to be used for active surveillance in vaccinated flocks.

  - **Recommendation**: The vaccination plan should only set out the use of diagnostic methods compliant with Points a or b of Point 1 of Article 6 of Commission Delegated Regulation (EU) No 2020/689,[15] while Members States are encouraged to conduct additional studies to collect field experience and validation data on alternative diagnostic methods in vaccinated establishments. The Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (WOAH, online-b) (Section 1.1.6. – Validation of diagnostic assays for infectious diseases of terrestrial animals and Section 2.2. – Validation of diagnostic tests), as well as the requirements of the EN ISO/IEC 17025[16] provide guidance for validating diagnostic methods to ensure they are compliant with the purpose they are intended for.
  - **Recommendation**: Since vaccinated birds, if exposed and infected, are expected to excrete lower levels of the virus than unvaccinated birds, the use of diagnostic methods with high sensitivity is recommended.

- Serological methods used to assess vaccination coverage, which are based on the detection of antibodies, can perform poorly in flocks that have received only replicating-vectored vaccines as these predominantly induce T-cell over humoral immune responses.

  - **Recommendation**: For each vaccine strategy-specific thresholds to estimate vaccination coverage as well as correlates of protection need to be identified based on experimental studies and field evidence.

- Serological methods usually have a lower specificity than molecular diagnostic methods. This compromises their use as a sole surveillance assay.

  - **Recommendation**: Serological results when aiming at demonstrating disease freedom must be confirmed with molecular virological investigations (i.e. adequate samples should be collected to allow for virological testing in DIVA-vaccinated flocks with seropositive monitoring results).

- Serological data obtained from laboratory trials performed in the EU demonstrated that accurate selection of antigens for HI tests is important for sensitive detection of vaccine-induced humoral immune responses (i.e. selection of antigens

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[15]Commission Delegated Regulation (EU) 2020/689 of 17 December 2019 supplementing Regulation (EU) 2016/429 of the European Parliament and of the Council as regards rules for surveillance, eradication programmes, and disease-free status for certain listed and emerging diseases. OJ L 174, 3.6.2020, pp. 211–340.

[16]EN ISO/IEC 17025:2017. General requirements for the competence of testing and calibration laboratories. International Organization for Standardization.

antigenically close to the one contained or expressed by the vaccine used) and for the differentiation of antibodies stimulated by HPAI infection in vaccinated birds from antibodies elicited by vaccination (i.e. antigens antigenically close to circulating field strains).

- **Recommendation**: Besides data collected in laboratory settings, field data are needed to understand antibody dynamics in the field.
- **Recommendation**: Continuous monitoring and improvement of test antigens tailored to the specific surveillance activities aims is needed to ensure the highest performances of HI assays.

- NP-ELISA assays can be used to detect past infection in vaccinated birds (DIVA approach) starting from 7 days post-infection with Se that range from 60 to 100% depending on the species and if compatible vaccines (i.e. vaccines that do not contain the AI-NP in the formulation) are used.

- **Recommendation**: Commercially available indirect ELISA have demonstrated superior performance compared with available competitive ELISA assays in both chickens and ducks and thus should be preferred if employed in surveillance activities and if the assay is validated for the target species.

- Reservation to using DIVA vaccination is linked to the low specificity of serological tests which increases risks of false-positive results.

- **Recommendation**: Seropositive results in DIVA-vaccinated flocks require independent confirmation by molecular assays carried out on swab samples.
- **Recommendation**: Continuous monitoring and improvement of serological tests tailored to the specific surveillance activities aims in vaccinated flocks, is needed to ensure the highest performances of these assays.

- Co-circulation of avian influenza viruses of low pathogenicity can interfere with clear-cut molecular and serological diagnostic surveillance measures in HPAI vaccinated as well as in non-vaccinated flocks.

- **Recommendation**: Molecular methods that allow identifying all avian influenza viruses, including LPAIV, down to the sub- and pathotype levels must be in place in official laboratories.

- Analysis of samples collected from the environment is cost- and time-saving compared with sampling and testing individual birds for HPAIV in premises suspected of infection. However, it is not yet suitable for surveillance, particularly when targeting early detection, as there is currently a lack of validation data and protocols in comparison to diagnostic performances with individual bird-derived samples in vaccinated flocks.

- **Recommendation**: Validation of environmental sampling methods and associated protocols for vaccinated flocks, in particular for use as part of an early detection strategy, are needed in the field; research studies aimed at filling these knowledge gaps are encouraged also in the context of zoos or confined establishments.

- Field experience in vaccinating countries has demonstrated that the use of unvaccinated fully susceptible sentinels can increase the risk of infection of the flock and that numerous practical problems reduce their advantages or hamper their implementation.

- **Recommendation**: Use of sentinels in vaccination plans is not recommended.

- Currently available commercial point-of-care diagnostic methods for HPAIV detection cannot be employed for surveillance in vaccinated flocks due to lack of validation. In general, they have lower performance (in particular lower sensitivity) compared with RT-qPCR.

- **Recommendation**: Further development and validation of point-of-care assays are needed in the field; research in that direction should be performed. Currently, the use of rapid antigen assays for active surveillance in vaccinated flocks is not recommended.

- Genetic and phenotypic characterisation tools are available for the accurate detection and characterisation of vaccine escape mutants.

- **Recommendation**: Genetic characterisation of all viruses identified from HPAIV detected in vaccinated flocks is highly recommended and NRLs should be prepared for this activity before beginning the vaccination campaigns.
- **Recommendation**: Viral isolation followed by phenotypic characterisation (e.g. evaluation of antigenic characteristics by HI and/or V-NT; adaptation to mammalian species; replicative behaviour; etc.) is recommended in a representative number of PCR-positive samples obtained from vaccinated flocks.

4.2 | ToR 3: Surveillance

General considerations

- The epidemiological unit for surveillance is the establishment, which may consist of one or several flocks (comprising all poultry of the same health status kept in the same enclosure).
- Sample sizes are provided at the flock level, meaning that if an establishment includes several flocks, sampling should be carried out within each individual flock. Detection of HPAIV in one flock within an establishment indicates infection for the entire establishment.
- Surveillance strategies for testing dead birds include random sampling of all dead birds up to a number of 5, 10 or 15. If fewer dead birds than this number are available, all available dead birds should be tested.
- We assumed the lowest case fatality rates of 20%, 5% and 20% in infected vaccinated chicken layers, ducks and turkeys, respectively, observed for vaccines considered as not effective in stopping sustained transmission (EFSA AHAW Panel, 2023). However, uncertainty remains in estimating these parameters accurately.
- The model relies on within-flock transmission parameters, yet uncertainty persists regarding these parameters and their accurate determination due to data limitations.

  - **Recommendation** for research: It is recommended to estimate case fatality in (a sample of) HPAIV outbreaks in vaccinated flocks to reduce the uncertainty of this parameter. Laboratory testing of dead and live birds individually can be used to obtain these estimates.
  - **Recommendation** for research: It is recommended to estimate within-flock transmission parameters during HPAIV outbreaks to reduce the uncertainty of the transmission parameters.

General conclusions

- Surveillance strategies based on sampling and testing dead birds have a higher probability of early detecting HPAIV than strategies based on testing live birds.
- Increasing the sample size above the numbers of 5, 10 or 15 dead birds provided does not increase the probability of HPAIV detection.
- The effectiveness of surveillance is increased by the repeated sampling in time.
- If HPAIV enters a flock between two sampling events, the proportion of infected birds will be highest close to the second sampling event. Consequently, testing birds found dead in the last 48 h yields the highest sensitivity.
- If, at a certain sampling event, no dead birds are present in the flock, it is probable that the infection has either not entered the flock or entered so recently that it cannot be detected yet. In the latter case, the surveillance strategy involving repeated sampling would result in the collection of dead birds in the next sampling event, leading to the detection of the infection entered in the previous sampling interval.

  - **Recommendation**: Sampling and testing dead birds should consist of dead birds found in the last 48 h.

- No definitive conclusion can be drawn regarding the duration of the surveillance after the cessation of vaccination in a flock, due to the current lack of knowledge on the correlate of protection and consequently on the duration of protective immunity in vaccinated poultry, particularly in long-living birds.

  - **Recommendation**: Vaccinated flocks should be under surveillance up to their replacement, with the sampling and testing of dead birds being the recommended option. Meanwhile, passive surveillance should be applied for unvaccinated flocks within the vaccination area.

4.2.1 | Surveillance for early detection at establishments for emergency vaccination

Strategies for early detection were considered effective if they resulted in more than 95% of the simulated outbreaks reaching detection probabilities above 99% (interpreted as leading to early detection). 'Passive reporting' and 'mortality threshold' strategies were considered effective if they stopped sustained between-flock transmission ($R_s < 1$).

Chicken layers

- The surveillance strategy described in Delegated Regulation (EU) No 2023/361, which involves testing 60 **live birds** for virus presence (**virological** testing) every 2 weeks during emergency vaccination to detect at least one infected bird given 5% or higher disease prevalence, was assessed as not effective for early detection since there was < 95% certainty that it would lead to the early detection of HPAIV in vaccinated chicken layer flocks.

- The alternative surveillance strategy of testing 60 **live birds** for antibodies presence (**serological** testing) every 2 weeks during emergency vaccination was also assessed as not effective since there was < 95% certainty that it would lead to HPAIV early detection in vaccinated chicken layers.
- The surveillance strategies based on '**passive reporting**' or on '**mortality thresholds**' were not effective for HPAIV early detection, resulting in sustained between-flock transmission ($R_s > 1$) and long mean detection times post-introduction (e.g. 31 days (95% CI: 25–43) and 28 days (95% CI: 22–39), respectively, for 20,000 size flock; 21 days (95% CI: 16–33) and 15 days (CI 95%: 10–26), respectively, for 2500 flock size).
- The alternative strategy of testing **dead birds**, when implemented for flocks ≥ **3000** birds, was effective for early detection with > 95% certainty (Table 10A).
- For flocks **< 3000** chicken layers, none of the surveillance strategies assessed was effective with > 95% certainty for early detection (Table 10B). The maximum certainty that could be reached for early detection, by sampling all up to a number of 10 or 15 dead birds weekly, was 90%.

  o **Recommendation**: For early detection of HPAIV outbreaks, in flocks ≥ **3000** birds, during emergency vaccination, molecular testing of dead chicken layers in a flock is recommended. A number of effective options have been identified according to flock size – testing all up to a number of 5, 10 or 15 dead birds weekly, up to 10 or 15 dead birds every 2 weeks or up to 15 dead birds every 3 weeks – and should be selected according to a country's specific circumstances and resources.
  o **Recommendation**: For flocks **< 3000** birds, it is recommended to test all up to a number of 10 dead birds weekly, which combined with molecular testing of live birds every 2 weeks, as recommended in the Delegated Regulation (EU) No 2023/361, would increase the level of confidence of early detection.

Ducks

- The surveillance strategy described in the Delegated Regulation (EU) No 2023/361, which involves testing 60 **live birds** for virus presence (**virological** testing) every 2 weeks during emergency vaccination to detect at least one infected bird given a disease prevalence of 5% or higher, was assessed as effective for early detection since there was > 95% certainty (specifically 97%) that it would lead to the early detection of HPAIV in vaccinated ducks flocks. The average detection time post-introduction was 14 days (95% CI: 11–23) in flocks of 2500 ducks, and 17 days (95% CI: 13–27) in flocks housing 6000 ducks, with these detection timeframes resulting in limited between-flock transmission ($R_s < 1$).
- The alternative surveillance strategy of testing 60 **live birds** for antibody presence (**serological** (DIVA) testing) every 2 weeks during emergency vaccination was also assessed as effective since there was > 95% certainty (specifically 97%) that it would lead to HPAIV early detection in vaccinated ducks. However, diagnostic performance of the serological tests is inferior compared with molecular tests, thus reducing our certainty in this strategy.
- A surveillance strategy based on '**passive reporting**' only was not effective for HPAIV early detection, resulting in sustained between-flock transmission ($R_s > 1$) and a longer mean detection time post-introduction of 21 and 23 days (95% CI: 17–30; 19–32), depending on flock size (2500 and 6000, respectively).
- A surveillance strategy based on '**mortality thresholds**' was effective for early detection, resulting in limited between-flock transmission ($R_s < 1$); additionally, in flocks of 2500 ducks, the average detection time post-introduction was the shortest – 10 days (95% CI: 7–18).
- The alternative strategies of testing **dead birds** were proven to be effective with > 95% certainty that it would lead to early detection of HPAIV in vaccinated ducks. The average detection time ranged between 14 and 17 days post-HPAIV introduction in the flock, depending on sampling frequency, sample size and flock size and this was accompanied by limited between-flock transmission ($R_s < 1$) (Tables 11A and 11B).

  o **Recommendation**: For early detection of HPAIV outbreaks during emergency vaccination, molecular testing of dead ducks in a flock is recommended. A number of effective options have been identified: (i) in flocks of 2500 ducks (representing flocks with < 6000 ducks), testing all dead birds up to a number of 5, 10 or 15 weekly, (ii) in flocks of ≥ 6000 ducks, testing all dead birds up to 5, 10 or 15 weekly, testing all dead birds up to 10 or 15 every 2 weeks, or testing all dead birds up to 15 every 3 weeks. These strategies should be selected according to country's specific circumstances and resources.
  o **Recommendation**: As an alternative to testing dead birds, testing of 60 live ducks every 2 weeks (by means of molecular or serological testing) in a flock can also be carried out, but it may present more challenges in terms of welfare consequences and logistics (e.g. catching, handling and restrain) as well as requiring more resources.
  o **Recommendation**: As an alternative to testing dead birds, a surveillance strategy based on mortality threshold can also be carried out, but its effectiveness depends on the availability and accuracy of information about mortality thresholds that could prompt farmers to report suspicions of HPAI.

Turkeys

- The surveillance strategy described in the Delegated Regulation (EU) No 2023/361, which involves testing 60 **live birds** for virus presence (**virological** testing) every 2 weeks during emergency vaccination to detect at least one infected bird given a disease prevalence of 5% or higher, was assessed as not effective for early detection since there was < 95% certainty that it would lead to the early detection of HPAIV in vaccinated turkey flocks.
- The alternative surveillance strategy of testing 60 **live birds** for antibodies presence (**serological** testing) every 2 weeks during emergency vaccination was also assessed as not effective since there was < 95% certainty that it would lead to HPAIV early detection in vaccinated turkeys.
- The surveillance strategies based on '**passive reporting**' or on '**mortality thresholds**' were not effective for HPAIV early detection in flocks ≥ 6000 turkeys, resulting in sustained between-flock transmission ($R_s > 1$) and long mean detection time post-introduction (e.g. at 27 days (95% CI: 31–38) and 24 days (95% CI: 19–34), respectively, for flock 18,000).
- The alternative strategies of testing **dead birds**, when implemented for flocks of ≥ 6000 turkeys, proved to be effective for early detection with > 95% certainty (Table 12A).
- For flocks < 6000 turkeys, the only effective surveillance strategy for early detection was based on the use of '**mortality thresholds**' (0.21%), resulting in limited between-flock transmission ($R_s < 1$) (Table 12B).

  - **Recommendation**: For early detection of HPAIV outbreaks, for flocks ≥ 6000 turkeys, during emergency vaccination, molecular testing of dead turkeys in a flock is recommended. A number of effective options have been identified – testing up to 5, 10, 15 or 20 dead birds weekly, up to 15 or 20 dead birds every 2 weeks or up to 20 dead birds every 3 weeks – and should be selected according to country's specific circumstances and resources.
  - **Recommendation**: For flocks < 6000 turkeys, the surveillance strategy based on mortality threshold should be carried out which combined with molecular testing of live birds every 2 weeks, as recommended in the Delegated Regulation (EU) No 2023/361, would increase the level of confidence of early detection; the effectiveness of mortality threshold depends on the availability and accuracy of information about mortality thresholds that could prompt farmers to report suspicions of HPAI.

Surveillance in peri-vaccination zone

- Based on the results from EFSA AHAW Panel (2021), it was assessed that in a 'surveillance zone', where there is an unvaccinated poultry population, the probability of transmission of HPAIV beyond a 10-km radius (jump spread) was below 0.04. The same radius can be applied to the peri-vaccination zone as a conservative estimate which increases the confidence that the probability of transmission beyond 10 km is lower than 0.04.

  - **Recommendation**: A 10-km peri-vaccination zone during emergency protective vaccination is recommended, to increase the confidence that the probability of jump spread is lower than 0.04 (4%).

- In relation to the type of surveillance in the peri-vaccination zone, the options depend on the vaccination status of the establishments.

  - **Recommendation**: For preventively vaccinated farms, it is recommended to apply the same surveillance strategy as for early detection of emergency vaccination. For those farms that are not vaccinated, the recommendations provided in EFSA Opinion on control measures for Category A diseases (EFSA AHAW Panel, 2021) are considered applicable. Therefore, passive surveillance is to be applied for gallinaceous poultry (chickens and turkeys) and bucket sampling for Anseriformes (ducks and geese), carrying out a weekly sampling of all available dead birds, preferably within the last 48 h.

4.2.2 | Surveillance for early detection and demonstrating disease freedom following preventive vaccination

- Molecular virological testing of all dead birds in vaccinated flocks up to a number of 15 every 30 days, coupled with passive surveillance in unvaccinated flocks, would lead to at least 99% confidence in disease freedom in the zone, assuming ≤ 5% prior prevalence of establishments infected. This was confirmed by the model in the three high-risk zones assessed, the Netherlands with vaccinated chicken layers, France with vaccinated ducks and Italy with vaccinated turkeys.

  - **Recommendation**: Molecular virological testing of up to 15 dead birds every 30 days in vaccinated flocks, while maintaining passive surveillance in both vaccinated and unvaccinated flocks, is recommended to effectively demonstrate disease freedom with > 99% confidence within high-risk zones for HPAIV infection.

- With the above testing scheme, the probability of early detecting at least one infected vaccinated establishment (EDse) in the time frame of 30 days was 92% for vaccinated chicken layers in the Netherlands, 74% for vaccinated ducks in France and 93% for vaccinated turkeys in Italy; reducing the sampling intervals from 30 days to 14 or 7 days increased the EDse to > 98% for chicken layer and turkey flocks and to 100% in duck flocks.

    ○ **Recommendation**: If the aim is to increase the early detection surveillance sensitivities, then it is recommended to reduce the sampling intervals.

- Reducing the proportion of vaccinated establishments to be tested to 25% would have a limited impact on the probability of freedom, remaining > 96% for chicken layers, 95% for ducks and > 96% for turkeys.
- Reducing the proportion of vaccinated establishments tested markedly decreases the early detection sensitivity (EDse). For instance, reducing the proportion of establishments to be tested from 100% to 50% would result in a decrease on EDse from 92% to 46% in chicken layers, from 74% to 37% in ducks and from 93% to 49% in turkeys.

    ○ **Recommendation**: For **chicken layers**, a number of effective options have been identified to maintain a level of early detection sensitivity > 92%, and probability of freedom > 99%, by testing all dead birds up to a number of 15 in 100% of the vaccinated establishments monthly; in 25% of the vaccinated establishments weekly – and should be selected according to country's specific circumstances and resources.
    ○ **Recommendation**: For **ducks**, a number of effective options have been identified to maintain a level of early detection sensitivity > 74% and probability of freedom > 98%, by testing all dead birds up to a number of 15 in 100% of the vaccinated establishments monthly; in 50% of the vaccinated establishments every 2 weeks – and should be selected according to the country's specific circumstances and resources.
    ○ **Recommendation**: For **turkeys**, a number of effective options have been identified to maintain a level of early detection sensitivity > 75% and probability of freedom > 98%, by testing all dead birds up to a number of 15 in 100% of the vaccinated establishments monthly; in 50% of the vaccinated establishments every 2 weeks; in 25% of the vaccinated establishments weekly – and should be selected according to country's specific circumstances and resources.

- In the high-risk zones, where unvaccinated and vaccinated establishments are present, passive surveillance in the unvaccinated establishments would contribute to increase the overall surveillance system sensitivity, and the confidence of disease freedom.

    ○ **Recommendation**: Maintaining passive surveillance efforts in unvaccinated establishments in vaccinated zones is recommended to enhance the overall sensitivity of the surveillance system.

4.2.3 | Surveillance for estimation of vaccination effectiveness

- The estimation of vaccination effectiveness (VE) under field conditions is a crucial element for (i) preventing and controlling epidemics, (ii) verifying the measures applied and (iii) justifying the investments. The effectiveness can differ among vaccination programmes.
- The assessment of vaccination effectiveness requires careful planning, a good data collection framework, follow up of all flocks in the vaccinated area as well as sufficient human resources to conduct the study, in order to ensure high quality of data and outcomes from the analysis.

    ○ **Recommendation**: Data from the vaccinated population in the field should be collected from each epidemiological unit considered, including at least outbreak (events, suspicion and confirmation date) and vaccination (events, date) data, and spatial coordinates of each epidemiological unit. Data should be assessed to estimate vaccination effectiveness. Assessing VE at 7 and 14 days post-vaccination would give an indication of the VE for emergency vaccination.
    ○ **Recommendation**: In case of HPAI outbreaks following vaccination, it is recommended to sequence the virus to estimate the likelihood of between farm spread in contrast to separate introduction from wild birds.

4.2.4 | Surveillance to assess level of immune response induced by vaccination

- Assessment of the level of immune response following vaccination of an establishment can be performed when virus circulation is absent or expected to be low in the zone where (preventive) vaccination was applied.

    ○ **Recommendation**: Assessment of level of immune response following vaccination of an establishment according to the vaccines used and the selected diagnostic test, should be done combined with an assessment of the absence of virus circulation in the flock.

- ○ **Recommendation**: To best detect the proportion of birds with a measurable immune response sampling should be done when the highest immune response is expected, e.g. between 21 and 28 days post primary or booster vaccination. If only vector-based vaccines are used (e.g. HVT-H5), immune response should be assessed at least 4 weeks (e.g. 4 weeks in chickens or 7 weeks in turkeys) after vaccination.

- Based on the R0 for within flock transmission quantified from experimental and field data, the minimum proportion of protected birds needed to stop transmission was estimated to be higher than 90% for ducks and turkeys, and 70% for chicken layers. Although there are limited estimates of R0 values for ducks and turkeys, transmission in these species is expected to be faster and more extensive (with 95% certainty) than transmission between chickens, hence the high level (> 90%) of herd immunity required.

  - ○ **Recommendation for research**: Further quantification of R0 values for the currently circulating HPAIV is recommended to have a more accurate estimate of the minimum proportion of birds that should be protected in a flock to stop transmission.

- There is no established correlate of protection, e.g. antibody titre against HPAIV, that could be considered as a proven indicator of protection for poultry species. In the meantime, assessment of the prevalence of birds showing an immune response following vaccination is assumed to provide an indication of sufficient protection and possibly herd immunity. For chickens, taking 20 samples per establishment would allow confirmation of this prevalence being higher than the 70% with 95% certainty. For turkeys and ducks, 40, 50 or higher number of samples might be needed to confirm that the prevalence of birds' immune response is higher than 90% with 95% certainty.

  - ○ **Recommendation**: At least 20 blood samples per establishment, tested with the most suitable diagnostic test depending on the vaccine used, are appropriate to determine whether vaccines are correctly used in the field.
  - ○ **Recommendation**: As there are no established antibody titres that can be used as a correlate of protection for poultry species, the (sero)positivity in an appropriate test could be used as a proxy measure of protection in vaccinated birds. To estimate the true level of protection (herd immunity), an animal level correlate of protection should be defined. If a certain antibody titre was to be shown as a good correlate of protection, then a serological survey (based on serological test) could be conducted.

4.2.5 | Surveillance in vaccinated captive birds

- In confined establishments such as zoos and in small ornamental bird establishments, the risk of HPAI transmission to poultry holdings is likely limited, warranting passive surveillance. However, ornamental bird establishments with more extensive trade activities may pose increased risks.
- There is limited knowledge about the dynamics of HPAI infection in vaccinated species reared in confined or captive bird species establishments (which are usually different from poultry species).

  - ○ **Recommendations**: Maintaining passive surveillance in vaccinated birds from confined and small ornamental bird establishments is recommended.
  - ○ **Recommendations**: For kept ornamental birds, certain movements (i.e. those with a high risk of HPAIV spread such as for trade, exhibitions, shows, etc.) may represent a risk, and testing should be carried out before the movement takes place, since the premovement clinical examination alone may not be sufficient.

4.3 | ToR 4: Restriction and risk mitigation measures

4.3.1 | Emergency vaccination

- For **emergency vaccination**, where HPAI outbreaks are present in the area, vaccinated establishments are already under surveillance, including laboratory examination. In Delegated Regulation (EU) No 2020/687, clinical inspection in the 72 h before movement and laboratory examination to demonstrate freedom from HPAI is considered part of the risk mitigation measures.

  - ○ **Recommendation**: To enable safe movement of vaccinated poultry, day-old chickens and hatching eggs deriving from such poultry, birds should be clinically inspected and all up to a number of 15 dead birds should be tested no earlier than 72 h before movement to demonstrate that they are free from HPAIV. Negative test results and the absence of clinical signs should be provided before movement.
  - ○ **Recommendation**: Given the ongoing surveillance applied after emergency vaccination for early HPAIV detection and to demonstrate that the establishment is free from HPAIV, testing could coincide with the sampling session of the surveillance in place.

4.3.2 | Preventive vaccination

- For **preventive vaccination**, in which no HPAI outbreaks are present, the surveillance described in Section 3.1.2.3 (testing all dead birds up to a number of 15 in vaccinated flocks every month, see Section 4.2.2) provides high certainty that the area is free from HPAIV, given negative results. Accordingly, if no outbreaks are detected and all establishments follow the surveillance protocol, the risk of HPAIV spread via birds' movement is considered negligible.

  - **Recommendation**: To enable the safe movement of vaccinated poultry, day-old chickens and hatching eggs deriving from such poultry, negative surveillance test results should be provided, and no clinical signs should be observed before the movement.
  - **Recommendation**: If the vaccinated establishment is not under surveillance, for example when fewer than 100% of vaccinated establishments are tested, vaccinated poultry, day-old chickens and hatching eggs deriving from such poultry should be clinically inspected and all up to a number of 15 dead birds should be tested no earlier than 72 h before movement. Negative test results and the absence of clinical signs should be provided before movement.

ABBREVIATIONS

AI	avian influenza
AIV	avian influenza virus
AR	adjusted relative risks
CVMP	Committee for Veterinary Medicinal Products
DIVA	Differentiating Infected from Vaccinated Animals
EMA	European Medicines Agency
FPV	Fowlpox virus
GMO	genetically modified organism
HDPA	high-density poultry areas
HI	haemagglutination inhibition
HP	highly pathogenic
HPAI	highly pathogenic avian influenza
HPAIV	highly pathogenic avian influenza virus
HVT	Turkey Herpesvirus
LP	low pathogenic
LPAI	low pathogenic avian influenza
LPAIV	low pathogenic avian influenza virus
NDV	Newcastle Disease Virus
NP-ELISA	nucleoprotein-based ELISA
R_h	between-flocks reproduction number
RR	relative risk
SAN	specific antibody negative
SLR	systematic literature review
SPF	specific pathogen free
TRL	technology readiness level
USDA	United States Department of Agriculture
VE	vaccine efficacy
WOAH	World Organisation for Animal Health

GLOSSARY

CSe_i	component sensitivity for the *i*-th surveillance system component. It is the average probability that a surveillance system component will return a positive surveillance outcome, given disease is present in the population at a level equal to, or greater than, the specified design prevalence
EDSe	early detection sensitivity of each SSC, which is the probability that the surveillance activity is able to correctly detect among the poultry establishments the epidemiological unit(s) affected by a new incursion within a specific time frame
Pfree	probability that the country is free from disease, given that the surveillance system did not detect the disease. Confidence of population freedom (= negative predictive value)
PriorInf	prior probability (also known as prior prevalence) that the poultry population at establishment level would be infected
SSC	surveillance system components, a surveillance activity that, in itself, can contribute evidence to disease freedom; it has the capacity to detect disease if/when it occurs Five SSCs were identified: broilers, fattening ducks, meat turkeys, breeders and laying hens
TotalSe	surveillance system sensitivity, which combines the *EDSe* calculated for each SSC

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CONFLICT OF INTEREST

If you wish to access the declaration of interests of any expert contributing to an EFSA scientific assessment, please contact interestmanagement@efsa.europa.eu.

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APPENDIX A

Mapping of ToRs 3 and 4 and Opinion's output

TABLE A1 Term of references (ToR) as they were received from the European Commission and the assessment methods and results relative to each ToR or sub-ToR.

ToR	Sub-ToR	Assessment method	Assessment results
3. Surveillance in the vaccinated zone and/or vaccinated establishments	3.1. Assess the **suitability and effectiveness of the reinforced surveillance** set out in Part 2 and in point 2 of Part 5 of HPAI Annex to the Delegated Regulation (EU) No 2023/361 **to early detect** infection in the vaccinated flocks and to **prevent spread** of the HPAI virus by movement of birds and their products from vaccinated establishments/flocks. The above assessment should include scenarios depending on different vaccination strategies.	By vaccination strategy: a. To early detect (Section 2.1.5.1) and demonstrate freedom from HPAIV during emergency vaccination: modelling HPAI infection dynamic in vaccinated flocks b. To early detect and demonstrate freedom from HPAIV during preventive vaccination (Section 2.1.5.2): scenario's tree model	By vaccination strategy (emergency protective/ preventive): a. R_s values and time between introduction and detection based on virology (prev infectious), serology (prev recovered), mortality (prev dead), given different sampling scheme (frequency and size) (Section 3.1.2.1) b. Sensitivity of HPAIV early detection given different sampling schemes (frequency and proportion of vaccinated establishments under surveillance) and confidence of freedom (Section 3.1.2.3)
	3.2. Taking into account the possible different surveillance approaches (e.g. serology, virology, conventional, DIVA, passive/active surveillance of vaccinated birds, use of sentinels) explore **and provide for alternative suitable surveillance** approaches/strategies indicating the **minimum level and duration of surveillance** required in a vaccinated establishment, including sampling schemes and testing procedures, to: a. ensure **early detection** of infection with HPAI viruses; b. be implemented as additional guarantees **to authorise the movement** of vaccinated and non-vaccinated poultry and poultry products within the vaccinated zone and from the vaccinated zone or establishment to outside that zone or establishment; c. **be implemented after cessation of vaccination** as necessary risk mitigation measure to authorise movement of birds and their products from those establishments, taking into account: • the relevant factors to influence the level and duration of protection following vaccination with different type of vaccines, • simultaneous presence in the establishment of vaccinated and unvaccinated animals.	By vaccination strategy (emergency protective/preventive): a. To early detect (Section 2.1.5.1) and demonstrate freedom from HPAIV during emergency vaccination: modelling HPAI infection dynamic into vaccinated flocks b. To early detect and demonstrate freedom from HPAIV during preventive vaccination (Section 2.1.5.2): scenario's tree model c. Qualitative assessment d. Descriptive analysis of diagnostic methods available for surveillance of HPAIV vaccinated birds (Section 2.1.4) Description of vaccination effectiveness (Article 9) based on relative risk measures; assessment of level of immune response induced by vaccination (Section 2.1.5.3)	By vaccination strategy (emergency protective/ preventive): a. Rs values and time between introduction and detection based on virology (prev infectious), serology (prev recovered), mortality (prev dead), given different sampling schemes (frequency and size) (Section 3.1.2.1) b. Sensitivity of HPAIV early detection given different sampling schemes (frequency and proportion of vaccinated establishments under surveillance) and confidence of freedom (Section 3.1.2.3) c. Timespan of surveillance to be applied after cessation of vaccination (Section 3.1.2.6) d. Indication of appropriate diagnostic methods tailored to the surveillance objectives and sample type (Section 3.1.1) Indication of options and data needed to assess vaccination effectiveness (Section 3.1.2.4) and level of immune response induced by vaccination (Section 3.1.2.5)
	3.3. Taking into account the **WOAH standards** (i.e. surveillance to be carried out in all vaccinated flocks) **explore and provide for alternative** suitable surveillance approaches/ strategies to be implemented in a vaccination zone to **demonstrate freedom** from HPAI based on representative sampling of the vaccinated (and not vaccinated) establishments within a vaccination zone.	For preventive vaccination: b. Scenario's tree model (Section 2.1.5.2)	b. Confidence of freedom given different sampling schemes considering different proportion of vaccinated establishments under surveillance (Section 3.1.2.3)

(Continues)

TABLE A1   (Continued)

ToR	Sub-ToR	Assessment method	Assessment results
4. Restrictions and risk mitigation measures to be applied in a vaccinated establishment or a vaccination zone	4.1. Assess the suitability of the restrictions and risk mitigation measures set out in Part 3 and in points 3 to 5 of Part 5 of HPAI Annex to the Delegated Regulation (EU) No 2023/361 **to prevent the spread** of HPAI viruses, enabling safe movement of poultry and their products following emergency and preventive vaccination, respectively. 4.2. Explore and **provide for alternative** (to those referred in 4.1.) suitable movement restrictions and risk mitigation measures required to prevent the spread of virus by movement of birds or their products from a vaccinated establishment/vaccination zone.	By vaccination strategy (emergency protective/preventive) (Section 2.2.1): Qualitative assessment of measures currently in place and demonstration of freedom as additional/alternative measure via modelling HPAI infection dynamic into vaccinated flocks (emergency vaccination) and scenario's tree model (preventive vaccination)	By vaccination strategy (emergency protective/ preventive): confidence of freedom given different sampling schemes (frequency, size, proportion of vaccinated establishments under surveillance) (Section 3.1.2.3)