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ANIMAL HEALTH RISK ANALYSIS

MANAGEMENT No. 4

# GUIDING PRINCIPLES FOR THE DESIGN OF AVIAN INFLUENZA ACTIVE SURVEILLANCE IN ASIA

*Designing active, comprehensive, risk-based avian influenza surveillance*

## SUMMARY

- Since the first detection of the Gs/GD **highly pathogenic avian influenza** H5N1 virus in 1996, avian influenza viruses (AIVs) have continued to diversify, generating new subtypes in both domestic and wild birds throughout Asia.
- In addition, the **zoonotic potential of AIVs** constitutes an ever-present threat to public health.
- Co-circulation of these different virus subtypes and strains provides the opportunity for further genetic reassortment between viruses, generating new, potentially **highly virulent strains with serious impacts on animal and human health** as well as on national economies in Asia and beyond.
- Given this complex situation, the Food and Agriculture Organization of the United Nations (FAO) suggests **risk-based, comprehensive surveillance approaches for AIV monitoring and early warning**.
- This guidance was developed by FAO in the context of ongoing active surveillance in Asia and can be **adapted by countries' veterinary services to update their AIV surveillance procedures**.

## BACKGROUND

Since the first detection of the Gs/GD highly pathogenic avian influenza (HPAI) H5N1 virus in 1996 in Southern China, many changes have occurred among avian influenza viruses (AIVs) in Asia in terms of subtypes, clades and distribution (Verhagen *et al.*, 2021). H5N1 became endemic in multiple regions in China and reassorted with other co-circulating viruses, either low or highly pathogenic, generating new subtypes in both domestic

and wild birds (e.g. H5N6, H5N8). H5N1 HPAI viruses and their descendants are still endemic in several countries in Asia, including Bangladesh, Cambodia, Indonesia, India, Nepal and Viet Nam (FAO, 2021a). H5N8 viruses of clade 2.3.4.4, originally detected in China in 2010, have been spreading widely since then, not only within Asia (e.g. Republic of Korea, Japan), but have also reached other continents (e.g. Africa, Europe, North America) through wild bird migrations (FAO, 2021a). Similar

## ACRONYMS

AIV	avian influenza virus
ES	environmental sampling
FAO	Food and Agriculture Organization of the United Nations
HA	hemagglutinin (gene)
HPAI	highly pathogenic avian influenza
LBM	live bird market
LPAI	low pathogenic avian influenza
NA	neuraminidase (gene)
RT-PCR	reverse-transcription polymerase chain reaction
USAID	United States Agency for International Development

events, although to a lesser extent, have been observed with the H5N6 HPAI virus that emerged in 2013 in China and since then has continuously circulated in the country, while also reaching new territories over the years (e.g. Europe, Middle East). In mid-2016, the emergence and spread of a new H5 HPAI subclade 2.3.4.4b originating in Central Asia led to significant losses for domestic poultry and wild bird populations across Eurasia and Africa (Khomenko *et al.*, 2018). Since then, periodic intercontinental waves of 2.3.4.4b viruses, mostly spread by wild bird migrations, have been occurring. During 2020–2021, new reassortants of the H5 HPAI clade 2.3.4.4b emerged, leading to virus diversification with virus subtypes including H5N1, H5N2, H5N3, H5N4, H5N5 and H5N6 (Verhagen *et al.*, 2021). Importantly, in February 2021, asymptomatic human infections of influenza A(H5N8) were reported in the Russian Federation, highlighting its zoonotic potential (WHO, 2021). While highly pathogenic strains of influenza virus are often in the spotlight, low pathogenic avian influenza (LPAI) viruses are also causes of concern. In 2013, LPAI H7N9 virus emerged in China, causing a significant number of human deaths and eventually evolving into an HPAI strain in early 2017 (Qi *et al.*, 2018). With the efficient launch of a nationwide vaccination programme in September 2017, China managed to control the disease, and both poultry and human cases dropped drastically shortly thereafter. However, H7N9 positive cases are still sporadically detected in poultry, indicating a persistence of the virus in the country, albeit at a low level. Lastly, other LPAI viruses can significantly affect people's livelihoods, e.g. by decreasing egg production in poultry in the absence of any other clinical sign, as seen with H9N2 LPAI virus (Lee *et al.*, 2013).

Endemicity of both LPAI and HPAI in domestic poultry on the one hand, and a pool of AIV in wild birds on the other, provide opportunities for genetic reassortment between different viruses. This generates new and potentially highly virulent strains with serious impacts on animal and human health as well as on national economies (e.g. production losses, vaccination programmes) in Asia and beyond, through trade or wild bird migrations (Sims *et al.*, 2017). To date, human cases caused by influenza A(H5) or A(H9) continue to be reported, particularly in

Asia (WHO, 2022). Given the current avian influenza situation, with increased emergence and co-circulation of new subtypes and continuous pandemic threats, the Food and Agriculture Organization of the United Nations (FAO) suggests risk-based, comprehensive surveillance approaches for avian influenza monitoring and early warning.

## PURPOSE AND SCOPE

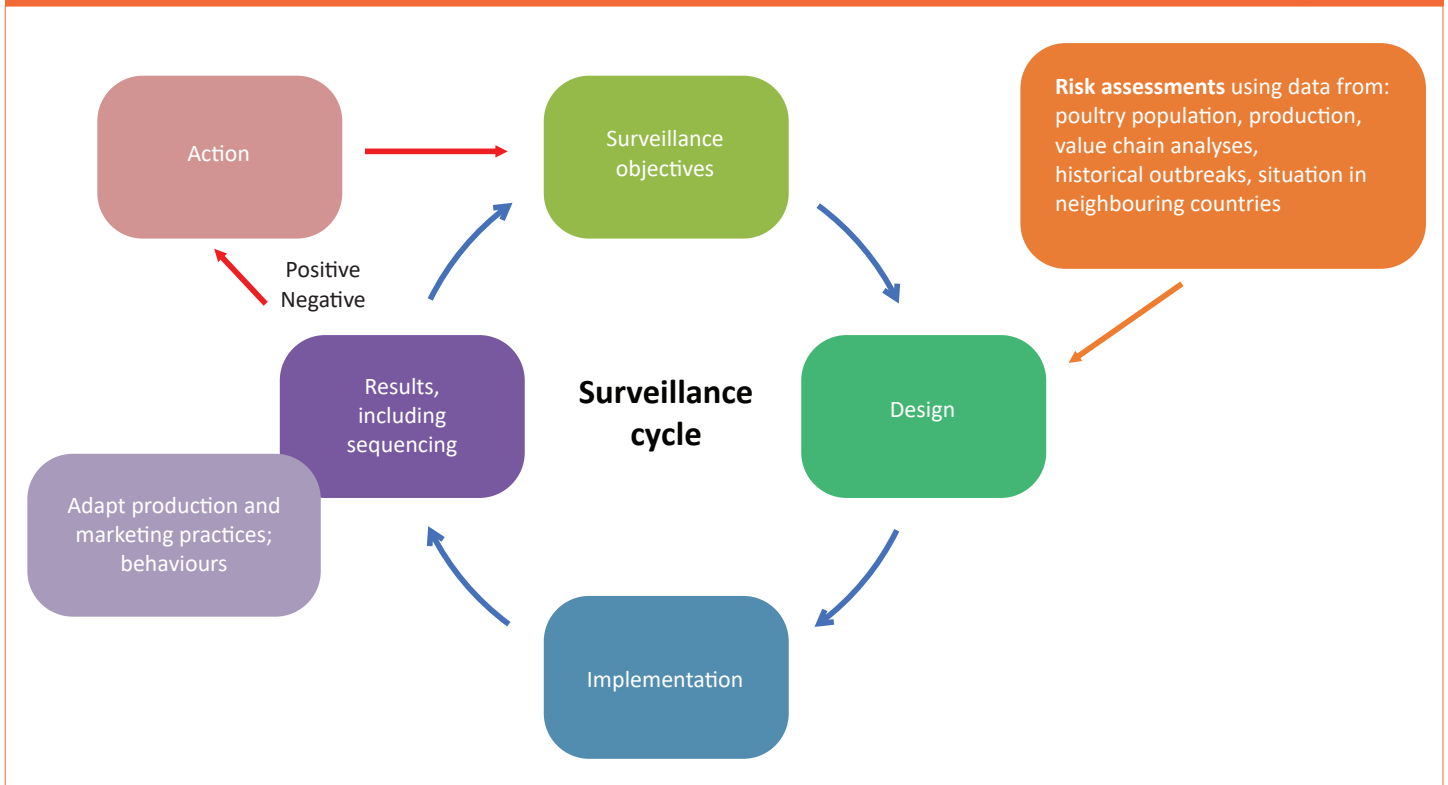
The purpose of this publication is to provide guiding principles for the development or update of **active, comprehensive, risk-based** avian influenza surveillance strategies, mainly targeting domestic poultry. This document is not intended to replace current surveillance methodologies that serve a country's needs. Rather, these guidelines should be used as a resource for surveillance planners and implementers (e.g. National Veterinary Services, FAO country teams, other international organizations, research institutions) to develop their own avian influenza surveillance protocols, adapted to the local context. Specific surveillance approaches are described in the paragraphs that follow.

**Active/passive:** *Active surveillance* relies on organized and planned collection and testing of samples, with a pre-defined sampling strategy, even in the absence of any clinical signs in the targeted population. Another type of surveillance is *passive surveillance*, which is based on the detection of suggestive clinical signs and systematic reporting of suspected cases. Only active surveillance is being addressed in this document.

**Comprehensive:** Comprehensive influenza surveillance aims to broaden the spectrum of viruses detected, rather than focusing on one particular subtype of avian influenza. The present document expands the scope of previous surveillance efforts to include a range of AIV subtypes of interest, including H5N1, H5N2, H5N6, and H5N8 HPAI virus subtypes and H7N9 and H9N2 LPAI virus subtypes, and subtypes that may emerge in the future.

**Risk-based:** Risk-based surveillance is a surveillance strategy that focuses on where the virus is most likely to be present. Instead of random sampling, knowledge of the disease and associated risk factors is used to identify areas, sites, periods of time, and animal species at higher risk of disease occurrence so that resources can be allocated accordingly. Using knowledge of disease risk factors to increase the chance of finding the virus therefore increases the cost-effectiveness of the surveillance programme. However, this strategy does not allow for virus prevalence in a certain area to be determined; instead, random sampling would need to be applied in this case. Nonetheless, when conducting risk-based surveillance it is crucial that the information on risk factors be correct and reliable; otherwise the active surveillance protocol may be biased and surveillance outputs misleading.

FIGURE 1. Surveillance cycle (FAO)



Surveillance planning is an iterative process (Figure 1) that must be framed within specific questions to answer (i.e. objectives). It is of utmost importance to design the surveillance before any implementation takes place. This process must be based on all relevant epidemiological data available, including value chain mapping, risk assessments, history of AIV circulation in the country as well as abroad, and other data. This information will help focus surveillance activities on certain animal species, type of premises and geographical areas, as well as optimize sample collection and diagnostic tests used in order to be more cost-effective. FAO guidance is available to support countries in drafting their own surveillance plans incorporating all operational aspects of surveillance for a specific animal disease or threat.<sup>1</sup>

Once surveillance is ongoing, the results obtained, including observations of production and marketing practices as well as risky behaviours, will inform decisions on where to target risk mitigation measures. In case of positive results, the next steps to take should be considered in the planning phase. Analysis of surveillance results should inform evidence-based prevention and control activities. Surveillance therefore provides support to disease prevention and control programmes. The objectives of surveillance must be periodically revised and the design adapted accordingly in order to correspond with the current situation and priorities of a country or a region.

## OBJECTIVES OF COMPREHENSIVE AVIAN INFLUENZA SURVEILLANCE

The common main objectives of avian influenza surveillance are to:

- 1) Assess where and which AIVs of interest (e.g. H5N1, H5N6, H5N8, H7N9, H9N2) are circulating in poultry value chains, including the frequency of infection in different poultry population subsets (e.g. farming systems, nodes of a value chain, geographical areas), to inform risk management;
- 2) Rapidly detect the incursion of AIV in previously unaffected areas or the emergence of new subtypes or variants in already affected areas, in order to allow for rapid and efficient containment;
- 3) Investigate risks of human exposure.

In addition, comprehensive risk-based surveillance allows for:

- 1) Monitoring the effectiveness of avian influenza control programmes;
- 2) Providing information on circulating strains for avian influenza vaccine-matching countries implementing vaccination;
- 3) Raising awareness (e.g. on biosecurity measures, signs of disease, reporting mechanisms) among different actors along the poultry value chain.

<sup>1</sup> See FAO's *Guidelines for designing animal disease surveillance plans*. (2021) [www.fao.org/3/cb3252en/cb3252en.pdf](http://www.fao.org/3/cb3252en/cb3252en.pdf)

These objectives will be achieved through the following activities:

- a) Identifying high-risk areas and sites, and designing an efficient sampling strategy by using data from value chain analyses and risk assessments;
- b) Collecting samples from different poultry species and production systems, including environmental sampling at live bird markets (LBMs);
- c) Testing collected samples using a standardized and validated reverse-transcription Polymerase chain reaction (RT-PCR) laboratory algorithm for the detection of all virus subtypes of interest;
- d) Characterizing and subtyping any H5, H7 or H9 RT-PCR positive sample;
- e) Performing full genome or partial – at least hemagglutinin (HA) gene – sequencing of new and re-emerging AIVs that are detected, as well as selecting endemic viruses, to track the evolution of the latter over time and provide vaccine-matching for countries where vaccination takes place;
- f) Advising, raising awareness and building trust with communities by providing information on surveillance activities, objectives and eventual consequences on production systems when positive cases are identified, sharing surveillance results, and advising farmers and traders on biosecurity measures, disease signs, reporting mechanisms, and other topics;
- g) Sharing surveillance results with the human health sector at country level, which can lead to joint multisectoral activities (e.g. joint risk assessments, co-design and joint implementation of poultry and human surveillance components), and sharing with institutions and other relevant stakeholders at regional and global levels.



Sampling poultry in a live bird market in Cambodia

## AREA AND SITE SELECTION

Risk-based surveillance is designed according to levels of likelihood of infection by the viruses of interest. Based on existing knowledge of avian influenza epidemiology and value chains, several criteria can be used to identify areas and sites with a higher likelihood of being infected. Surveillance should therefore be preceded by a thorough risk assessment, using up-to-date information on the disease situation and poultry value chains, to identify sites which would both effectively describe the burden of already circulating viruses and optimize the chances to detect incursion or emergence of new strains.

### Area selection

Priority should be given to areas of the country that:

- have a history of avian influenza outbreaks;
- are known to have different AIVs circulating (high-risk areas for emergence of novel viruses);
- have wild bird congregation sites (wetlands) or are on a migratory flyway;
- are bordering affected (or potentially affected) area(s);
- have a high poultry density as compared to other areas;
- have a high number and frequency of poultry movements/trade;
- import live poultry from affected (or potentially affected) area(s);
- import live poultry from unknown origins (e.g. through informal trade);
- operate important live bird markets where significant volumes of poultry congregate;
- have a high demand for poultry meat (high human to chicken population ratio).

### Site selection

Poultry production systems in Asia come in many different sizes and forms, from village-based, local breed, scavenging poultry-rearing to industrialized, highly integrated broiler production systems.

Poultry gathering, trading and sometimes slaughtering in LBMs is a common practice in many countries in Asia. This poses important biosecurity challenges and increases the risk of human infection. LBMs have been shown to play a critical role in AIV spread, and human infection caused by AIVs (Wan *et al.*, 2011; Wang *et al.*, 2020) and therefore should be a priority site when collecting samples for surveillance.

### Live bird markets

LBM selection should be guided by poultry value chain knowledge and previous risk assessment exercises. Based on results from case control studies and known risk factors, sampling efforts should particularly focus on LBMs (both wholesale and retail markets) which:



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Conducting oropharyngeal swabbing in a duck

- are located in affected (or potentially affected) area(s);
- have direct trade links with affected (or potentially affected) area(s);
- are geographically close to affected (or potentially affected) area(s);
- are most highly connected in live bird trade networks;
- have a large throughput of poultry;
- have a large number of traders;
- are sourcing birds from different origins;
- have a high diversity of different poultry and other domestic or wild bird species;
- have repeatedly tested positive for influenza A viruses in the past;
- have on-site slaughtering;
- have low or no biosecurity practices, including poor implementation of disinfection and hygiene measures, unsold birds reoffered for sale the following day, no segregation of bird species, among others.<sup>2</sup>

### **Farms, villages or poultry-gathering sites**

Farms, village backyard farming households, and other poultry-gathering sites can be included in the sampling plan if located in or near an affected (or potentially affected) area, including sites where wild birds congregate, if these sites are known to import poultry from affected (or potentially affected) area(s), or if they play an important role in the domestic or cross-border poultry value chain. However, when conducting systematic surveillance in farms, there is a risk that the sampling team introduces or spreads the virus to non-affected farms, particularly if biosecurity measures are weak. It is recommended that sampling only be conducted on farms that show excessive poultry mortality or where poultry show clinical signs suggestive of HPAI, and that good biosecurity practices be observed while sampling and when moving between farms.

<sup>2</sup> See FAO's Animal Health Manual on Biosecurity for Highly Pathogenic Avian Influenza for details: [www.fao.org/3/i0359e/i0359e.pdf](http://www.fao.org/3/i0359e/i0359e.pdf)

## **Slaughterhouses**

Any slaughterhouse or any site where poultry is slaughtered within a selected area should be considered for inclusion in the sampling plan. Slaughterhouse surveillance allows for easy collection of samples for laboratory testing and enables data to be collected from birds of a variety of origins at reduced cost.

Slaughterhouses are particularly interesting for serological sampling, as they allow testing at the end of the production cycle, thus increasing the chances that the sampled bird has encountered the virus in its lifetime and will test positive. Blood samples should preferably be collected while the animal is bled.

**Note:** When sampling in LBMs, poultry gathering or slaughtering points, it is important to collect information on the geographic origin of the birds (acknowledging that tracing back can be difficult) – see Section 10. The detection of any non-endemic virus should lead to the implementation of containment measures as per approved national action plans – for example: market closures or movement controls; further investigation in the field (backward and forward tracing of infected populations); and the addition of in-contact sites or areas to sample.

Once relevant areas and sites have been selected for the implementation of active surveillance, field sampling teams should regularly collect samples from the same identified areas and sites to ensure consistency in compiling data and to allow for analysis and comparability of the surveillance findings over time. If the epidemiological situation evolves or if the poultry value chains change, adjustments can be made to the active surveillance design; in any case, active surveillance should be conducted using a defined protocol and a long-term approach (i.e. at least several consecutive months for each site).

## **SAMPLE TYPE**

### **Virological samples**

#### **Bird sampling**

##### **Criteria for bird selection**

###### **Species**

Chickens and ducks should be the primary target species at LBMs, farms or other poultry-gathering sites because of their significant role in AIV epidemiology in Asia. However, turkeys, quail, pigeons and farmed waterfowl other than ducks may also carry AIV (Spickler, 2018) and should be included in the sampling scheme where the context suggests it to be relevant (e.g. relative importance in the value chain). Generally, it is recommended that high-risk species within a market be sampled

using a sampling method that is representative of the site. For example, if a market has 60 percent chickens, 20 percent ducks, 10 percent quail and 10 percent geese, then the sampling should roughly follow this proportion. In farms, the production type linked with higher risk of AIV should be sampled, with the possibility to also sample other relevant species present on site such as pigs,<sup>3</sup> as per the agreed surveillance protocols of the country.

#### *Clinical signs*

Clinical signs associated with AIV infections in poultry caused by highly pathogenic viruses include severe respiratory distress with excessively watery eyes and sinusitis, cyanosis of the combs, wattle and shanks, depression, lameness, coughing, sneezing, neurologic signs, diarrhoea, haemorrhages, or bruise-like discoloration on skin (Spickler, 2008). LPAI infections usually present with more subtle clinical signs (e.g. ruffled feathers, reduced egg production, reduced food or water intake). When virus detection is the objective of surveillance, clinical signs may be used as criteria for bird or flock selection, especially for birds sampled at village/farm level. In general, ducks and other waterfowl tend to show few clinical signs, if any (CFSPH, 2016).

When collecting samples from a specific site, **priority should be given to dead or sick-looking birds**, as these clinical signs may reflect ongoing HPAI or comorbidity with LPAI and other pathogens. If the number of dead and sick birds is not sufficient to allow collection of the pre-determined sample size, then healthy birds should also be sampled. For example, in a surveillance programme where the computed sample size is 30 birds per site, if only 5 dead birds and 12 sick birds could be identified during sampling in an LBM, then 13 apparently healthy birds should be sampled in addition to the 5 dead and 12 sick birds in order to reach the target of 30 animals sampled. These figures represent guiding principles, with an estimated observed prevalence based on an apparently healthy population – if sick/dead birds are noted during the sampling round, the sampling number can decrease. It is recommended that countries adjust their sampling scheme based on expected AIV prevalence in healthy or sick animals.

Please note that if the objective of surveillance is to assess prevalence of the disease, a representative sample should be taken instead.

#### **Samples to collect and pooling**

For virological surveillance in birds, oropharyngeal swab samples should be prioritized, as this is the main route for shedding of H7N9 and H5N1 virus subtypes. Cloacal swabs should be included when surveillance is also targeting LPAI.

In order to reduce testing costs, swabs can be pooled together in the laboratory, following these rules:

- Only pool samples from the same species;
- Only pool samples from the same epidemiological unit (e.g. one stall in an LBM, one house/barn in a farm, one batch in a collector yard);
- According to existing scientific literature, pooling of around 5 (but not more than 11) samples is acceptable and confirmed to safeguard testing sensitivity (Spackman *et al.*, 2013);
- Only pool samples of a same type (if other swabs are also collected, e.g. cloacal or environmental).

**Note:** *It is recommended that all animal samples be kept separate and that samples be pooled only at the laboratory so that if a pool tests positive for one or several avian influenza viruses, it will be possible to determine exactly which individual sample(s) actually contained which virus(es). This is especially important for pools that contain more than one virus or subtype. For example, if an H5N6 virus is present in one individual sample of the pool and an H9N2 virus in another one, if testing for the HA and neuraminidase (NA) genes is only done at the pool level it will be difficult to determine which subtypes are present, since several combinations of subtypes would be possible, such as H5N2, H5N6, H9N6 or H9N2.*

*Environmental samples can (and should) be pooled in the field, as described below.*

#### **Environmental sampling**

If relevant to the objectives of the surveillance, environmental samples should be collected at the different sites of interest; for example, LBMs, collector yards, farms and slaughterhouses/ slaughter points. They are obtained by swabbing cages, wastewater drains, faeces, wastebins, processing or display tables, and baskets.

Samples from the same site can be pooled in the field (five samples per pool is recommended). It is recommended that sampling be undertaken equally from the different parts of the market (i.e. delivery area, slaughtering/processing area and sale/customer area). If one objective of the surveillance is to investigate virus loads in different areas of the market or site (and thus human exposure risk), then environmental samples should be pooled by area.

As an indication, a study conducted in Indonesia revealed that the following sites showed the highest chances for detecting the virus: chopping or slaughtering boards; processing tables after de-feathering; baskets holding poultry; wastebins; scales; and tables for poultry display (Indriani *et al.*, 2010).

Advantages and limitations of environmental sampling are presented in Annex 1.

<sup>3</sup> Dogs and cats have also shown susceptibility to AIV and may be sampled, although their role in avian influenza epidemiology is unknown.



Chicken slaughtering area in a live bird market

**Note:** The way markets are managed can be important when deciding which samples to prioritize. For example, while drinking water samples appear to be more sensitive than live bird samples, they are not available in markets where birds are not kept long enough to be supplied with drinking water.

### Serological samples

Serological surveillance involves poultry serum sample collection and testing for avian influenza antibodies. Unlike virus detection, which suggests an ongoing infection, detecting specific avian influenza antibodies only shows that the animal has, on at least one occasion during its lifetime, been exposed to the virus (either by natural infection or through vaccination). Since serological assays commonly used are either pan-AIV- or HA-specific, they usually provide limited information on the exact subtypes implicated. Because serology relies on detecting antibodies that are built up over time following infection, it is not a very efficient tool for early detection of cases; it does, however, provide the opportunity to screen a population and identify which viruses may have been circulating in this population (unless vaccination was or is being used). Such information can help adapt the surveillance scheme or control programmes to specific contexts.

**Slaughterhouses/slaughter points:** Serological screening in slaughterhouses/slaughter points provides an opportunity to collect information from many different populations and origins at a low cost and is highly recommended when and where possible/appropriate.

**Farms:** Serology at farms is a lesser priority, especially in countries where farms have low biosecurity measures in place, as the sampling team could involuntarily introduce the virus into the site. However, farms importing birds or day-old chicks directly or indirectly from infected or high-risk countries can be included in serological screening.

**LBM:** Because of the high turnover of poultry and the lack of efficient traceability in most markets, serological screening in LBMs is not suggested in the context of our surveillance objectives.

It is **NOT** recommended that serum samples be pooled.

**Note:** In countries where avian influenza is endemic, the use of serology may be limited. For non-endemic countries, positive serology results may serve as a trigger for additional screening that aims to detect any current AIV circulation in animal populations. Serological testing may also help detect the use of vaccines during post-vaccination monitoring or in scenarios where vaccination against avian influenza is prohibited but believed to be occurring nevertheless.

## SAMPLE SIZE

### Sample size for virological swabs

**Note:** This section addresses sample size only when the objective is to detect the virus. It is not applicable to sampling designs aimed at determining virus prevalence or proving freedom of disease, both of which require a substantially larger sample size.

When designing a surveillance scheme, it is essential to consider its cost and feasibility. The sensitivity of a surveillance system increases as the number of sampled animals increases, but so do costs, including human/logistical resources needed to implement surveillance activities. A good surveillance system must be cost-effective and practical, two attributes that should always be considered when designing the sampling strategy.

The sample size depends mainly on the expected prevalence in the sampled flock, the desired flock sensitivity (i.e. the confidence level targeted to ensure that infection has not been missed; 95 percent confidence is generally used), and the sampling design. The lower the expected prevalence, the higher the number of samples required and therefore the higher the cost.

Increasing flock sensitivity also implies an increased sample size. Based on the surveillance objectives, a balance needs to

**TABLE 1** Flock sensitivity according to true virus prevalence and sample size

True prevalence in the sampled site		10%	5%	2%
Flock sensitivity	60 samples (12 pools)	>99%	95%	69%
(chances to detect at least one positive)	50 samples (10 pools)	>99%	91%	62%
	30 samples (6 pools)	95%	77%	44%

Test sensitivity: 98%; test specificity: 100%; pool size: 5; total population unknown.  
Source: Ausvet Epitools® - Sergeant, 2018.

be found between the expected prevalence, the desired flock sensitivity, and the resources and capacities available to collect and test the samples.

Furthermore, considering that environmental samples would be collected in addition to poultry samples at the same locations, the chances of detecting the virus would increase, as information sources are increased for each site.

For 100% test specificity the formula applied in Table 1 is:

#### Flock Sensitivity

$$= 1 - (1 - [\text{Test sensitivity}] \times (1 - (1 - [\text{True prevalence}])^5))^{number\ of\ pools}$$

Different online tools exist to assist in fine-tuning your sampling strategy such as Ausvet Epitools,<sup>4</sup> or the United States Department of Agriculture's Animal and Plant Health Inspection Service Animal Sample Size Calculator.<sup>4</sup> A table showing the population sensitivity based on the animal population and sampling size is provided in Annex 2.

<sup>4</sup> [www.aphis.usda.gov/aphis/ourfocus/animalhealth/ceah-toolbox/animal-sample-size-calculator](http://www.aphis.usda.gov/aphis/ourfocus/animalhealth/ceah-toolbox/animal-sample-size-calculator)



Sampling ducks at a farm

The concept of flock sensitivity is based on the assumption that individuals in a flock are mixing homogeneously and that the 30 samples are taken in a randomized method. However, this may not always be the case. This is why it is suggested that 30 samples be collected from each of the chicken and duck populations (“flocks”) present at a site if the animals are kept separately.

**Note:** When sampling LBMs with a large number of mixed animals,<sup>5</sup> for a good cost-effectiveness ratio it is recommended that a minimum of 30 oropharyngeal samples [6 pools of 5 samples] representative of bird species relevant for avian influenza epidemiology and their ratio on site be collected, as well as a minimum of 15 environmental samples [3 pools of 5, one per area]. If bird species are strictly kept separately, 30 oropharyngeal samples [6 pools of 5 samples] may be collected for each of the chicken and duck populations along with the 3 pools of environmental samples. Overall, the sampling size depends on the size of the selected LBM, laboratory storage and testing capacities, and financial resources in the country. If other poultry species (e.g. turkeys, quail, pigeons, farmed waterfowl) present on site are considered important for the national or regional epidemiological context, they should also be sampled. As mentioned previously, samples should be collected in priority from live birds that are sick or recently dead birds to enhance virus detection probabilities.

While 30 samples are considered the minimum sample size for larger populations in the context of these guidelines, if the number of birds on a given site is smaller than 30, all birds present should be sampled. It is not recommended that other sites be included in the area (e.g. neighbouring farms) to reach 30 samples, as the additional sites are not representative of the initial site investigated and would bias the interpretation of the results. For bird populations between 30 and 300 animals, 30 samples (6 pools of 5) can be considered adequate to reach the 95 percent confidence level (see Annex 2).

#### Sample size for serological samples

Sample size for serological screening follows the same statistical rules as for virological screening. The sample size depends on the sero prevalence expected, the desired flock sensitivity, and the sampling design. Flock sensitivity is slightly reduced because the sensitivity of serological tests is lower than that of RT-PCR tests. (However, it can be expected that in many cases the sero-prevalence will be significantly higher than the viro-prevalence, particularly for LPAI viruses or HPAI viruses in ducks.)

<sup>5</sup> By large number of animals, the guidelines refer to more than 300 hundred birds present on site, considering 95 percent confidence levels and 98 percent test sensitivity, and an expected prevalence of 10 percent.



**TABLE 2** *Flock sensitivity of the serological screening according to sero prevalence and sample size*

True sero prevalence in the sampled population		10%	5%	2%
Flock sensitivity	60 samples	>99%	94%	68%
(chances to detect at least one positive)	50 samples	>99%	91%	61%
	30 samples	94%	76%	43%

Test sensitivity: 95%, total population unknown.

Source: Epitools\*.

For serological screening of large poultry populations, it is recommended that at least 50 samples be collected per site (Table 2) (note: serological testing is less costly than virological testing).

**Note:** When interpreting serological results, it is important to consider the possibility of a flock having been vaccinated, either legally or illegally, and thus resulting in positive serology.

## SAMPLING FREQUENCY FOR VIROLOGICAL AND SEROLOGICAL SURVEILLANCE

Active surveillance usually compares to a longitudinal study, consisting of a series of sampling rounds. The frequency of these rounds depends on several factors, mainly:

**Risk level:** When the risk of virus introduction and/or virus spread is high, the sampling frequency should be increased accordingly. The level of risk can also evolve depending on several factors (e.g. season, national holidays, festivities).

**Financial resources available for surveillance:** Sample collection and testing are costly, and the sampling frequency will directly affect the cost of the surveillance programme.

**Human resources:** Both field capacities and laboratory testing capacities need to be assessed to make sure that the additional labour created by the surveillance does not overwhelm current capacities. In addition, sampling frequency (and total sample size for each round) needs to be agreed upon with the laboratory involved, to ensure that its logistical capacities (including storage at the appropriate temperatures) and human resources can manage the reception and testing of all samples in a reasonable time frame (ideally in less than 72 hours after sampling).

As an example, in the case of avian influenza surveillance in a country with a high risk of virus presence, it is recommended that there be a higher sampling frequency (e.g. every two weeks) during the high risk seasons (November to February and July to

August), compared to the low risk-seasons (March to June and September to October) (e.g. once a month).

To reduce resource needs and increase cost-effectiveness, rounds with live-bird plus environmental sampling can be alternated with rounds in which only environmental samples are taken. For example, LBM environmental surveillance may be conducted all year round (i.e. “BASELINE”), while additional samples from live birds can be taken during rounds in high-risk seasons and in the same and additional high-risk provinces (i.e. “ENHANCED”).

## DATA COLLECTION

When conducting sampling in the field, accurate data should be collected about the selected site, the poultry populations tested, and the samples taken. Items highlighted in Table 3 below are **minimum requirements**.

Samples collected in the field must be clearly labelled on the tube/vial with a unique number that can easily be linked to the following minimum information:

- species
- type of sample
- if pooled sample, number of individual samples inside the pool
- sampling date
- location.

**TABLE 3** *Data requirements for conducting sampling in the field*

Site information	Type of site (e.g. LBM, slaughterhouse, collection point, production farm, parent stock/breeding farm, hatchery) Name and location of the site (city, county/district, province) GPS coordinates Name, address, telephone number and email of the site manager Date of the sampling Total number of samples collected Name and contact (phone) of the sampler Production type and avian influenza vaccination status (for farms)
Epidemiology information	Total poultry population at the site Poultry species present at the site (type and number) Are species mixed or segregated? List sources of poultry, together with movement frequency List destinations of poultry, together with movement frequency List biosecurity measures in place Is poultry slaughtered on site?
Sample information	Poultry samples: species, breed, type and age of the animal Environmental samples: type of sample (e.g. cages, baskets, chopping boards, processing table, drinking water) Traceability, number of animals or farm/location of origin (if available)
Case information	Case history, if known (e.g. clinical signs) Overall morbidity and mortality

It is essential to store all the data collected during sampling in an organized database (e.g. at minimum a spreadsheet software such as Microsoft Excel) to ensure that all information relative to sampled sites, animals or the environment can be consulted and used later on.

## TRANSPORT OF SAMPLES

Environmental samples can be pooled in the field (around 5, maximum 11 samples per pool; only pool together samples of a same type, same species, same site, and same epidemiological unit), while oropharyngeal swabs should be kept in individual tubes (to be pooled only at the laboratory). All samples must be properly packaged, labelled, and stored at 4°C for transport.

Oropharyngeal, cloacal and environmental swabs should be transported in a virus transport medium.<sup>6</sup> If no transport medium is available, options include using sterile phosphate buffered saline or thoroughly moistening the swab with liquid sample material (do not use ethanol). Samples must be kept between 2°C and 8°C and protected against ultraviolet light (avoid exposure to direct sunlight) until arrival at the laboratory.

Timely delivery to the laboratory is critical for an effective surveillance programme: Samples should be tested within 72 hours after sampling and any positive findings followed up using a previously agreed action plan. The laboratory should be notified well in advance of planned sampling dates and numbers of samples to be received.

## LABORATORY TESTING

### Virology

This section aims to provide a cost-effective algorithm for the detection and characterization of the main avian influenza subtypes of interest in Asia, namely H5N1, H5N2, H5N5, H5N6, H5N8, H7N9 and H9N2.

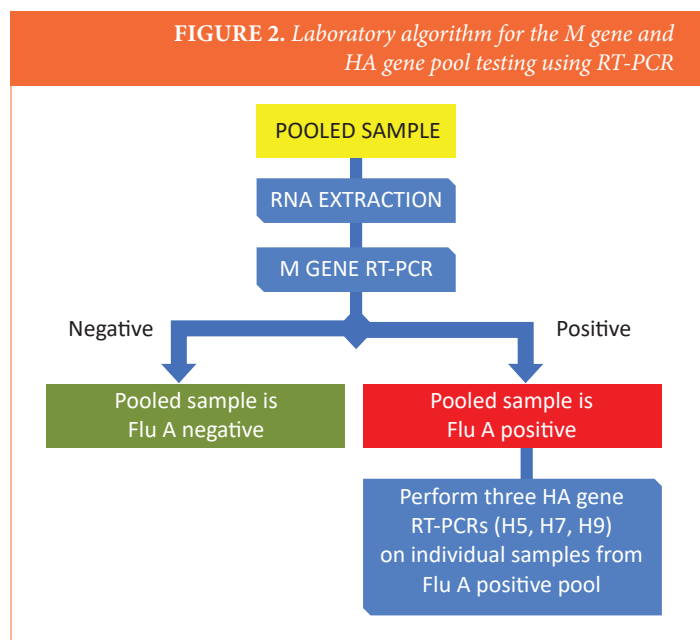
**Note:** Only samples from the same sampling site (i.e. same poultry farm or LBM), same sample type, same species and same epidemiology unit (i.e. same poultry flock or same market stalls) should be pooled.

### Matrix gene and HA gene pool testing

Matrix gene (or M gene) testing will detect all influenza A positive samples, without giving any information on the subtype. This first step aims at reducing the number of samples that will need to be tested further. Samples or pooled samples that test positive for M gene are then submitted to three additional

<sup>6</sup> Follow relevant regulations when transporting specimens: e.g. national regulations on the transport of biological substance when domestic transport is involved; International Air Transport Association regulations when air transport is involved ([www.iata.org/](http://www.iata.org/)).

FIGURE 2. Laboratory algorithm for the M gene and HA gene pool testing using RT-PCR

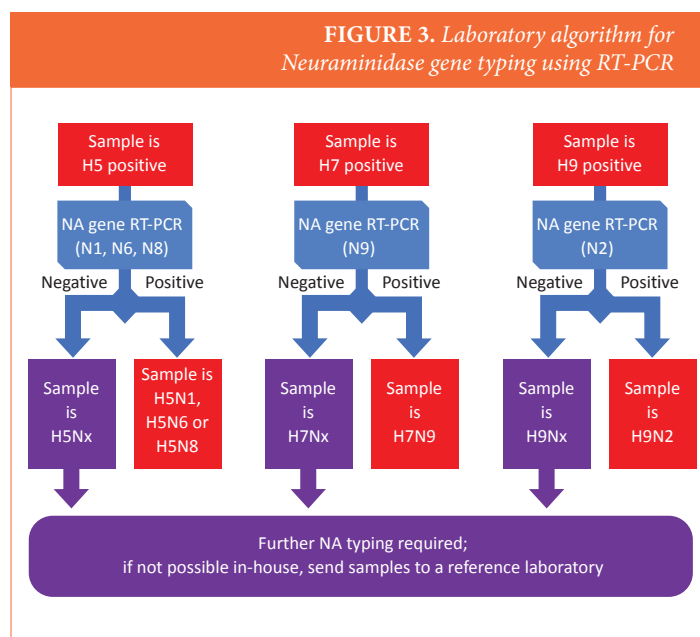


RT-PCR tests in order to determine the HA type (Figure 2). Ideally RT-PCR for HA gene detection should be conducted on individual samples and not on the whole positive pool as it can make identifying specific subtypes challenging. Therefore, it is important to determine the M gene positive samples from a given pool. When detected, H5 or H7 positive samples should undergo pathotyping (i.e. determining if they are low or highly pathogenic AIVs). If the laboratory cannot perform the pathotyping, positive samples can be shipped to any reference laboratory able to do so.

### Neuraminidase RT-PCR analysis of H5, H7 and H9 positive samples

Depending on the resources available, samples that tested positive for one of the three HA genes of interest (H5, H7 and H9) can be further characterized for NA gene identification (Figure 3). NA

FIGURE 3. Laboratory algorithm for Neuraminidase gene typing using RT-PCR





Live poultry for sale at a bird market

types to be tested depend on the type of HA identified. Ideally, NA typing is performed on individual samples instead of pools in order to correctly identify subtypes involved, especially in situations where multiple subtypes are co-circulating. NA typing on pools may be considered; however, it may give inconclusive results on the subtypes circulating if a pool of samples contains more than one subtype.

For samples that test positive for M gene but negative for all three HA genes of interest (H5, H7 and H9), it is recommended that a representative selection of these samples (different species, different areas, different types of sites) be sent for full genome sequencing in order to characterize the other avian influenza subtypes that are potentially circulating.

In case a sample tests positive for more than one type of HA gene, it should be tested for all NA gene types relevant to the positive HA genes (e.g. if the sample tests positive for both H7 and H9, it should be tested for both N9 and N2).

### Full genome sequencing

If a non-endemic virus is detected, virus isolation and full genome sequencing should be performed. In case this cannot be conducted locally, a sample submission to international reference laboratories can be facilitated by FAO (EMPRES-shipping-service@fao.org). In the case of endemic viruses, virus isolation and full genome sequencing are also recommended for a strategic selection of positive samples (e.g. vaccine matching, monitoring of circulating strains), especially if there is evidence of viral genetic changes in the local, regional or global avian influenza epidemiological context.

### Serology

It is recommended that Hemagglutinin Inhibition testing using the main HA subtypes be utilized to determine the antibodies against HA subtype(s) present in the serum.

Recommended antigens to use include updated antigens of subtypes currently circulating in the country or region, or antigens of subtypes with pandemic potential (mainly H5, H7 and H9). The use of other antigens from older H5N1 clades that do not circulate anymore in the region, or from other H7Nx virus subtypes, may result in false negative results. It is crucial to always include positive and negative controls during testing (using homologous antigens and antibodies) to allow for better interpretation of the results or to troubleshoot laboratory testing issues, if needed.

Assays to determine NA subtypes in serum samples are difficult to perform. For serum samples that tested positive for at least one type of HA representing a particular interest (such as an H7 seropositive sample in an area supposed to be free of H7Nx viruses), and in case the local laboratory does not have the capacity, sample referral for further characterization of the NA type is highly recommended.

**Note:** *In case a low seroprevalence for avian influenza is expected (in countries or areas with no or low-level avian influenza circulation), it is recommended that an initial influenza A screening be conducted using a multiscreen ELISA, and that Hemagglutinin Inhibition tests only be conducted on samples that tested positive, thus reducing costs.*

## SAMPLE STORAGE AT THE LABORATORY LEVEL

### Before testing

Samples should be tested immediately upon reception or stored at 4°C until tested. If testing is not possible within 72 hours after sampling, virological samples (environmental and oropharyngeal swabs) must be frozen and stored at -70°C to retain viral viability. Serum samples can be stored at -20°C.

### After testing

All samples (positive and negative) must be kept and stored for at least 12 months, ideally until the end of the project. Virological samples must be frozen at -70°C and serum samples can be stored at -20°C. All stored samples must be clearly labelled on the tube/vial with a unique number that can be traced to the following minimum information:

- identification number
- species
- type of sample
- sampling date
- location.

## COMMUNICATION WITH SURVEILLANCE STAKEHOLDERS

Surveillance is most effective when stakeholders have a sense of ownership and involvement. This collaborative spirit can be fostered through best practices in public relations and risk communication before, during and after surveillance activities.

For example, building trust with communities well in advance can increase the likelihood of positive reception and collaboration when activities actually begin. Announcing sampling visits (when technically advisable to do so) can foster stakeholder buy-in. Addressing stakeholder concerns such as risks posed by avian influenza and consequences of outbreaks (i.e. poultry loss, human infection, economic impact) and explaining the value of surveillance as part of prevention and early control can increase collaboration and access. Above all, surveillance planners and implementers should maintain a continuous dialogue with the different stakeholders, listening to their concerns and modifying activities, where appropriate, to address them.

Furthermore, field surveillance provides a good opportunity to raise awareness about the risks posed by avian influenza to stakeholder livelihoods, and the disease/risk mitigation measures people can take to reduce these risks.

Surveillance results should be summarized on a regular basis and outcomes of the analysis shared and discussed with the stakeholders at all levels. When compiling and summarizing results, interpretation in the national and local (e.g. LBM or farm management) context is crucial. The results of the analysis and their presentation must be useful for decision-makers.

Any actions that would be implemented following the detection of positives should be discussed and agreed with concerned stakeholders in advance.



Wild bird area in Cambodia

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## ACKNOWLEDGEMENTS

This guideline is an output of the project “Support for early warning and control of avian influenza A viruses with pandemic potential in Asia” (OSRO/GLO/302/USA) under the Emerging Pandemic Threat Program (EPT-2) funded by the United States Agency for International Development (USAID).

Production of this guideline was made possible by the generous support of the American people through USAID. The contents are the responsibility of FAO and do not necessarily reflect the views of USAID or the government of the United States of America.

## Annex 1

### ADVANTAGES AND LIMITATIONS OF ENVIRONMENTAL SAMPLING FOR AVIAN INFLUENZA SURVEILLANCE<sup>7</sup>

FACTORS	DESCRIPTIONS OF ADVANTAGES
Cost-effectiveness	Cost-effective; simple and rapid procedure, minimal training and equipment is required; thus, sample size can be expanded at low cost.  Techniques for demonstrating viable virus through embryonated egg inoculation in certain environmental sampling (ES) (e.g. fresh faecal samples) are well-established; many studies have successfully isolated virus from a range of ES.
Flexibility and simple operation	Flexible; adaptable to wide range of sampling size, timing, frequency and location, and applicable in a number of contexts including value and supply chains.  Previous examples highlight the effectiveness of ES use either on its own in wild bird surveillance, or as a component of large-scale risk-based surveillance systems in both low- and high-resourced settings, overcoming logistical challenges that are often encountered in broad-scale surveillance.
Acceptability to relevant personnel in LBM	Acceptable to traders and stall vendors in LBM.
Bird welfare and minimized impacts on wild bird	Bird welfare; trapping, capturing or handling of birds are not required for ES sampling. As a non-invasive technique, it minimizes the effects on animal welfare and trades in markets, and reduces the impacts on wild bird communities.
Risk of aerosolization	Safer; ES reduces the likelihood of virus aerosolization.
Understanding to biology and distribution	ES can be representative of wider geographical areas compared to location-specific sampling sites for live birds.
Successful virus isolation and phylogenetic analysis	Studies have demonstrated successful virus isolation and phylogenetic analysis with ES, as well as a good correlation with subtypes detected in the environment.

FACTORS	DESCRIPTIONS OF LIMITATIONS	SOLUTIONS
Loss of species-specific information	ES may result in the loss of species-specific information on virus-shedding source, limiting its utility in taxonomic-level analyses.	Utilize mitochondrial DNA barcoding
Lack of information on the source of the virus	Due to the segmented genome of AIV, ES may be unable to provide information on the specific combination of gene segments but provide only information on single gene sequence at a time, making reconstruction of parental gene constellations a challenge in mixed samples. Subtype or strain characterization in the event of several co-circulating subtypes or strains can be challenging as well.	Utilize fresh faecal sample  Collect samples from single species flocks  Observe faecal morphology prior to sample collection
No collection of relevant biological metadata	As some ES collected cannot represent birds at the individual level, it provides limited biological metadata which is valuable for advanced epidemiological or risk factor analysis, especially when applying to wild birds.	
Limited timeline information	ES provides limited information on the time of virus deposition; the detection does not necessarily represent the viruses presented in birds at a particular time, due to accumulation and persistence within the environment, especially for mud and sewage samples.	
Suitability for certain surveillance objectives	Surveillance objectives for which ES may not be suitable include those that require individual or host population information, the characterization of co-circulating subtype combination, trace-back of infected flocks, estimation of prevalence, and incidence or any absolute measures of AIV infection within a specific population.	

<sup>7</sup> Created based on Hood, *et al.*, 2021

## Annex 2

### POPULATION SENSITIVITY FOR VARYING SAMPLE AND POPULATION SIZE

	n = 5	n = 10	n = 15	n = 20	n = 30	n = 50	n = 75	n = 100	n = 200	n = 300	n = 500	n = 1000
N = 50	0.4029	0.664	0.8246	0.9169	0.9881	1	NA	NA	NA	NA	NA	NA
N = 100	0.3949	0.6435	0.7961	0.8871	0.9692	0.9988	1	1	NA	NA	NA	NA
N = 200	0.3911	0.6339	0.7828	0.8729	0.9584	0.9964	0.9999	1	1	NA	NA	NA
N = 300	0.3898	0.6308	0.7785	0.8683	0.9547	0.9953	0.9998	1	1	1	NA	NA
N = 500	0.3889	0.6283	0.7751	0.8646	0.9517	0.9942	0.9996	1	1	1	1	NA
N = 1000	0.3881	0.6265	0.7726	0.8619	0.9494	0.9934	0.9995	1	1	1	1	1
N = 5000	0.3875	0.625	0.7706	0.8597	0.9476	0.9927	0.9994	0.9999	1	1	1	1
N = 10000	0.3874	0.6249	0.7703	0.8594	0.9474	0.9926	0.9994	0.9999	1	1	1	1
N = 100000	0.3874	0.6247	0.7701	0.8592	0.9472	0.9926	0.9994	0.9999	1	1	1	1
N = 1000000	0.3874	0.6247	0.7701	0.8591	0.9471	0.9926	0.9994	0.9999	1	1	1	1
N = inf	0.4029	0.6435	0.7871	0.8729	0.9547	0.9942	0.9996	1	1	1	1	1

Test sensitivity = 98 %; test specificity = 100%; true prevalence = 10 %; n: sampling size; N: population size; N = inf: considered large population. For a sampling size of 30 (n=30), the population sensitivity would be very similar to a population of 300 (N=300) onwards, with an approximate population sensitivity of 95% (confidence level).

Source: Sergeant, E.S.G., 2018. EpiTools Epidemiological Calculators. Ausvet. <http://epitools.ausvet.com.au>

#### NOTES



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## RISK ANALYSIS IN ANIMAL HEALTH

Risk analysis is a procedure, which we all do intuitively in our everyday life as we also do in our professional work to assess the risk of any hazard or threat. In animal health, risk analysis has been most widely used as a decision tool to help select the most appropriate health interventions to support disease control strategies, guide disease surveillance and support disease control or eradication strategies.

It should be remembered that risk is not equal to zero and never stays static. Risk changes as drivers or factors of disease emergence, spread or persistence change, such as intensification of livestock production, climate change, civil unrest and changes in international trading patterns. Risk analysis should therefore not be seen as a “one off” but as good practice for animal health systems as part of their regular activities. Therefore, the risk analysis process should be repeated and updated regularly.

Risk analysis comprises the following components:



**Hazard identification:** the main threats are identified and described.



**Risk assessment:** risks of an event occurring and developing in particular ways are first identified and described. The likelihood of those risks occurring is then estimated. The potential consequences or impact of the risks if they occur are also evaluated and are used to complete the assessment of the risk.



**Risk management:** involves identifying and implementing measures to reduce identified risks and their consequences. Risk can never be completely eliminated but can be effectively mitigated. The aim is to adopt procedures that will reduce the level of risk to what is deemed to be an acceptable level.



**Risk communication:** an integrated process that involves and informs all stakeholders within the risk analysis process and allows for interactive exchange of information and opinions concerning risk. It assists in the development of transparent and credible decision-making processes and can instil confidence in risk management decisions.

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### Required citation

FAO. 2022. *Guiding principles for the design of avian influenza active surveillance in Asia – Designing active, comprehensive, risk-based avian influenza surveillance*. FAO Animal Health Risk Analysis – Management, No. 4. Rome.

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