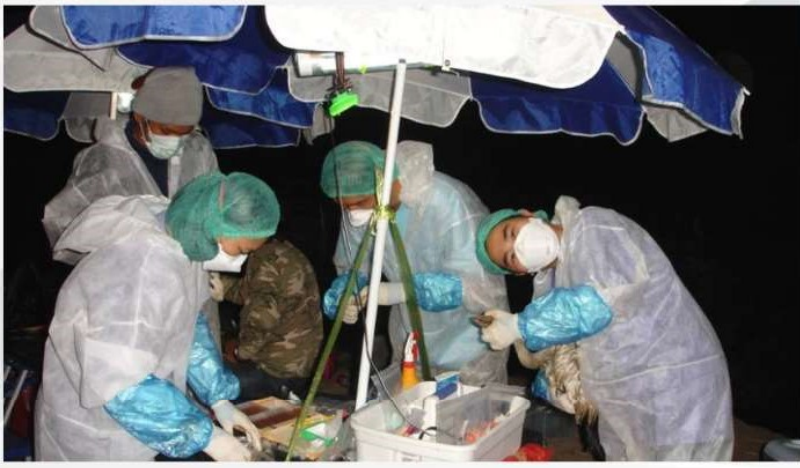
TRAINING CURRICULUM ON SAMPLE COLLECTION FOR AVIAN INFLUENZA AND ENVIRONMENTAL SURVEILLANCE



Executive Summary

The development of a comprehensive curriculum for wildlife sample collection aims to enhance wildlife health surveillance, focusing on both live wild birds and carcasses, as well as remains of small and large mammals, including marine mammals. This training program is designed to equip participants with practical knowledge and skills, beginning with wildlife species identification and census techniques, which are essential for supporting national and regional wildlife health monitoring initiatives.

The curriculum places significant emphasis on safe and effective capture methods for live wild birds to facilitate sample collection for Avian Influenza (AI) surveillance. It also outlines procedures for sample collection, handling, transportation, and submission to laboratories. Additionally, participants will receive training in environmental sampling techniques, incorporating standard methods for detecting AI and utilizing environmental DNA (eDNA) for other pathogens, all while adhering to internationally recognized best practices based on biosafety guidelines.

This initiative has been developed in collaboration with prominent national and international institutions, including the Faculty of Veterinary Science and the Faculty of Tropical Medicine at Mahidol University in Thailand; the Thailand Institute of Molecular Biosciences at Mahidol University; the Centre National de la Recherche Scientifique (CNRS) in France; the Atlantic Veterinary College at the University of Prince Edward Island in Canada; the Canadian Wildlife Health Cooperative; the U.S. Geological Survey; the Singapore National Parks Board (NParks); the World Organisation for Animal Health (WOAH); Wildlife Health Australia; the Australian Centre for Disease Preparedness - CSIRO; and the Armed Forces Research Institute of Medical Sciences (WRAIR-AFRIMS).

The primary target audience consists of wildlife veterinarians, foresters, biologists, rangers, and other personnel involved in wildlife sample collection for AI and other pathogen surveillance. The curriculum is also structured to support a "Train the Trainers" approach, enabling participants to share their knowledge and skills with others in their respective countries.

This training manual will serve as a valuable resource, particularly within the Asia-Pacific region, contributing to the enhancement of regional capacities in wildlife disease surveillance, the early detection of emerging pathogens, and the promotion of safe sampling practices across a diverse range of wildlife species, along with biosafety and biosecurity in field practices and laboratory diagnosis.

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Chapter I: Introduction

The global landscape of infectious diseases is increasingly shaped by zoonotic pathogens that emerge at the human-animal-environment interface. Among these, Avian Influenza (AI) viruses, particularly High Pathogenicity Avian Influenza (HPAI) strains like H5N1, represent a persistent and evolving threat. Recent pan-continental outbreaks have resulted in unprecedented mortality in wild bird populations, devastating poultry industries, and causing sporadic but severe human infections. This underscores a critical reality: AI is not merely an agricultural issue but a significant concern for global health, food security, and biodiversity conservation. The ability of these viruses to mutate and reassort creates a constant risk of novel strains with pandemic potential, making robust surveillance an indispensable tool for early warning and rapid response.

To effectively monitor Avian Influenza, we must look to its natural reservoir. For the purposes of this curriculum, wildlife is defined as non-captive animal populations living in their natural or semi-natural habitats, free from direct human control or management. This scope is critical because it distinguishes these animals from those in managed settings like zoos, farms, or domestic environments. Our focus is primarily on species that act as key players in the viral ecosystem. Examples include:

- **Migratory Waterfowl**: Ducks, geese, and swans that travel vast distances along established flyways, capable of carrying and disseminating AI viruses across continents.
- **Shorebirds, Gulls, and Terns**: Species that congregate in large numbers at coastal and wetland sites, creating opportunities for viral transmission and amplification.
- **Resident Wild Birds**: Non-migratory birds that can act as local reservoirs or bridge species, facilitating spillover from wild populations to domestic poultry.

While birds are the primary focus, it is also important to recognize that mammals such as foxes, seals, and mink can be infected, acting as potential mixing vessels for viral evolution and indicators of broader ecosystem contamination. Surveillance in these non-captive populations is our first line of defense in detecting viral circulation and genetic changes.

Effective AI surveillance is not a single activity but a combination of strategies. This curriculum is built around two principal approaches:

- **Mortality Event Investigation (Reactive Surveillance)**: This is often the most visible sign of HPAI circulation. A mass mortality event where an unusual number of sick or dead animals are found in a specific location and time is a critical alarm bell. Responding requires a rapid, systematic, and biosecure approach. A key challenge is determining how to sample effectively. The intuition behind sample size calculation in this context is not to test every carcass, but to collect a statistically representative

set of samples to confirm the causative agent with high confidence. This involves careful field preparation, use of appropriate personal protective equipment (PPE), and standardized collection of high-quality tissue and swab samples that can yield a definitive diagnosis.

- **Targeted Surveillance (Proactive Surveillance)**: This strategy involves planned monitoring of apparently healthy, high-risk wild populations to detect the virus before it causes die-offs or spills over into poultry or humans. This is fundamental for early warning. It requires identifying key geographic locations (e.g., major wetlands, migratory stopover sites) and target species. Here, the intuition behind sample size calculation is different. It is driven by the goal of detecting the virus if it is present at a certain minimum prevalence within the population. For example, "How many birds must we sample to be 95% confident of detecting the virus if at least 5% of the population is infected?" This proactive approach provides invaluable data on which strains are circulating, where they are, and how they are changing over time.

The Asia-Pacific region is a global hotspot for the emergence and spread of Avian Influenza. It is home to major migratory bird flyways (like the East Asian-Australasian Flyway), some of the world's highest densities of poultry production, and countless points of close contact between wildlife, domestic animals, and humans. For these reasons, building robust, in-country capacity for wildlife disease surveillance is not just a regional priority but a global imperative. A chain of effective surveillance is only as strong as its weakest link. Equipping local and national personnel with standardized, best-practice protocols for sample collection, data management, and biosecurity is essential for generating reliable data that can be.

The complexity of Avian Influenza (AI) demands a perspective that transcends single disciplines. The One Health approach, which recognizes the deep interconnection between human, animal, and environmental health, provides this necessary framework. However, to be effective, this concept must be translated into a functional system. As Lajaunie and Morand (2025) argue, the development of ‘One Health Observatories’ is a critical step in this direction. These observatories are not just data repositories; they are structured platforms designed to collect, share, and analyze integrated data from diverse sectors. For a region like Southeast Asia, a recognized hotspot for emerging infectious diseases due to its high biodiversity and intense human-animal interfaces. Such observatories are essential for moving from a reactive to a proactive stance against threats like AI.

Adopting a One Health mindset fundamentally improves the design and execution of surveillance programs. The primary contribution of One Health is breaking down institutional silos. It mandates that wildlife biologists, veterinarians, and public health officials work together. This synergy creates a more complete operational picture. For example, a wildlife agency's data on migratory bird routes becomes critical intelligence for veterinary services to enhance biosecurity along those flyways. Instead of asking siloed questions, a One Health approach asks integrated ones. The question is no longer just "Is the virus present in this bird?" but rather, "What strain is it, how is it evolving, what is its proximity to poultry farms and human settlements, and what environmental factors might

be driving its spread?" This provides a far more nuanced and actionable understanding of the actual risk. By sharing information and coordinating activities, resources can be deployed more strategically. An early detection of HPAI in a wild bird population can trigger targeted, localized surveillance in nearby poultry farms, rather than broad, expensive national testing. This efficiency is critical, especially in resource-limited settings.

Robust AI surveillance in wildlife is a cornerstone for putting the One Health concept into practice. The data generated directly serves all three pillars of the framework. Surveillance in wild birds provides the earliest possible warning of new or evolving AI strains that could pose a threat to humans. This "sentinel" function gives public health authorities invaluable lead time to assess pandemic potential, prepare diagnostic tools, and develop risk communication strategies. Early detection of HPAI circulation in wildlife allows veterinary services to warn farmers, implement enhanced biosecurity measures, and prevent devastating outbreaks in poultry. This protects a vital food source, safeguards national economies, and preserves the livelihoods of millions. HPAI has caused unprecedented mass mortality events in wild bird and even mammal populations. Surveillance is the primary tool we have to quantify this impact, identify species at high risk, and understand the ecological consequences. This data is essential for conservation agencies to develop strategies aimed at protecting vulnerable wildlife and preserving biodiversity.

The One Health framework provides the collaborative strategy for effective surveillance, while the data from that surveillance provides the evidence needed to drive coordinated action across the human, animal, and environmental health sectors.

When we think of AI, we naturally focus on the host: the infected bird. However, the virus does not exist in a vacuum. Infected birds, particularly waterfowl, shed vast quantities of virus into their surroundings through feces and respiratory secretions. This viral material contaminates the environment, which can then act as a "silent reservoir." The AI virus, especially in aquatic environments, can remain viable for extended periods, days or even weeks in cold water. This means that lakes, ponds, wetlands, and the surrounding soil and sediment can become contaminated reservoirs where the virus persists. A healthy bird can then become infected simply by drinking or foraging in this contaminated environment, without ever coming into direct contact with another infected bird. Environmental surveillance, therefore, is the systematic collection and testing of environmental samples (e.g., water, soil, sediment, fresh feces) to detect the presence of the pathogen itself.

Traditionally, AI surveillance has overwhelmingly focused on sampling animals, both wild and domestic. This is intuitive and provides a direct link between the virus and a specific host. In contrast, environmental surveillance has often been a neglected component. The intuition behind this neglect is understandable. It can feel like searching for a needle in a haystack. A positive water sample does not tell you which specific bird shed the virus or when. However, viewing it this way misses the point. A better analogy is a crime scene investigation: while interviewing witnesses (sampling birds) is crucial, examining the scene for forensic evidence (environmental sampling) can reveal that a culprit was present, even if they are no longer in sight. By focusing only on the animals we can catch, we may be missing a huge piece of the transmission puzzle that is silently waiting in the environment.

Integrating environmental surveillance into AI monitoring programs is not just an academic exercise. It provides critical, actionable intelligence that is difficult to obtain through animal sampling alone. In large wetlands or remote areas, capturing a statistically significant number of birds can be logistically challenging and expensive. A few strategically collected water samples can detect the presence of the virus in the area with far less effort, serving as a highly efficient early warning that the virus is circulating locally. Consistent positive results from a specific pond or waterway can identify it as an environmental "hotspot" for viral transmission. This information is invaluable for focusing resources, such as targeted animal surveillance, enhanced biosecurity recommendations for nearby farms, or public health advisories. Moreover, environmental sampling is non-invasive. It does not require the capture, handling, and potential stress of wild animals, which is a significant advantage for animal welfare. It can also be safer and require less specialized training and equipment than capturing live birds. Systematic environmental testing helps answer critical questions about the virus's lifecycle. How long does it survive under different temperature and pH conditions? How does it spread through waterways? This data is fundamental for building more accurate risk models and understanding the true dynamics of an outbreak.

This training curriculum is not a theoretical exercise. It is the distillation of decades of collective field and laboratory experience from a diverse group of international experts, including veterinarians, wildlife biologists, epidemiologists, and public health professionals. It is designed to be intensely practical, translating complex epidemiological concepts into actionable field protocols. By drawing on lessons learned from past outbreaks across different continents, this training provides participants with a comprehensive and pragmatic toolkit to establish or enhance AI surveillance programs within their own countries, ultimately strengthening the global network of defense against this critical threat.

Scope

This curriculum offers a comprehensive guide for collecting wildlife samples, aimed at building capacity for wildlife health surveillance in the Asia-Pacific region. It consists of Standard Operating Procedures (SOPs) and structured learning modules that explain how to effectively learn, apply, and evaluate these procedures. The practical components focus primarily on avian species, covering both carcasses and live animals. Additionally, it includes training on sample collection from small and marine mammal carcasses. The curriculum also addresses topics such as animal census techniques to support wildlife population assessments, appropriate tools and methods for capturing live wild birds based on species and habitat, and the collection of various sample types for virus detection. It also includes guidance on selecting and using appropriate personal protective equipment (PPE) based on risk identification and biosafety levels, ensuring safe field and laboratory practices. Furthermore, it introduces environmental sampling for detecting avian influenza (AI) viruses and the use of environmental DNA (eDNA) techniques to identify other pathogens in the environment. This curriculum can serve as a training-of-trainers (ToT) framework to enhance regional capacity for wildlife health surveillance.

Objectives

The goal of this curriculum is to provide a practical guide for training individuals involved in wildlife sample collection for disease surveillance or authorized research in their respective countries. The target audience includes wildlife veterinarians, foresters, biologists, rangers, and other related professionals responsible for field sample collection. This curriculum aims to equip them with standardized knowledge and skills in wildlife sample collection that can be applied to avian influenza (AI) surveillance and related wildlife research. It is developed from experiences and data gathered in the Asia-Pacific region to ensure it reflects real field practices and the regional context. The manual structure was shown in Figure 1.1.

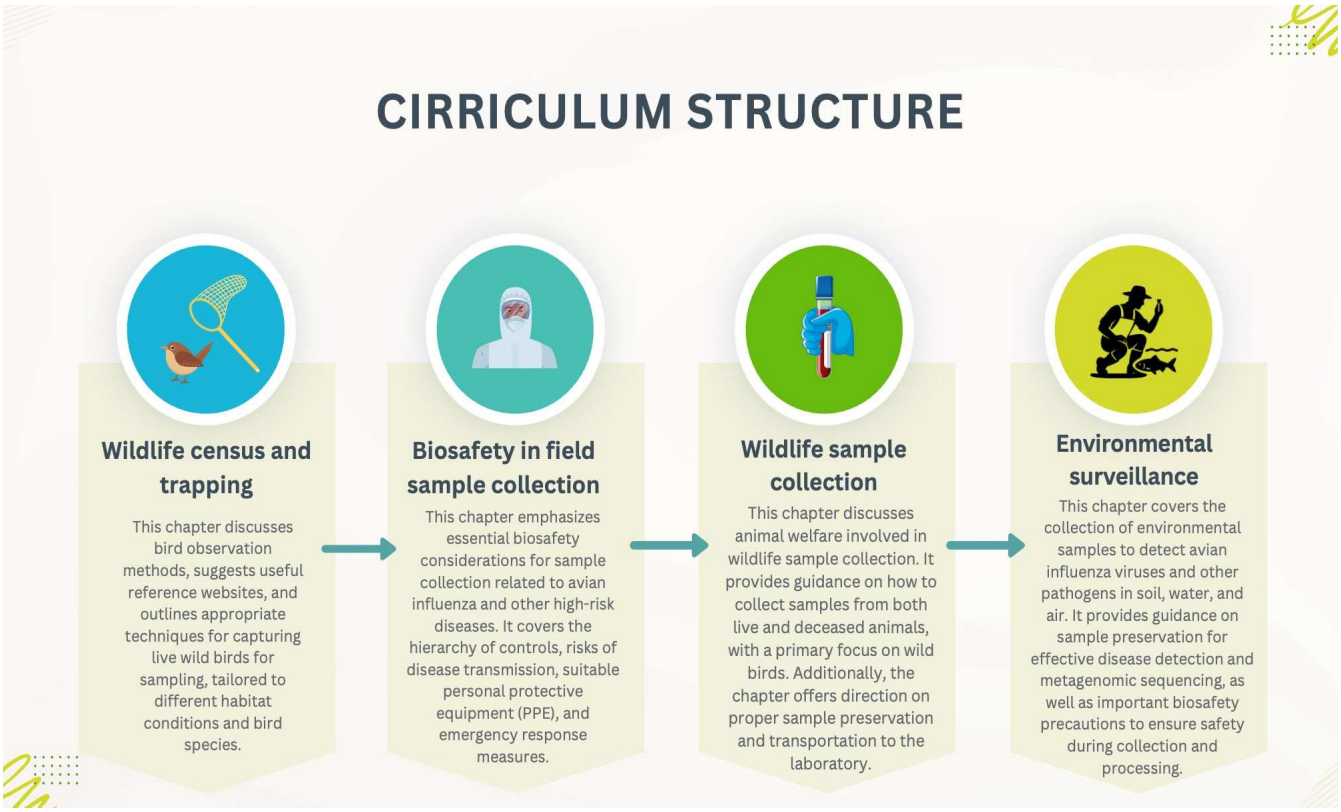

Figure 1.1. Curriculum structure

Chapter II: Wildlife Census and Trapping

Pre-course Materials

- Species identification
- Animal census techniques: overview of bird census concepts for avian influenza monitoring
- Techniques for wild bird trapping

Skills and Knowledge to be Acquired

- Learning outcomes:
  - Identification of wild bird species
  - Animal abundance estimation
  - Trap setup
- Contents:
  - Tools demonstration (Mist nets, Cannon net, and Hand net)
  - Hands-on session: trap installation and monitoring

Evaluation

- Practical: correct trap setup and safe release
- Quiz: species identification, animal census, and trap selection (See at the end of this session)

Content

Species identification

Methods of Bird Species Identification in the Field

Wearing dull-colored clothing helps you blend into the surroundings. This makes it easier to get closer to the birds. Do your homework before setting out. Studying the habits of the birds you hope to see will help you choose the best location and the right time of day when they are most likely to appear. Once you are prepared, you can begin applying various field identification techniques to recognize different bird species more effectively.

1. Visual Observation

Birds can be seen in virtually any part of the natural surroundings, even in urban areas. Direct field observation focusing on diagnostic morphological characteristics, including plumage color, body size and shape, wing and beak patterns, and behavior. This method allows observers to distinguish species based on visible traits that are often unique to particular taxa.

2. Binoculars

Birdwatching does not require complicated tools or expensive gear. With nothing more than your eyes, you can already enjoy the presence of birds around you. However, with just a single pair of binoculars, the experience becomes far more rewarding. Handheld binoculars bring birds closer, allowing you to notice the fine details of their plumage, subtle movements, and behavior, offering an intimate view of the avian world that would otherwise remain unseen to the naked eye.

When selecting binoculars, one should take into account not only the magnification power but also how closely the instrument can focus. The performance of binoculars depends on two main factors: magnification power and the diameter of the objective lens, measured in millimeters, as the lens affects the image produced. Such specifications are typically indicated in the product details. For example, binoculars described as 8x45 offer eight times the magnification of the naked eye and incorporate a 45 mm objective lens, which plays a crucial role in focus adjustment.

Beyond magnification and lens size, practical aspects also matter when choosing binoculars. Weight is important; a lighter pair is preferable for extended use and should feel balanced in the hands. For people with larger hands, very small models may be difficult to adjust and uncomfortable to hold, so the focusing system should be smooth and easy to operate. Eyecups must provide comfort and, for those wearing glasses, allow a full field of view. High-quality designs often feature nitrogen-filled chambers to prevent fogging from condensation, and the overall build should be durable with a protective casing for field use.

3. Telescopes (Spotting scope)

A spotting scope is equipped with lenses similar to those in binoculars, but is better suited for prolonged use. For example, when observing a nest or watching the fine details and behaviors of birds perched for long periods, the scope can remain fixed in place without the need to hold it constantly while waiting. A spotting scope provides a longer identification range than binoculars, particularly in wetlands, coastal zones, and open habitats where birds are often distant from the observer. It can be mounted securely using either a bench clamp fitting or a tripod, ensuring stability during extended observation.

4. Photography

Bird photography greatly enhances species identification by capturing fine details such as plumage patterns, beak, wing, and leg shape, and even flight behavior; features that may be overlooked in the field. Photos serve as reliable records for later verification or comparison with field guides, databases, and expert references. It is also widely used for data reporting in citizen science platforms, e.g., eBird, BirdLife International, GBIF, iNaturalist, etc.

In the past, birdwatchers relied on 35 mm SLR cameras with telephoto lenses or camcorders attached to scopes to record sightings. These tools allowed stills, video, and even bird songs to be documented, but they were heavy, film-dependent, and required extra accessories like spare batteries. Modern bird photographers often use digital mirrorless or DSLR cameras paired with telephoto lenses (300-600 mm) to capture distant birds clearly without disturbance. Advanced models offer fast autofocus and subject-tracking specifically for birds, making it easier to capture sharp images in motion. Additional tools, such as digiscoping with spotting scopes, tripods, and high-speed memory cards, further support detailed and consistent documentation, making photography an essential aid for accurate bird identification.

5. Acoustic Identification (Bird Calls and Songs)

Bird vocalizations are an essential tool for species identification, as many birds can be recognized more easily by sound than by sight. Each species has distinct songs and calls that reveal its identity, behavior, and even breeding status. Learning these vocal patterns allows birders to detect species hidden in dense vegetation, active at night, in low-light conditions, or far beyond visual range.

Identification through recognizing and recording vocalizations has become increasingly effective with the use of portable audio recorders and mobile applications equipped with spectrogram analysis, providing accurate identification and documentation of bird sounds.

6. Field Notes and Sketching

Recording notes or sketches of birds in the field helps capture key traits such as posture, flight behavior, and habitat use, complementing visual and acoustic observations. Start sketches with quick pencil lines and refine them if time allows; water-soluble pencils are useful for adding color with a bit of water and a brush. If a bird cannot be identified immediately, note its colors, markings, bill shape, and the length of its neck and legs. Estimate its size relative to familiar species and consider which family it may belong to. These records can later be compared with field guides or other references to confirm identification.

Bird Identification Resources

There are many resources available to help with bird identification, including printed bird guides (Figure 2.1), online references, and mobile apps designed specifically for bird ID (Table 2.1). These tools can provide pictures, sounds, and key features to make identification easier in the field.

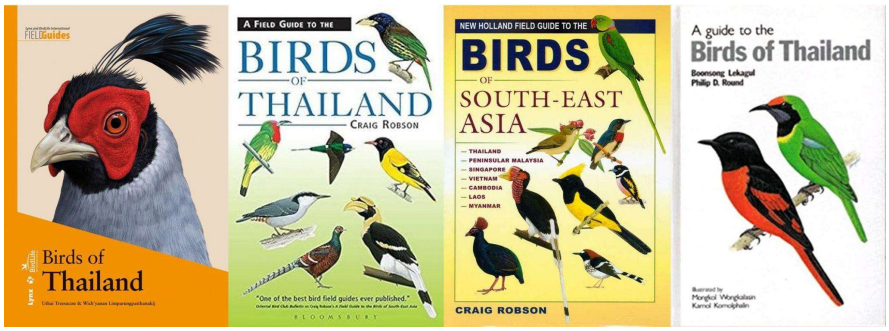

Figure 2.1 Example of Bird Guides for Species Identification for South-East Asia and Thailand

Table 2.1 Sources of Wild Bird Species Identification by Websites and Mobile Applications

Bird ID Resources	Website	Mobile application	URL
eBird (by Cornell Lab of Ornithology)	√	√	- https://ebird.org/home - https://ebird.org/explore - https://ebird.org/about/ebird-mobile
Merlin Bird ID (by Cornell Lab of Ornithology)	√	√	- https://merlin.allaboutbirds.org/ - https://www.allaboutbirds.org/guide/Merlin/id - https://merlin.allaboutbirds.org/download/
BirdLife International	√	-	- https://www.birdlife.org/ - https://datazone.birdlife.org/
GBIF (Global Biodiversity Information Facility)	√	-	- https://www.gbif.org/
iNaturalist	√	√	- https://www.inaturalist.org/
IUCN Red List	√	-	- https://www.iucnredlist.org/
BCST (Bird Conservation Society of Thailand)	√	-	- https://www.bcst.or.th/ - https://www.bcst.or.th/report-archives/

Geography

Geography plays a vital role in birdwatching, as the type of landscape, elevation, and vegetation influence which bird species can be found in a particular area. Understanding these features helps birdwatchers know where to look and what species they are most likely to encounter.

Habitat

Habitat is essential in birdwatching because each bird species depends on specific environments for food, shelter, and nesting. Knowing the habitat helps birdwatchers predict which species are likely to be present in a given area.

- **Community, Urban Parks, and Orchards**

  Birds that adapt well to human environments are common here, including sparrows, magpie-robins, starlings, barbets, pigeons, and bulbuls. In the cooler season, some uncommon migratory species may also appear.

- **Grasslands and Farmlands**

  These open habitats provide feeding grounds for various grassland and water-associated birds such as reed warblers, red-winged prinias, weavers, wagtails, and even the iconic Sarus crane.

- **Freshwater Wetlands**

  Lakes, ponds, rivers, and canals host herons, storks, crakes, cormorants, and migratory waterfowl that arrive during the winter months.

- **Dry Dipterocarp Forest**

  Dominated by species like *Shorea obtusa* and *Dipterocarpus tuberculatus*, these forests are home to woodpeckers, jays, nuthatches, parrots, and blue magpies.

- **Mixed Deciduous Forest (MDF)**

  With a diverse range of tree species, these habitats are vital for birds such as pheasants, orange-breasted trogons, laughing thrushes, and leafbirds.

- **Tropical Evergreen Forest (TEF)**

  Both dry and moist evergreen forests support high bird diversity. Hornbills are iconic here, along with bulbuls, flycatchers, broadbills, fairy bluebirds, and minivets.

- **Montane and Pine Forest (MPF)**

  Above 1,000 meters, these upland habitats, especially in northern mountains, harbor species like sibias, sunbirds, giant nuthatches, pheasants, and great tits.

- **Peat Swamp Forest (PSF)**

  Characterized by buttressed tree roots, these wetlands shelter specialties such as mangrove blue flycatchers, fuffy-backed tit babblers, and reddish scops owls.

- **Lowland Tropical Forest**

  Below 200 meters, dense rattan and palm undergrowth provides refuge for rare forest species, including Gurney's pittas, red-crowned barbets, and black hornbills.

- **Coastal Zone / Seashore**

  Sandy beaches, tidal flats, salt pans, and shrimp farms attract waders and egrets, with many migratory shorebirds arriving in winter.

- **Mangrove Forest (MF)**

  Along estuaries and tidal flats, mangroves support specialized birds such as collared kingfishers, gerygones, mangrove pittas, and mangrove blue flycatchers. In addition, these areas serve as nesting grounds for cormorants and herons, while during the winter season, various forest bird species may also be observed.

- **Offshore Island**

  These islands are important feeding and breeding grounds for seabirds including terns, frigatebirds, and boobies. They also serve as nesting sites for several species of terns, while forested islands provide habitats for species such as the Nicobar pigeon and pied imperial pigeon.

Observations

1. Distribution

Many protected areas remain poorly studied, with limited species lists and few birdwatchers visiting them. Exploring under-observed regions, such as the North and the South-West, may reveal new records or range extensions.

2. Numbers

Estimating bird density in forests is difficult, but simple counts can still be highly valuable. Reports of waterbird colonies, large flocks of migrants, or unusual concentrations provide essential data for conservation planning.

3. Breeding and Seasonality

Insect-eating birds generally breed in the late dry to early wet season, while waterbirds nest from late wet season to January. However, detailed data are scarce, making every nesting record valuable. Observers must take care not to disturb birds or expose nests to predators.

4. Feeding behavior

Knowledge of tropical bird diets is still limited. More records are needed on which trees provide fruit and flowers at different times, and how insectivorous species forage for prey.

5. Migration

Precise migration timing is poorly understood. Careful, repeated observations in the same location, such as temples, marshes, or small habitats, can reveal arrival dates, peak passage, and departure times (Alderton, 2002; Lekagul, 2005).

Animal Census Techniques: Overview of Bird Census Concepts for Avian Influenza Monitoring

Animal census represents a fundamental tool in wildlife ecology and ornithological research, providing quantitative data on animal populations across diverse ecosystems. Understanding population dynamics, species composition, and abundance patterns is essential not only for conservation management but also increasingly critical for public health monitoring, particularly in studying potential disease occurrence such as avian influenza in birds. This section examines the principles, methodologies, applications and potential limitations of bird census techniques, with emphasis on their role in avian influenza surveillance systems. Most of the content is outlined and complied from Bibby et al (1992).

Surveys and censuses provide essential population estimates for conservation and disease surveillance. Surveys collect data from sample areas using incomplete counts, yielding population indices or density estimates requiring statistical adjustment. Censuses conduct complete counts across entire study areas, providing absolute population numbers (Figure 2.2). Population data enables disease risk assessment by identifying species

abundance, distribution, and congregation patterns—critical for understanding disease ecology and transmission dynamics.

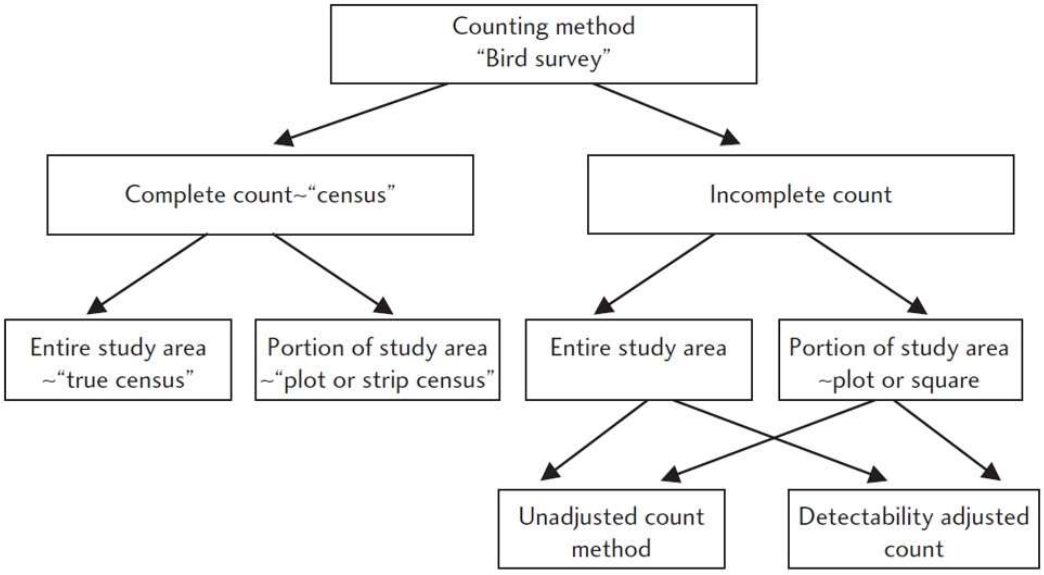

Figure 2.2. Bird surveys encompass two primary approaches: complete counts (censuses) that enumerate all birds within entire study areas, requiring no statistical adjustment for observation bias; and incomplete counts that sample portions of study areas or the entire area but fail to detect all individuals present. Incomplete counts can be analyzed either as raw, unadjusted data or preferably using statistical methods that account for variable detection probability. This distinction is fundamental to selecting appropriate analytical approaches for population estimation, as true censuses provide absolute population numbers while incomplete counts require detectability corrections to yield valid population estimates (Thompson, 2002; Gregory et al., 2004).

Bird census methodologies have evolved significantly since beginning in early ornithological studies (Bibby et al., 1992; Gregory et al., 2004). Traditional approaches relied on non-scientific observations and qualitative descriptions of bird communities. The professional bird census emerged in the early twentieth century with systematic approaches such as point counts and transect walks, which were later refined through statistical frameworks and standardized protocols (Ralph et al., 1981; Ralph et al., 1995; Buckland et al., 2001).

Comprehensive bird census data enables ornithologists, ecologists and scientists to:

- Monitor population trends and detect changes in species abundance over time
- Assess the health of ecosystems and environmental quality
- Guide conservation priorities and resource allocation
- Establish baseline data for impact assessments following environmental disturbances
- Track the distribution and movement patterns of migratory species

In terms of emerging role in disease surveillance, the recognition of wild birds as potential reservoirs and vectors for avian influenza has elevated the significance of bird census data. Population surveys now serve a dual purpose: understanding ecological dynamics while simultaneously providing epidemiological data critical for disease monitoring and early warning systems.

Techniques of Bird Census

1. Point Count Method: Point counts involve stationary observers recording all birds detected within a defined radius during a specified time period (typically 5-10 minutes). This method is particularly effective for territorial and vocally active species.

Advantages: Relatively simple to implement, less labor-intensive than other methods, and provides reliable density estimates for vocally conspicuous species.

Disadvantages: Biased toward auditory and visually detectable species; may underestimate cryptic or silent birds; effectiveness varies with time of day, season, and weather conditions.

Application to Avian Influenza Surveillance: Point counts at water bodies and wetland sites can systematically monitor waterfowl congregations, which are key risk zones for avian influenza transmission.

2. Transect Walk Method: observers walk predetermined routes at consistent speeds while recording all birds observed within specified distances on either side of the transect line. This method covers larger areas than point counts.

Advantages: Covers broader geographic areas efficiently; appropriate for detecting changes in spatial distribution; useful for landscape-scale monitoring.

Disadvantages: May miss stationary or cryptic species; observer bias affects detection probability; challenging in dense vegetation or rough terrain.

Application to Avian Influenza Surveillance: Transect walks connecting multiple water bodies and agricultural areas can map bird movement corridors and identify high-risk zones for disease transmission.

3. Mist-Netting and Capture-Mark-Recapture Methods: fine nets capture birds for direct examination, banding and biometric measurements. This hands-on approach provides detailed individual data including health status, body condition, parasites and disease indicators.

Advantages: Enables pathogen sampling and disease screening; provides precise morphometric data; allows for individual tracking through leg bands; permits assessment of physical health condition.

Disadvantages: Labor-intensive and time-consuming; requires specialized training and permits; may cause stress to captured birds; capture rates influenced by species behavior and netting site characteristics.

Application to Avian Influenza Surveillance: Mist-netting at selected study/surveillance sites enables direct screening for avian influenza virus through cloacal and oropharyngeal swabs; provides critical data on infection prevalence, viral load, and strain identification.

4. Acoustic Monitoring and Passive Sampling: automated recording devices capture bird vocalizations without direct observer presence. This technique is increasingly used for biodiversity monitoring and can provide continuous data collection across temporal scales.

Advantages: Eliminates observer bias; enables 24-hour monitoring; reduces disturbance to birds; cost-effective for long-term deployment.

Disadvantages: Requires computational resources for data analysis; species identification can be challenging, although progress has been made in machine learning techniques and open‑source data; may not capture silent birds; equipment costs and maintenance requirements.

Application to Avian Influenza Surveillance: Provides continuous presence/absence data at surveillance sites; when integrated with environmental DNA analysis, could potentially detect viral genetic material in the environment.

5. Visual Surveys and Specialized Counts: Techniques such as waterbird counts, raptor migration counts and roost counts employ targeted visual observation often in specific habitats or during particular times.

Advantages: Targets specific taxonomic groups or ecological communities; can be conducted from accessible vantage points; provides species-specific abundance data.

Disadvantages: Limited geographic coverage; observer skill variability; influenced by weather and visibility conditions.

Application to Avian Influenza Surveillance: Systematic waterbird roost counts identify congregation sites where transmission risk is elevated; migration counts track movement of potentially infected individuals.

6. Drone Technology for Bird Counts: Drones enable rapid, less-invasive bird surveys across large areas with minimal disturbance.

Advantages: expanded geographic coverage, cost efficiency, 24-hour thermal imaging, and automated data collection.

Disadvantage: requires specialized equipment and software for data processing, regulatory constraints, weather dependency, species identification limitations, and inability to capture specimens.

Application to Avian Influenza Surveillance: drones can be very good at identifying waterbird congregation hotspots, detecting mortality events, monitoring migration corridors, and guiding ground-based mist-netting efforts. However, drones complement but cannot replace traditional census methods requiring actual specimen collection for pathogen testing. Integration of drone surveys with ground-based sampling creates efficient, spatially-targeted surveillance approach.

Categories of Census Objectives

1. Stock-taking Assessments

Stock-taking represents the simplest census approach, addressing basic questions about which birds occur at specific locations. For avian influenza surveillance, this might involve establishing baseline species inventories at key habitats of interest e.g., wetlands or agricultural areas. This approach works particularly well for single-species surveys across extensive areas. For instance, documenting waterfowl congregation sites across a province

requires only basic presence/absence data with approximate numbers. The key assumption is that given adequate effort, most species can be reliably detected, though achieving equal coverage for all species remains virtually impossible—some birds are inherently difficult to detect regardless of effort invested.

2. Distribution Mapping

Distribution studies map where species occur without necessarily counting individuals. Modern approaches typically use standardized maps to document presence/absence systematically. For surveillance purposes, distribution mapping identifies high-risk zones where waterfowl concentrate or where wild birds overlap with poultry production areas. Coverage uniformity proves critical - maps showing bird distributions often reflect observer effort distribution. Sites near urban centers or research stations typically receive more attention than remote areas, potentially creating misleading patterns. Successful distribution studies either achieve reasonably uniform coverage or explicitly measure and report survey effort, allowing readers to distinguish between true absence and lack of survey coverage.

3. Population Monitoring

Monitoring population trends over time requires more rigorous approaches than simple stock-taking. For disease surveillance, for example tracking waterfowl numbers helps identify periods of elevated transmission risk when bird densities peak. The challenge lies in distinguishing genuine population changes from natural fluctuations due to weather, breeding success or observer variation. Effective monitoring demands repeatable methods applied consistently across years. The counts need not be perfectly accurate—systematic errors that remain constant over time still permit trend detection. However, sampling design becomes crucial: study sites must represent the broader population of interest, not just convenient or accessible locations. Non-random site selection limits inference and may miss important population changes occurring in unsampled areas.

4. Habitat Assessment

Understanding bird-habitat relationships helps predict where species occur and how land-use characteristics changes might affect populations. For surveillance, this translates to identifying landscape features associated with waterfowl concentrations or wild bird-poultry interface zones. Such studies require substantial data because bird communities vary along multiple environmental gradients simultaneously. Small sample sizes risk confounding effects—apparent patterns might reflect unmeasured variables rather than hypothesized

relationships. Successful habitat studies either employ large sample sizes across environmental gradients or use experimental approaches controlling for confounding variables.

Census errors and bias

Precision: Dealing with Random Variation

Precision refers to the repeatability of measurements—how closely repeated counts cluster around their average. Even using perfect methods, counts from different plots will vary simply because birds are not uniformly distributed. This random variation decreases as sample size increases, but the relationship follows diminishing returns: doubling precision requires quadrupling sample effort.

For example, consider measuring waterfowl density across multiple wetlands. Individual sites might support anywhere from 50 to 150 birds per hectare, even in seemingly uniform habitat. A single count provides little confidence about the true average density. Ten counts yield a reasonable estimate, while 100 counts would be extremely precise but likely impractical. The key is balancing precision needs against available resources.

Stratified sampling can improve precision efficiency. Rather than randomly selecting sites across a diverse region, first categorize habitats (permanent wetlands, seasonal wetlands, rice fields), then sample within each category. This approach often yields more precise estimates than purely random sampling, particularly when bird densities vary substantially among habitat types.

Accuracy: Addressing Systematic Bias

While precision concerns random errors that average out with large samples, accuracy involves systematic biases that persist regardless of sample size. Almost all census methods exhibit some bias—they consistently over- or under-estimate true values. Common sense suggests counts typically fall below actual numbers since detecting every bird remains unlikely, though some biases can inflate counts.

Multiple bias sources affect bird censuses including:

Observer bias stems from varying skill levels, motivation and sensory capabilities. Experienced observers detect more species and individuals than novices. Age-related

hearing loss significantly impacts detection of high-frequency bird calls. Training and standardization help minimize observer effects, but differences persist.

Method bias arises because different techniques detect species with varying efficiency. Point counts favor vocal species, while mist-netting captures secretive understory birds missed by visual methods. No single method provides unbiased counts across all species.

Effort bias reflects the general principle that greater effort yields higher counts. Walking transects slowly detects more birds than rapid surveys. Standardizing effort (speed, duration, area covered) ensures comparability, though determining optimal effort levels requires balancing thoroughness against practical constraints.

Habitat bias occurs because detection probability varies among vegetation types. Birds in open wetlands are more conspicuous than those in dense emergent vegetation. This particularly affects surveillance when comparing sites with different habitat structure.

Species bias recognizes that detectability varies dramatically among species. Conspicuous waterfowl are easily counted, while secretive rails in marsh vegetation are routinely missed. This differential detectability complicates community-level assessments.

Temporal biases include seasonal, daily, and weather effects. Breeding seasons maximize detectability through territorial singing, while dawn chorus periods yield highest activity levels. Wind and rain reduce both bird activity and observer effectiveness. Standardizing survey timing minimizes but cannot eliminate temporal biases.

Integration with Disease Surveillance

For avian influenza monitoring, census data serves multiple functions beyond simple population counts. Identifying high-risk periods when waterfowl concentrate and characterizing interface with other wildlife should help guiding intensive sampling schedules. Documenting species composition indicates which taxa require targeted surveillance based on known virus susceptibility. Mapping movement patterns between roosting and feeding areas reveals transmission pathways.

The challenge lies in balancing ecological monitoring needs with epidemiological sampling requirements. Census protocols could generate data suitable for both conservation assessments and disease risk evaluation without compromising either objective. This might

mean conducting standard ecological counts while simultaneously noting sick or dead birds or timing census efforts to coincide with disease sampling periods.

Conclusion

Successful bird census programs recognize that perfect counts remain difficult, all methods involve compromises and limitations. The goal is not perfection but rather fitness for purpose, generating data adequate for answering specific questions within resource constraints. By understanding fundamental principles of precision and accuracy, recognizing bias sources, and carefully matching methods to objectives, surveillance programs can be informed by ecological studies while producing valuable information for both conservation and disease monitoring.

For avian influenza surveillance in Southeast Asia, this means accepting that waterbird counts will never be complete, that detection probability varies among species and sites, and that weather and logistics will force departures from ideal protocols. Despite these limitations, well-designed census programs provide essential data for understanding disease ecology and guiding public health responses. The key lies in acknowledging limitations while maximizing information value through thoughtful study design, consistent implementation, and appropriate interpretation recognizing inherent uncertainties in wildlife population assessment.

Introduction of Wild Bird Trapping

Bird trapping remains a critical component of avian research, conservation, and wildlife health monitoring. When conducted ethically and systematically, trapping facilitates the acquisition of essential data on population dynamics, disease prevalence, migration patterns, and ecology. This handbook aims to provide comprehensive guidance on the selection, deployment, and safe use of three commonly utilized bird trapping techniques—mist nets, cannon nets, and hand nets. It is intended for researchers, veterinarians, conservation practitioners, and students involved in fieldwork requiring the live capture of birds.

The scope of this section encompasses planning and preparatory procedures,practical deployment methods, and post-capture handling protocols. Emphasis is placed on ensuring the welfare of captured birds and minimizing capture-related stress or injury. Although specific techniques may vary according to species and environmental context, the general principles and procedures described herein are broadly applicable across a wide range of avian taxa.

In summary, this section provides detailed and practical information to facilitate the humane and efficient use of mist nets, cannon nets, and hand nets for the live capture of birds (Figure 2.3). By adhering to the principles and protocols presented herein, field practitioners can conduct trapping activities responsibly and generate high-quality scientific data while safeguarding the welfare of avian populations.

Figure 2.3 Wild bird trapped by mist net and cannon net

General principles of bird trapping

Ethical considerations are fundamental to all trapping activities. All procedures described in this handbook should be conducted in accordance with relevant national and institutional animal care and use guidelines. Researchers must obtain appropriate permits from governmental and institutional regulatory bodies prior to the commencement of fieldwork. Furthermore, trapping should only be undertaken when scientifically justified and

when the anticipated benefits clearly outweigh the potential risks to the birds. Safe work practices are essential to protect both personnel and wildlife. Field teams must evaluate environmental hazards, utilize appropriate personal protective equipment, and maintain clear communication during trapping operations. Regular training in handling techniques and emergency procedures is strongly recommended to reduce the likelihood of injury and to ensure effective response in unexpected situations.

Bird trapping should be integrated within a broader framework of ecological responsibility. Investigators must select trapping locations to avoid unnecessary disturbance of nesting sites and other sensitive habitats. Capture activities should be scheduled to coincide with periods of minimal stress for the target species, considering breeding seasons, weather conditions, and diurnal activity patterns.

Mist Nets

Overview of mist net trapping

Mist nets are fine, nearly invisible mesh nets widely used in ornithological and chiropteran studies. They are especially effective for capturing small to medium-sized passerine birds but are also employed for bats and other flying animals. The nets are typically suspended between poles at varying heights in habitats where target species are active, such as forest edges, understory pathways, or near water sources. When birds or bats fly into the net, they become gently entangled in the mesh and can be safely extracted by trained personnel. Mist netting provides researchers with a non-lethal method to capture live animals for banding, sampling, or behavioral studies (Figure 2.4).

Figure 2.4 Setting Mist Nets for Wild Bird Capture

Equipment

- Mist nets

It is common to use 3-5-layer nets, which are widely available. The mesh size depends on the size of the bird, with mesh sizes typically ranging from 30 mm to 60 mm, which are commonly used for small to medium-sized birds (Figure 2.5).

- Poles

The material used for the poles depends on factors such as convenience in sourcing, transportation, and usage, including detachable aluminum poles or bamboo. The length of the poles depends on the terrain and the habitat characteristics of the birds.

- Nylon cords/ropes

Used for tying the poles to trees or anchors.

- Stakes/pegs

Used together with ropes to secure the poles.

- Bird bags

These are cloth or lightweight, breathable bags with seams sewn on the outside or reversed seams when in use to prevent thread from getting tangled in the bird's beak, legs,

or body. Only one bird should be placed in each bag. The bags should be of bright, easily visible colors to avoid using dark colors, as dark colors absorb heat, which could be harmful to the birds.

- Net-cutting equipment

Operators should carry sharp scissors to cut the net in case a bird cannot be extracted from the net.

Figure 2.5 Field Techniques: Mist Netting for Wild Bird Studies

Site selection

The choice of mist netting site depends on the target bird species and the characteristics of the surrounding habitat. Nets are typically positioned along feeding areas, natural flight paths, or within habitats where birds are most active. Selecting shaded locations is preferred, as reduced light minimizes the visibility of the net and increases capture efficiency. In addition, shaded sites help reduce heat stress on captured birds, ensuring better welfare during handling.

Precautions

Areas exposed to direct sunlight, open spaces, or strong winds should be avoided, as these conditions increase net visibility and reduce capture efficiency. Nets should also not

be set up during extreme weather conditions, such as excessive heat, cold, heavy rain, or strong winds, which can endanger both birds and researchers (Figure 2.6). In addition, mist nets should not be placed near nesting areas during the breeding season, as this may cause stress to the birds and inadvertently attract predators to the nests.

Figure 2.6 Safety Precautions for Mist Netting and Wild Bird Capture

Setting up mist nets

1. Clear the area before setting up the net by removing any obstacles on both sides and the ground, such as shrubs, grass, branches, or debris. Ensure that the area where the net will be deployed, when blown by the wind, does not get tangled with any obstructions.

2. Attach one end of the net to the pole. If the pole has pointed ends for inserting into the ground, insert the pole vertically into the ground. You may use ropes to secure the pole to trees, branches, or anchors to keep it stable. Then, gradually extend and stretch the net to its full length and attach it to the pole.

3. Arrange the loops at both ends of the net in order. Spread out the net and make sure the spacing between the layers of the net is appropriate, depending on wind conditions and the target bird species. If the spacing between layers is too wide, birds may bounce out when they run into the net, and if the spacing is too narrow, the birds may get severely entangled.

4. If multiple nets are being set up in a row, you can use the same poles. Attach the loops of each net to the poles in sequence and alternate between each net, for example, attach loop 1 of net 1 to loop 1 of net 2, loop 2 of net 1 to loop 2 of net 2, and so on for all layers.

Monitoring and checking mist nets

Mist nets must be checked frequently every 10–15 minutes to minimize the risk of injury, predation, or overheating of captured birds. Birds caught in the net should be removed within 10 minutes, and the process of removing the birds should not take more than 5 minutes. Operators should always carry net-cutting tools when checking the nets. Handling should only be done by trained personnel to ensure safe extraction and reduce stress on the animals.

Nets should be closed when not actively monitored and must be taken down at the end of each field session to prevent accidental captures of non-target species, such as nocturnal animals. To avoid entanglement injuries, nets should be properly maintained, with damaged mesh repaired or replaced promptly. Additionally, researchers should ensure that permits and ethical approvals are in place and that all activities follow animal welfare guidelines.

Taking down mist nets

1. Closing the nets when not in use. Gather all the nets, separating the top layer from the other layers. Then, shake or roll up the three lower layers of the net and bring the top layer down to be level with the others. Use ropes to tie the net at both ends of the loops and tie the net at several points (approximately 1 meter apart) to prevent birds or other animals, especially bats, from getting tangled in the net.

2. Taking down the nets. Inspect the net to ensure no items are stuck in it. Then, gather all four layers of the net together, tie the loops at both ends of the net, and remove the loops from the pole. Slowly walk and gather the net to its full length. Once the net is collected, place it in separate bags for each section, or tie each section of the net with rope to keep them separate.

Precautions

- Be careful not to let the net touch the ground during installation, as it may become entangled with obstacles on the ground.

- The lowest layer of the net should not be obstructed by grass or other obstacles, as this may make it difficult to see any birds caught in the net, which could be dangerous for the birds.

- The net is prone to becoming tangled with various objects. Operators should wear clothing without buttons or zippers and avoid wearing jewelry that could get caught in the net while working.

Safe Removal of Birds from Nets

Safe removal of birds from mist nets requires patience, care, and proper technique to ensure the safety of both the bird and the handler. Begin by approaching the bird calmly to avoid causing further stress or struggle, which can increase the risk of injury. Gently but securely hold the bird, ideally around the body with its wings folded against its sides, to prevent flapping. Start the removal process at the part of the bird that is least entangled, typically the feet or tail, and work methodically toward the head. Carefully tease the net away from feathers and limbs, avoiding sudden movements. If necessary, use blunt-tipped scissors to cut the net, but only as a last resort to prevent injury. Throughout the process, minimize handling time to reduce stress. Once the bird is free, examine it for signs of injury or fatigue. Healthy birds can be released immediately, while those that appear injured should be taken to a licensed wildlife rehabilitator for care. Proper training in mist net

handling is essential, and inexperienced individuals should not attempt removals without supervision (Figure 2.7).

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Because the technique involves explosives/pyrotechnics, it requires formal training, proper permits, careful planning, and strict attention to safety and animal welfare (Figure 2.8).

Figure 2.8 Cannon Net for Wild Bird Trapping

Equipment and trigger systems

- Cannon

There are three types: 2-cannon, 3-cannon, and 4-cannon, depending on the size of the net. The cannon consists of three main components:

• Cannon made of steel, mounted on a rectangular steel base.
The base has slots for inserting black powder cartridges.
• Steel base at the rear of the launching barrels, rectangular in shape.
• Rubber pads, two pieces, used to cover the steel base,
sized to match the base.

- Cannon net

The net constitutes a component that spreads outward under the force of the projectiles. It is designed with specific features, including pouches folded along the back edge and elastic cords attached to the rear margin, which are secured to the ground with pegs. The dimensions of the net and the mesh size vary according to the target bird species. In general, mesh sizes are classified as wide or narrow, each presenting distinct advantages and disadvantages (Table 2.2).

Table 2.2 Comparison of Mesh Types Used in Cannon Net Setting

Mesh type	Advantages	Disadvantages
Wide mesh	- Allows good airflow and offers minimal wind resistance. - Suitable in wet environments, as birds can extend their heads through the mesh to breathe.	- Birds experience greater difficulty moving under the net. - Bird removal is also more challenging.
Narrow mesh	- Facilitate bird movement under the net. - Removal of birds from the net is easier.	- Provides greater wind resistance. - Unsuitable for wet environments, as the net may press birds down, increasing the risk of drowning.

- Cartridge and plate

A stainless steel or rust-proof steel cylinder, open at the top and with a small hole at the bottom to insert the igniter. It is used to contain black powder. Once the black powder is loaded, a plate is placed on top to seal it. The projectile is loaded at the base of the cannon barrel and connected to the firing box via electrical wires attached to a terminal strip.

- Projectile (Figure 2.9)

A device launched from the cannon to spread the net. It is made of steel and is slightly longer than the barrel. The upper end has a loop for attaching to the net's loops.

Figure 2.9 Projectile Equipment for Cannon Net Setting

- Black powder

A low-pressure explosive is used as the propellant to launch the projectile from the cannon. The amount of black powder used depends on the net size and the number of cannons.

- Firing cable and cable

Electrical wires are used to connect each cannon to the ignition point.

- Scales

Used to weigh the black powder.

- Angle Gauge

A tool for measuring the angle of the barrel and projectile placement. Typically, it measures the angle between the barrel and the ground. The appropriate firing angle depends on the wind direction and the size of the target bird.

- Igniter

A small wire with one end connected to the firing box wires and the other end containing an easily ignitable compound that produces a spark when heated. It is inserted into the small hole at the bottom of the black powder cartridge.

Figure 2.10 Other Equipment for Cannon Net Setting

- Chocolate block electrical / Terminal strip

  A device used to connect two electrical wires together.

- Circuit tester

  A tool used to test electrical continuity and ensure there are no short circuits after wiring the barrels. Before connecting the wires, the ignition switch must be in the off position. After connecting, test whether the circuit is complete by switching to the test position. If the light turns on, the circuit is complete.

Caution: Do not stand in front of the barrels when testing the circuit.

- Firing box

  Responsible for sending electrical current through the wires to the ignition system. The box is positioned at the firing location and must be operated by an experienced and licensed individual (Figure 2.12).

Figure 2.11 Fire Box for Controlling the Cannon

- Keeping cages

Made from lightweight, breathable fabric and divided into several compartments for

separating different bird species. The cages are placed on the ground and secured using metal loops sewn into the fabric.

- Pegs and knocker
- Jiggler

A rope used to drive birds into a safe area. Small pieces of cloth are tied along the rope every 1–2 meters. The rope is typically placed about 50 centimeters in front of the net.

- Wrench

Site Preparation and Baiting

The trapping site should be selected in an area where large groups of birds naturally congregate. The site must not be prone to flooding after the net has been deployed, to minimize the risk of drowning. Baiting is commonly employed to attract and concentrate birds within the capture zone. The type of bait should be chosen according to the dietary preferences of the target species and distributed in a manner that encourages regular feeding. To ensure effective habituation, baiting should commence several days prior to trapping, allowing birds to become accustomed to the site and reducing the likelihood of disturbance during capture.

Safety Measures (for birds and operators)

To minimize risks to birds, nets should never be deployed in areas prone to flooding, excessive heat, or hazardous terrain. The capture process should be closely monitored, and birds must be promptly removed from the net to prevent injury, overheating, or drowning. Handling should follow established animal welfare guidelines, using appropriate restraint techniques to reduce stress and physical harm.

For operators, cannon nets pose risks due to the explosive charges and the rapid deployment of heavy projectiles. Only trained personnel should handle and fire the equipment. Safety precautions include maintaining a clear firing zone, wearing appropriate protective gear (e.g., eye and ear protection), and following standardized firing protocols. All operators should remain at a safe distance from the net during deployment and avoid standing directly behind the projectiles.

Setting the cannon net system

1. Select and prepare the site by first ensuring it is not prone to flooding and that it is regularly used by large groups of the target bird species. Choose an area that offers both visibility and safety for net placement. Before setting up, carefully clear the immediate vicinity of any debris or hazards such as rocks, holes, or sharp objects that could harm captured birds or interfere with the mist net. This preparation helps ensure both the effectiveness of the capture and the safety of the birds and researchers involved.

2. Assembling the launching cannons

- Insert the igniter, with the end containing the explosive compound, into the small hole at the rear of the cartridge. Seal the rear hole of the cartridge securely.
- Then, weigh the black powder according to the net size and the number of cannons being used. For example:
- Net size: 10 x 15 meters. Using two cannons, use 12:12 grams of black powder.
- Net size: 10 x 18 meters. Using three cannons, the proportions are 14:15:14 grams.

- Net size: 10 x 25 meters. Using four cannons, the setup is 14:15:15:14 grams.
- After weighing, seal the top of the cartridge tightly with a plate to prevent powder leakage.
- Insert the loaded cartridge and projectile into the rear end of the launching cannon.
- Place the rubber pad with three holes between the base and the cannon. Then cover the barrel base with the rubber pad with two holes.
- Thread the electrical igniter wires through the rubber pad to prevent them from being pinched or damaged.
- Tighten the bolts between the launching barrel and its base securely.

3. Layout and locate capture area

- Spread the net evenly over the selected capture area, making sure it will settle flat and function properly when deployed.
- Locate cannon and net position
- Fix the rear edge of net, rig the ground anchors and elastic returns by staking elastic cords or tie lines to ground pegs placed at the rear edge of the net. These will help control how the net spreads and recoils during deployment. Double-check that all net pouches, seams, and elastic attachments are properly positioned, untangled, and unobstructed to allow for smooth deployment.
- Furl the net, ensuring it is positioned 1 meter in front of the cannons (Figure 2.12).

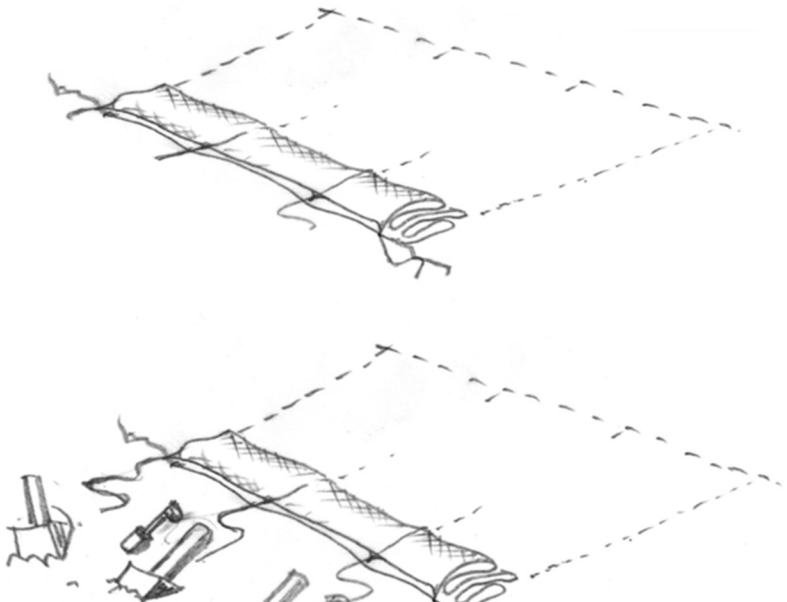

Figure 2.12 Preparing Nets and Assembling Launchers

4. Setting the launching cannons

- Dig a hole to place the launching barrel, positioning it at an angle to the ground using an angle gauge. Typically, the cannon is set at an angle of 10–30 degrees from the ground, depending on the terrain, wind direction and strength, and the type of bird species.
- For smaller birds, the angle is generally narrower than for larger birds. Use natural materials that match the surrounding environment to conceal the cannon completely.
- Setting the cannons and attaching the net involves different procedures depending on the number of barrels used, as outlined below:
- *Two cannons*
- Position the two cannons 5 meters from the edge of the net. The cannon openings should face the corners of the net when it is fully deployed, both to the left and right.
- *Three cannons*

- The outer cannons should be set 5 meters from each edge of the net. The cannon center should be placed 1 meter from each edge of the net. The outer cannon openings should be angled toward the corners of the net, while the center cannon should be positioned to fire perpendicular to the base of the net.
- *Four cannons*
- The outer two cannons should be set 5 meters from the net edges, while the two center cannons should be placed 3 meters from each edge. The outer cannons should point toward the corners of the net, and the center cannons should be aimed perpendicularly at the base of the net.

5. Camouflaging the Cannons

Conceal the launching cannons using materials that blend with the local environment, such as: sand when working on a beach, soil when working in salt flats or fields. Ensure the materials used for camouflage are not too heavy, as this may prevent the net from deploying fully or affect its spread.

6. Assembling and camouflage the net

- Set the jiggler 50 centimeters in front of the net. The safe zone for the birds is a distance of 50–100 centimeters from the front edge of the net.
- Attach the net's rope loops to the greased loops of the projectiles, then insert the projectiles into the cannons (Figure 2.13).

Figure 2.13 Attaching Nets to Projectiles (Launch Assembly)

- Use natural materials such as branches, leaves, or vegetation from the area to

camouflage the net, taking care to match the surrounding substrate. Avoid using shiny, brightly colored, or contrasting materials, as these can alert birds and reduce capture success. Proper camouflage helps increase the net's effectiveness while minimizing disturbance to the birds (Figure 2.14).

Figure 2.14 Site Preparation for Cannon Net Setting

7. Carry out final positioning and camouflage, adjust the net so it lies naturally and flat across the deployment area, blending it into the surrounding terrain using natural materials. Ensure that all parts of the launching mechanism are hidden from the birds' line of sight. Proper setup and concealment increase capturing success while minimizing stress and risk to the birds.

Pre-firing safety checks

- Confirm all personnel know their roles and safe positions; establish a single designated firing officer.
- Ensure the firing zone is clear of people, pets, and non-essential equipment.

- Check wind direction and strength; abortion if conditions are unsafe (strong gusts, sudden weather changes).
- Inspect all knots, anchors, and the shooting tube stability one final time.

Firing and Capture

- Wait for birds and deploy
- Allow birds to enter and feed in the capture area undisturbed. Monitor quietly from a concealed, safe position.
- When target birds are within the capture zone and all safety checks are satisfied (ideally centered and not too close to the net's edge), the designated firing officer activates the system to launch the net.

Post-Capture Handling and Extraction

Immediate post-deployment actions approach the net only after ensuring projectiles have settled and it is safe to do. It is essential to begin bird removal without delay to minimize stress and reduce the risk of injury or, in certain environments, drowning. The welfare of the birds must be the highest priority throughout the extraction process. Begin by working from the outer edges of the net toward the center, removing one bird at a time using slow, controlled, and deliberate movements. Carefully disentangle each bird, ensuring that the wings, feet, and head are freed without pulling feathers, twisting limbs, or causing undue stress. Once removed from the net, each bird should be placed in a quiet, well-ventilated holding container or soft cloth bag to allow it to rest undisturbed until all individuals have been safely extracted. Throughout this process, all birds should be closely monitored for signs of injury, exhaustion, or distress. Any bird exhibiting abnormal behavior or physical trauma should be promptly referred to as a licensed wildlife rehabilitator or veterinarian. Adhering to best practices for post-capture handling is critical to ensuring the birds' safety and well-being, and supports ethical standards for research, conservation, or rehabilitation efforts.

Hand Nets

Overview of Hand Net Use

Hand nets are essential tools used to catch and handle animals, particularly in situations where precision and control are crucial. They're commonly used in Avian, wildlife management, research, and animal control. In bird banding and monitoring, hand nets help researchers catch birds for banding, weighing, and measuring, providing valuable insights into population dynamics and behavior. Additionally, hand nets play a crucial role in rescue and rehabilitation efforts, enabling wildlife rehabilitators to catch and care for injured or orphaned birds, increasing their chances of survival and successful release. They're particularly useful for catching birds in nesting sites, dense vegetation, or small enclosures, where other capture methods may be ineffective (Figure 2.15). By prioritizing bird welfare and minimizing stress, researchers and conservationists can use hand nets effectively and humanely in various avian contexts.

Figure 2.15 Wild Bird Trapping by Hand Net

Selection of Hand Nets

Hoop Size should be selected based on the species and situation. Hoop sizes may range from 15–30 cm for small birds up to 90–180 cm for larger birds such as waterfowl.

Net Material preferably be made of knotless nylon to prevent feather damage, although monofilament and multifilament materials may be used in other contexts. Pole type may be telescoping poles (aluminum or fiberglass) provide flexibility and reach, while fixed-length poles offer stability. Before use, equipment must be inspected for damage, disinfected, and prepared alongside holding bags or containers for secure transfer.

Capture Techniques and Tips

During capture, handlers should approach the bird calmly and quietly to avoid alarming it. The net should be positioned behind, above, or to the side of the bird, and then move swiftly and decisively to enclose it. Once captured, the bird should be allowed to settle at the bottom of the net and supported gently from beneath without pressing it against the hoop. Birds must be transferred promptly to a holding bag or secure container, with care taken to avoid entanglement of feathers, wings, or legs. Handling time should be minimized, and birds should never be left unattended in a net. Safety and welfare are paramount throughout all stages of the procedure. The smallest effective net should always be used, and the number of capture attempts should be limited to prevent exhaustion or stress. Staff must be trained in low-stress handling techniques to avoid injury to both birds and handlers. All captures should be documented, including the date, location, species, and purpose of capture, along with any injuries or stress responses observed

Hand Nets

https://www.youtube.com/watch?v=Ju3qEru8pe0

Quiz

Question 1: When conducting a comprehensive bird survey in a mixed deciduous forest (MDF), which combination of identification methods would provide the most robust and scientifically rigorous data collection?

A) Relying solely on visual observation and basic field notes
B) Integrating multiple techniques, including acoustic recording, photography, systematic field notes, and habitat-specific observation protocols
C) Using only binoculars and a standard field guide
D) Depending exclusively on mobile application identification tools

Correct Answer: B) Integrating multiple techniques, including acoustic recording, photography, systematic field notes, and habitat-specific observation protocols

Question 2: In designing a comprehensive avian influenza surveillance program using bird census methods, which integrated approach would most effectively balance ecological monitoring with epidemiological sampling requirements?

A) Implementing a multi-method approach that combines point counts, mist-netting, acoustic monitoring, and drone surveys, with strategically selected sites representing diverse habitats and potential disease transmission zones
B) Relying solely on point count methods in easily accessible locations
C) Conducting visual surveys only during peak migration seasons
D) Using exclusively drone technology for population estimation

Correct Answer: A) Implementing a multi-method approach that combines point counts, mist-netting, acoustic monitoring, and drone surveys, with strategically selected sites representing diverse habitats and potential disease transmission zones

Question 3: When addressing potential sources of bias in bird census methods for avian influenza surveillance, which combination of strategies most comprehensively mitigates systematic errors?

A) Ignoring bias and assuming all counts are equally accurate
B) Conducting surveys only during optimal weather conditions
C) Relying exclusively on experienced observers
D) Implementing standardized protocols that address observer, method, habitat, species, and temporal biases through:
- Consistent training programs

- Stratified sampling designs
- Multiple detection methods
- Standardized survey timing and effort
- Explicit documentation of survey limitations

Correct Answer: D) Implementing standardized protocols that address observer, method, habitat, species, and temporal biases through:
- Consistent training programs
- Stratified sampling designs
- Multiple detection methods
- Standardized survey timing and effort
- Explicit documentation of survey limitations

Question 4: When designing a comprehensive bird capture protocol for avian influenza surveillance, which integrated trapping approach would most effectively balance scientific objectives with animal welfare considerations?

A) Relying exclusively on mist netting in easily accessible locations
B) Implementing a multi-method approach that combines mist nets, cannon nets, and hand nets, with carefully selected sites, standardized protocols, and explicit welfare guidelines
C) Conducting captures only during peak migration seasons without considering species-specific sensitivities
D) Using the most aggressive capture techniques to maximize sample collection

Correct Answer: B) Implementing a multi-method approach that combines mist nets, cannon nets, and hand nets, with carefully selected sites, standardized protocols, and explicit welfare guidelines

Question 5: In evaluating the potential risks and methodological challenges of cannon net trapping for avian influenza surveillance, which combination of factors demonstrates the most sophisticated understanding of field research protocols?

A) Implementing a comprehensive approach that integrates:
- Detailed site selection criteria
- Precise baiting strategies
- Rigorous safety protocols for both birds and operators
- Careful consideration of environmental conditions
- Standardized post-capture handling procedures
B) Relying on minimal preparation and improvising during the capture process
C) Focusing solely on the technical aspects of net deployment
D) Prioritizing capture efficiency over animal welfare

Correct Answer: A) Implementing a comprehensive approach that integrates:
- Detailed site selection criteria
- Precise baiting strategies
- Rigorous safety protocols for both birds and operators
- Careful consideration of environmental conditions
- Standardized post-capture handling procedures

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Chapter III: Biosafety in Field Sample Collection

Pre-course Materials

- **Introduction to Biosafety section**
  - Introduction and learning outcome
  - Assessment and certification
  - Masking Competition (**evaluation - pre workshop**)
  - Module Overview
  - Biosafety Quiz (**evaluation - pre workshop**)
  - Biosafety module discussion space
- **Biosafety main course**:
  - Introduction to HPAI globally and Asia PAcific specifically
  - Biosafety Vs Biosecurity
  - Biosafety technical page (Originally created for SARS CoV-2 at the human wildlife interface with colleagues from University of Minnesota, Stamford University, IUCN SSC on Great APes and the Pan African Sanctuary Alliance
  - Modified Heirachy of Controls for Field Settings
  - Personal Protective Equipment (PPE)
  - Occupational Hygiene and PPE (**Evaluation Assignment**)
  - Technical face covering brief
  - Managing Risk
  - Managing risk example: Infectious disease biosafety in an African Wildlife Sanctuary Setting (with colleagues from University of Minnesota, Stamford University, IUCN SSC on Great APes and the Pan African Sanctuary Alliance
  - Mapping the Risk (**Evaluation assignment**)
  - Field Sampling collection protocols (being edited - to combine into sample collection module)
  - Emergency response and reporting (Being edited - to combine into following module).
  - Biosafety Quiz (**evaluation post workshop**)
  - Contingency planning for a respiratory disease outbreak **(Evaluation assignment**)
  - ADDITIONAL section FYI only: Introduction to All Hazards Approach to Disaster Management.

Skills and Knowledge to be Acquired

- Learning outcomes:
    - Understand why particular biosafety measures are recommended in the context of potential risks and their mitigation in the larger health system
    - Have knowledge of basic HPAI epidemiology and risk management and where knowledge resources for same can be found
    - Be proficient in developing and enacting a biosafety plan for HPAI under field conditions.

By the end of this module, (veterinary trained learners highlighted) will be able to:

- Understand the principles of biosafety and biosecurity in wildlife fieldwork (or Describe Core principles of biosafety and biosecurity in wildlife field settings)
- Apply risk-based approaches to (conduct risk-based) sample collection in suspected HPAI outbreaks
- Implement (and supervise) appropriate PPE and hygiene protocols (decontamination measures)
- Modify the Hierarchy of Controls for field settings
- Navigate emergency response and reporting procedures in your country or region.

Materials requests for biosafety part of the live workshop:

- UV reactive powder (e.g see here)
- UV lamps (to detect the UV powder)
- PPE as described in the course
- Flip charts, markers and post-its.

***For full course, see Canvas: https://umnadvet.instructure.com/courses/732

Evaluation

- Pre workshop:
    - Masking competition (donning and doffing) by organisation
    - Biosafety quiz by individual
- During workshop
    - Practical: PPE checklist (observer-based), and proper donning/doffing PPE
    - Biosafety quiz (at end)

- Occupational Hygiene and PPE (**Evaluation Assignment**)
- Mapping the Risk (**Evaluation assignment**)

Content

Introduction to HPAI globally and Asia Pacific specifically

Veterinarian Focus

- Clinical presentation in wild birds and mammals
- Diagnostic sampling priorities (tracheal/oral swabs, tissues)
- One Health coordination with public health labs

Ranger Focus

- Identifying unusual mortalities in wild populations
- Safe surveillance of wetlands and roost sites
- Early-warning reporting channels

Key Resources as required reading before the onsite course:

- Overview of HPAI H5N1 clade 2.3.4.4b and its global spread. Updated joint FAO/WHO/WOAH public health assessment of recent influenza A(H5) virus events in animals and people
- Links to an external site.
- Assessment based on data as of 1 July 2025

At the present time, based on available information, FAO-WHO-WOAH assess the global public health risk of influenza A(H5) viruses to be low, while the risk of infection for occupationally or frequently exposed (e.g., with backyard poultry) persons is low to moderate depending on the risk mitigation and hygiene measures in place and the local avian influenza epidemiological situation. Transmission between animals continues to occur and, to date, a growing yet still limited number of human infections are being reported. Although additional human infections associated with exposure to infected animals or contaminated environments are expected to occur, the overall public health impact of such infections at a global level, at the present time, is considered minor. The assessment could change if and when additional epidemiological or virological information becomes available.

This risk assessment from FAO, WHO and WOAH updates the assessment of the risk of zoonotic transmission (for example, animal to human) considering additional information made available since the previous assessment of 17 April 2025. This update is limited to the inclusion of additional information being made available globally. Due to the potential risk

to human health and the far- reaching implications of the disease on the health of wild birds, poultry, livestock and other animal populations, timely notification to global authorities and the use of a One Health approach are essential to tackle avian influenza effectively, to monitor and characterise virus circulation, to prevent transmission within species and to new species to reduce spread among animals, and to prevent human infections from exposure to animals.

- Wildlife Health Australia H5 Bird Flu public page
- Links to an external site.
- - an example of a one-stop-shop specific to Australia's situation, giving scope as a potential template.

H5 bird flu, also known as *high pathogenicity avian influenza H5N1 clade 2.3.4.4b*, is a strain of the avian influenza virus. The virus has been spreading globally since 2021 through the movement of wild birds, with unprecedented impact. H5 bird flu can also affect other animals, and millions of wild animals worldwide have now died due to the virus. **H5 bird flu has not been detected in Australia**. However, H5 bird flu may arrive in Australia via wild bird movements year-round, with the annual Spring (August – November) migration of wild shorebirds from the northern hemisphere being the most likely way for H5 bird flu to arrive here. This page contains information and resources for H5 bird flu. In addition, we recommend you review the document High Pathogenicity Avian Influenza and Wildlife: Advice for veterinarians and animal health professionals

- Open access online laboratory-based courses from the CSIRO (via the Australian Centre for Disease Preparedness for biorisk - they are laboratory based but might be relevant for some participants. Access here.
- The Pathogens and Animal Toxins Act of Thailand 2558 (2015).
- Thailand's risk profile and migratory bird pathways (Ratanakorn et al., 2012;Ratanakorn et al., 2018)
- Updated guidance on HPAI - environmental update for USAP personnel (USAP - United States Antarctic Program). An example on communications from history:

T**here is a high risk that Highly Pathogenic Avian Influenza (HPAI H5N1) will arrive in the Southern Ocean during the 2023/24 austral summer.** Current outbreaks of this disease have resulted in the deaths of hundreds of thousands of seabirds around the world. **The introduction of this virus into the Southern Ocean may result in high mortality among affected seabird and marine mammal populations in Antarctica.** There is a heightened risk of HPAI introduction or spread by infected migrating seabirds, as well as by contaminated boots, clothing, and equipment from humans. In response, the USAP is requiring enhanced biosecurity protocols in the Antarctic this season, including pre-landing surveillance, decontamination of boots and gear between seabird colonies, reporting of suspected HPAI outbreaks to NSF OPP Environmental (OPP-ENV@nsf.gov), and enhanced management of areas impacted by HPAI.

*Importance of early detection and One Health coordination Example in action from NRM: H5 HPAI preparedness: The role of Wildlife Health Australia'

Beginning of Course Content

Clinical presentation in wild birds and mammals

Wildlife Resource: See High Pathogenicity Avian Influenza (HPAI) and Wildlife in Australia. A RISK MITIGATION TOOLBOX FOR WILDLIFE CARE PROVIDERS WITH A FOCUS ON HPAI H5 2.3.4.4b.

Avian influenza is potentially zoonotic. Contact with sick or dead birds should be avoided where possible. Precautions should be taken when handling or sampling potentially infected birds.

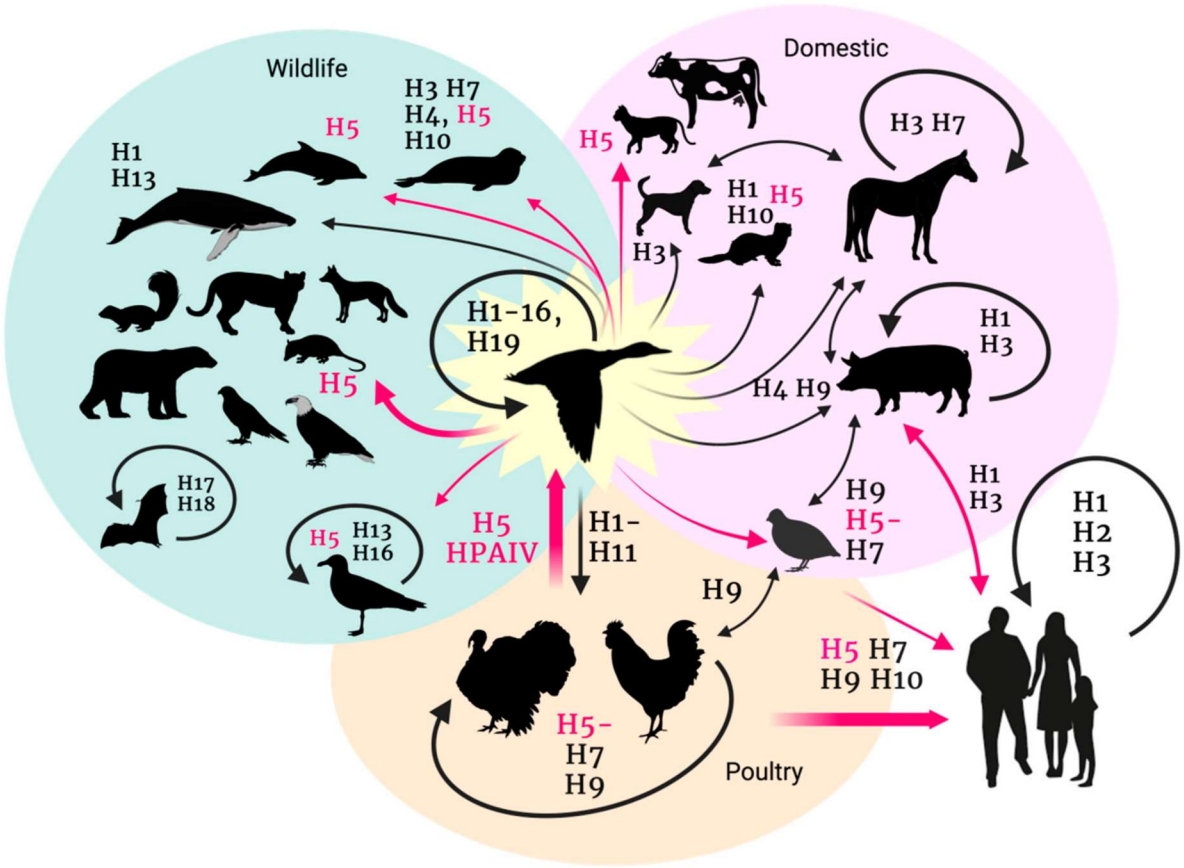

Figure 3.1 Avian Influenza (AI) in Animals and Humans

From Graziosi, G., Lupini, C., Catelli, E., & Carnaccini, S. (2024). Highly Pathogenic Avian Influenza (HPAI) H5 Clade 2.3.4.4b Virus Infection in Birds and Mammals. *Animals*, *14*(9), 1372. https://doi.org/10.3390/ani14091372 This paper provides an insight into clinical signs of HPAI seen in wildlife.

The following material on clinical signs is taken from Wildlife Health Australia's High Pathogenicity Avian Influenza and Wildlife: Advice for veterinarians and animal health professionals This is updated regularly, so please check for updates.

Clinical signs: Wild birds

HPAI should be considered as a differential diagnosis in the following scenarios for wild birds:

- Small groups or clusters (5 or more) of sick or dead wild birds of any species
- Individual or less than 5 sick or dead wild birds: seabirds, waterbirds, shorebirds or birds of prey
- Any other bird species with signs of avian influenza infection as outlined below
- Infected live birds may show a wide range of clinical signs, including:
    - neurological signs (ataxia, paralysis, seizures, tremors, abnormal posture)
    - respiratory signs (conjunctivitis, increased nasal secretions, oedema of the head, dyspnoea)
    - gastrointestinal signs (diarrhoea)
    - ocular signs.
    - sudden death, including the potential for mass mortality events
- Some species or individuals may be asymptomatic or show only very mild clinical signs. In some cases, birds may die suddenly without displaying any clinical signs
- While there are other diseases of wild birds that can cause these clinical signs, HPAI should be excluded to ensure that HPAI can be detected as soon as possible, and any associated risks to animal and human health can be managed.

Clinical signs: Wild mammals

- HPAI should be considered as a differential diagnosis in any event of sick or dead wild marine mammal, predator or scavenger species with signs of avian influenza infection as outlined below, or that may have been in contact with affected wild birds.
- Infected wild mammals may show a wide range of clinical signs, including:
    - neurological signs (ataxia, paralysis, seizures and tremors)
    - respiratory signs (increased nasal and oral secretions, dyspnoea, tachypnoea)
    - sudden death, including the potential for mass mortality events.
- Some species or individuals may be asymptomatic or show only very mild clinical signs. In some cases, animals may die suddenly without displaying any clinical signs.

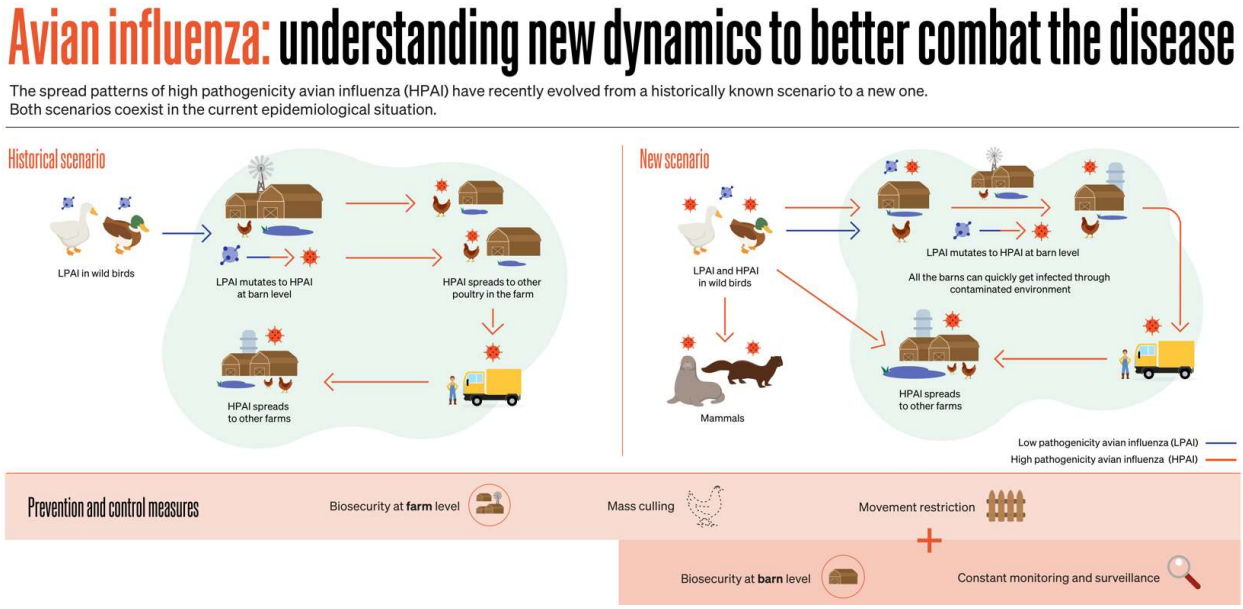

Figure 3.1 Avian Influenza: Understanding New Dynamics to Better Combat the Disease

From https://www.woah.org/en/disease/avian-influenza/.

Species Impacted:

Wildlife: HPAI H5 clade 2.3.4.4b (commonly referred to as H5 bird flu) can cause mass mortalities in many species of wild birds, as well as other terrestrial and marine mammal species, and may represent a population level threat to some wildlife hosts, impacting biodiversity. Other strains of HPAI are less likely to cause disease in wild birds and mammals. Low pathogenicity AI strains are unlikely to cause disease in wild birds or other wildlife species but are important due to the potential of LPAI H5 and H7 subtypes to mutate into HPAI forms when introduced to poultry.

Domestic Animals: A range of domestic species are susceptible to AIVs including poultry, swine, horses, cattle, dogs and cats.

Livestock species other than poultry have been affected: WOAH case definition of HPAI in cattle (March 2025).

Avian influenza is also a major concern for public health. Whenever avian influenza viruses circulate in poultry, sporadic cases of avian influenza in humans are sometimes identified.

The FAO has listed species globally that have been affected by Avian Influenza Viruses of zoonotic potential. As at June 2025, the **list of species** affected by H5Nx HPAI (so this includes all H5 variations) can be found at Global Avian Influenza Viruses with Zoonotic Potential situation update.

Overall updates via FAO with relevant global **maps**, and regularly updated, can be found here.

Table 3.1 Incubation periods in humans of various respiratory infections, including influenza: The time between exposure to when infectious (in days). Note this is potentially before symptoms are seen (Lessler, 2009)

	Range	Central tendency
Adenovirus	4-8	6
Coronavirus		
Human (non-SARS)	2-5	3
SARS-associated	2-10	5
Influenza	1-4	2
Measles	8-14	10
Parainfluenza	2-6	4
Respiratory Syncytial Virus	3-7	5
Rhinovirus	2-4	2

Diagnostic Sampling Priorities

If sample collection is required during HPAI surveillance, you will be advised on appropriate collection and laboratory submission protocols.

- A primary diagnosis of avian influenza is usually via qPCR testing of oropharyngeal and cloacal swabs. Using plain sterile swabs, collect samples individually from the cloaca and oropharynx and place in tubes containing viral transport media.
- You may also be advised to collect a range of post mortem tissues, or to submit the whole carcass to the laboratory.
- All samples and carcasses must be stored at 4oC prior to submission.
- WOAH example: Practical guide for authorised field responders to HPAI outbreaks in Marine Mammals

One Health Coordination and communication

Review the following document on the do's and do nots for good scientific communication regarding HPAI and wildlife

TASK: Reflect on how and why these suggestions may or may not work where you live. How would you modify them and why?

More information and resources on HPAI and wildlife:

General HPAI advice and information from WHA- Fact Sheet on AIV and Australian wild birds.

H5 bird flu and human health

The interim Australian Centre for Disease Control has information on bird flu, the situation in Australia, international travel tips and why getting your annual flu vaccine is important on their website

Communicable Diseases Network Australia (CDNA) - National guidelines for avian influenza: protecting people who work with birds and wildlife

Public Health Association of Australia webinar - What individuals need to know to prepare for the likely arrival of HPAI H5N1 in Australia from a One Health perspective

WHO guidelines on influenza in animals - Public health resource pack for countries experiencing outbreaks of influenza in animals (March 2024)

World Organisation for Animal Health & IUCN SSC Wildlife Health Specialist Group - Avian Influenza and Wildlife: Risk management for people working with wild birds

Links to an external site.

International HPAI guidelines from government agencies

World Organisation for Animal Health (WOAH) General resources on avian influenza; Considerations for emergency vaccination of wild birds against HPAI in specific situations; Practical guide for authorised field responders to HPAI outbreaks in marine mammals, with a focus on biosecurity, sample collection for virus detection and carcass disposal

WOAH & IUCN SSC Wildlife Health Specialist Group - Avian Influenza and Wildlife: Risk management for people working with wild birds

Agreement on the Conservation of Albatrosses and Petrels - Guidelines for working with albatrosses and petrels during the HPAI H5N1 panzootic

Convention on the Conservation of Migratory Species of Wild Animals Conference of the Parties - Resolution 14.18: Avian Influenza

FAO - Wild bird HPAI surveillance sample collection manual

Journal article - The risk of highly pathogenic avian influenza in the Southern Odean: a practical guide for operators and scientists interacting with wildlife (Dewar 2023)

Ramsar Convention on Wetlands

Wetland Disease Manual Guidelines for Assessment, Monitoring and Management of Animal Disease in Wetlands

Handbook 4: Avian influenza and wetlands - Guidance on control of and responses to highly pathogenis avian influenza

Biosafety Vs Biosecurity

- Definitions and distinctions
- Prevention Through Design and Hierarchy of Controls
- Risk-based systems mapping for field operations

Core Concepts

- **Biosafety**: deals with the accidental or unintentional release of biological agents.Biosafety focuses on biological risks within laboratory environments, while biosecurity addresses biological risks associated with non-laboratory environments.
- **Biosecurity**: focuses on preventing the intentional or negligent release of biological materials or the acquisition of dangerous knowledge and technologies. It is the way we stop the introduction and spread of harmful organisms such as viruses, bacteria, animals, plants, pathogens and insects into our country and how we manage the impacts of those already here.
- Systems mapping to pinpoint transmission nodes

The general public uses these terms interchangeably, but there are important differences that matter in a professional setting.

Biosafety has varying definitions depending upon the context, but a good general definition is, ***"methods that are used to stop a biohazard from spreading out of a given place or causing harm"*** (Cambridge dictionary). In this context a **biohazard** is something such as a disease or chemical that may harm people, animals, or the environment (Cambridge dictionary). The Australian Department of Health describes **biosecurity** as all the measures taken to minimise the risk of infectious diseases caused by viruses, bacteria or other microorganisms entering, emerging, establishing or spreading in a defined area (country, city, laboratory, building, etc.), potentially harming human or animal populations, food security and economy (see here for an Australian example). Sometimes, the two are used differently geopolitically, in which case **biosafety** aims to protect public health and the environment from accidental exposure to biological agents, and **biosecurity** deals with the prevention of misuse through loss, theft, diversion or intentional release of pathogens, toxins and any other biological materials. Recently, biosecurity has merged into the UN/WHO Global Health Security Agenda as a discipline, or specialty, creating some confusion.

A quick review of biosafety levels (BSLs)

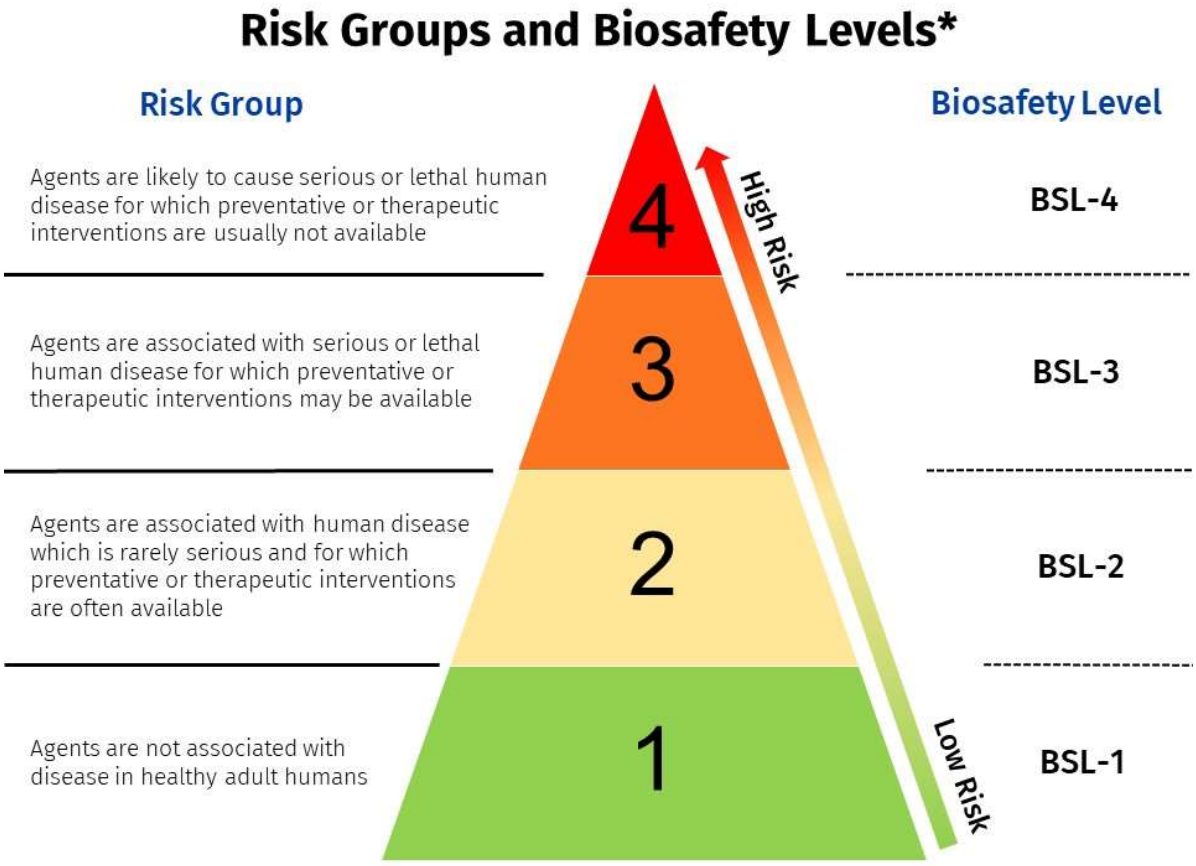

Figure 3.2 Risk groups and Biosafety Levels

Risk Groups and Biosafety Levels (Figure 3.2):

- **Risk Group 1 (RG1) / Biosafety Level 1 (BSL-1):**
    - **Risk Group:** Agents are not associated with disease in healthy adult humans.
    - **Biosafety Level:** BSL-1 labs are suitable for work with agents that pose minimal potential hazard to laboratory personnel and the environment.
- **Risk Group 2 (RG2) / Biosafety Level 2 (BSL-2):**

    - **Risk Group:** Agents are associated with human disease which is rarely serious and for which preventative or therapeutic interventions are often available.
    - **Biosafety Level:** BSL-2 labs are for work involving agents that pose moderate hazards to personnel and the environment, requiring specific practices and equipment.
- **Risk Group 3 (RG3) / Biosafety Level 3 (BSL-3):**
    - **Risk Group:** Agents are associated with serious or lethal human disease for which preventative or therapeutic interventions may be available.
    - **Biosafety Level:** BSL-3 labs are used for work with indigenous or exotic agents that may cause serious or potentially lethal disease through inhalation.
- **Risk Group 4 (RG4) / Biosafety Level 4 (BSL-4):**
    - **Risk Group:** Agents are likely to cause serious or lethal human disease for which preventative or therapeutic interventions are usually not available.
    - **Biosafety Level:** BSL-4 labs are designed for diagnostic work and research on dangerous and exotic agents that pose a high risk of aerosol-transmitted infections and fatal disease, often without available vaccines or treatments.

Note that WHO/ WOAH recommends a minimum BSL-3 for H5N1 testing.

Follow this link to the US-CDC Quick Learn Lesson of BSL's to help you to recognise different biosafety levels.

The following video focuses on BSL-3 - in particular the N95 mask and its use. Further information on specific face coverings is found later in the module here.

Biosafety Technical Page - respiratory infections at the human wildlife interface. Focus: SARS CoV-2 and HPAI

*Note: These notes were originally produced for the Pan African Sanctuary Alliance by a team that Steve Unwin was a part of and that **included** Dominic Travis and others (University of Minnesota) and Lynne Gaffikin (Stanford) . Steve has reviewed and modified for here.*

Executive Summary: Respiratory infections at the human-wildlife interface

The purpose of this technical page is to introduce the concept of biosafety, how to implement biosafety practices at the wildlife-human interface in “range country captive centers,” settings, (hereafter referred to as centers). It was originally conceived to introduce how COVID-19 impacts and challenges effective and safe biosafety practices in these centers. We have updated it here to include examples on HPAI and other pathogens to

expand the remit to free-ranging populations as well. We also hope you will find useful information that is relevant to all zoonotic respiratory disease situations. **Our goal is for information provided here to promote human and animal health and safety.**

Introduction of Terms: Here we discuss biosafety as a professional term, and how biosafety and biosecurity compare as professional practices. Both biosafety and biosecurity are focused on the ideas of prevention and control of health hazards (in this case infectious diseases). There are many strategies to prevent or control the spread of an infectious disease. These have been described holistically within the "Hierarchy of Controls" framework, created by the United States Centers for Disease Control and Prevention (CDC) and United States National Institute of Occupational Safety and Health (NIOSH). An overall approach to designing a system with the above in mind is called "Prevention Through Design", and involves a systems, risk-based approach to building all levels of the hierarchy into a safety plan for any workplace.

Risk-Based Approaches to Biosafety: We also introduce risk analysis, and how understanding risk influences biosafety. The field of Infectious Disease Risk Analysis was created to support decision making in the face of uncertainty. In the case of emerging infectious diseases, the lack of information (and resulting high degree of uncertainty) can make disease management confusing and frustrating. The COVID-19 pandemic was a good example of the need for a risk-based approach to support prevention and control efforts. Systems mapping can also highlight areas of concern for disease transmission, followed by analysis of how those critical points in the system can be managed to mitigate the risk disease spread.

Designing a Risk-Based Biosafety Program for Prevention and Control of infectious respiratory disease at the Human/Wildlife Interface: Uniquely complex settings, such as wildlife centers, involve specific risks associated with human-animal interactions and subsequent disease transmission. Therefore, characterizing risk in this setting can be challenging. Exposure pathways at the human-animal interface can be classified as very high, high, medium, or lower risk, all based on "likelihood of exposure." To help effectively prevent or reduce disease transmission, it is important to understand the transmission routes of respiratory infections.

Core Biosafety Measures: This section of the technical document is focused on understanding and implementing the Hierarchy of Controls. Once all the risks and critical points of disease transmission have been identified using the systems mapping and risk-based approach to biosafety, prevention and control measures can be created and implemented in the workplace. The control and prevention methods discussed herein specifically concern COVID-19 (but are useful for any highly transmissible zoonotic disease), and how to keep both humans and animals safe as they navigate their work/living environment. Some points of discussion (within the so-called hierarchy of controls) include, elimination controls, engineering controls, administrative control methods, and personal protective equipment (PPE).

PPE Training Program: In this section, we will provide a link to an online training resource that dives deeper into COVID-19, HPAI, biosafety, and working closely with animals on a day-to-day basis. Completion of this training will help ensure your understanding of facts about biosafety with respiratory pathogens, as well as provide the skills and knowledge to implement occupational health and safety best-practices to avoid infection with SARS-CoV-2 and other zoonotic respiratory infections. Competency in biosafety and clinical practices is important in reducing the incidence of disease in situations in which humans and wildlife frequently interact with one another very closely.

In summary, this technical document provides information on biosafety and zoonotic respiratory infections. Herein, we provide information and resources to practice system mapping according to risk level, and how to implement prevention and control methods to limit the spread of disease. We also plan to provide a training tool that anyone working with animals can use to improve their skills and better understand best practices in maintaining both human and animal health in a work environment.

Introduction to Biosafety and Biosecurity

Both biosafety and biosecurity are focused on the ideas of prevention and control of health hazards (in this case infectious diseases). **Prevention** can be defined as the precautionary measures implemented to halt or prevent disease transmission or prevalence in a population. Prevention strategies, such as vaccinations, prevent infection from other individuals or the environment and are used to reduce the spread of an incoming risk. **Control** can be defined as the reduction of disease incidence, prevalence, morbidity or mortality to a locally acceptable through deliberate efforts (Centers for Disease Control and Prevention, accessed 11/08/2025). Control strategies, such as treatment for a specific pathogen or disease, are used to reduce the spread of disease from an infected individual to a non-infected individual. The use of diagnostic tests that identify specific pathogens or infectious agents, help provide evidence on how well your prevention and control strategies are working.

There are many strategies to prevent or control the spread of an infectious disease. These have been described holistically within the “Hierarchy of Controls” framework below (Figure 3.3).

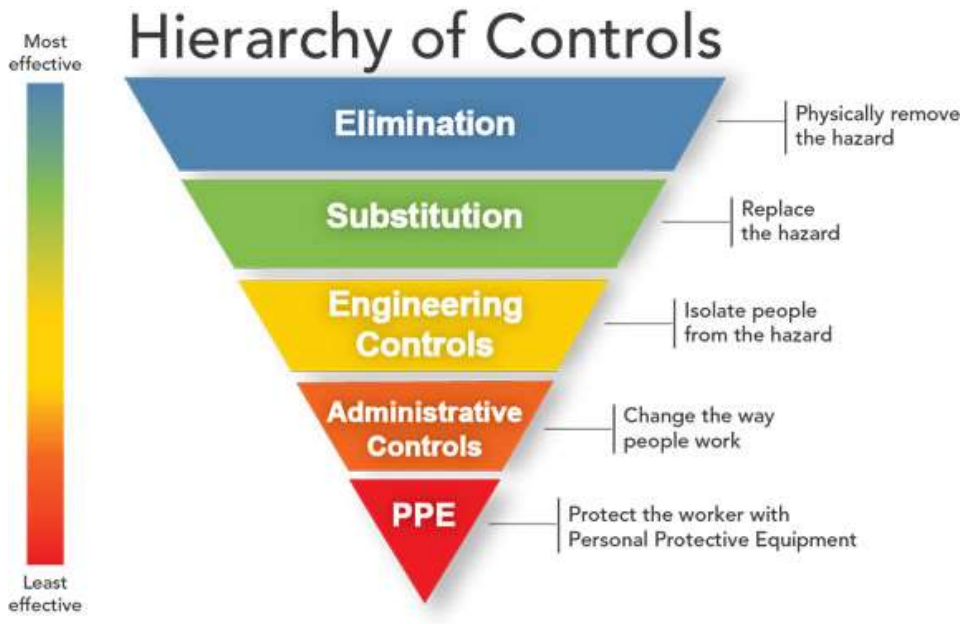

Figure 3.3 CDC/NIOSH Hierarchy of Controls.

Source: accessed August 11 2025

This model, created by the U.S. Centers for Disease Control and Prevention (CDC) and the U.S. National Institute for Occupational Safety and Health (NIOSH), is a system used to deploy effective controls (risk management) within an organization, workplace, or community to identify the most effective ways to control a hazard (Source accessed August 11 2025). The control methods at the top (wide part of the pyramid) are generally considered to be the most effective, while those at the bottom (tip of the pyramid) are the least effective when it comes to reducing risk. Specific examples of risk reduction measures in each category include:

- **Elimination:** proper disinfection (chemical, application method, concentration and time) to achieve elimination of infectious agents.
- **Substitution:** this category often applies more to the use of hazardous chemicals (often for disinfection) that may pose toxic threats. In these cases, substituting less risky chemicals (or treatments for instance) can decrease overall occupational risk. The use of bleach versus other potential cleaning methods for food safety in a sanctuary setting is an example often discussed.
- **Engineering Controls**: animal exhibit, or clinic/hospital, design is an example of specific attempts to create a safer human-animal interface in a sanctuary setting.
- **Administrative Controls:** these methods are focused on HOW people work within the engineered system above to minimize the spread of disease through adjusted workflow. Some examples include differentiating between the “dirty” and “clean” parts of a lab, clinic or animal enclosure; the order in which a person dons and takes off PPE; how animal food is delivered, cleaned, stored and prepped are all examples of adjusting workflow.

- **Personal Protective Equipment (PPE)**: this includes techniques for creating physical barriers to the release of a disease agent from one individual or blocking an agent's ability to find a portal of entry into a susceptible individual. Examples include wearing gloves, face masks, face shields, or safety goggles when working in an environment that favors or enhances disease transmission.

An overall approach to designing a system with the above in mind is called “Prevention Through Design”, and involves a systems, risk-based approach to building all of the above into a safety plan for any workplace (Source: Centres for Disease Control and Prevention) - this includes both infectious and non-infectious hazards like chemicals, toxins, etc. The specifics of this program may be overkill for a sanctuary/center or field setting (eg. they are often designed for larger hospitals, factories, or office buildings), but the principles and “design thinking” are relevant to any type of work and may be taught and practiced to increase the effectiveness of disease risk management strategies at the human-animal interface.

Risk Based Approaches to Biosafety

The field of Infectious Disease Risk Analysis was created to support decision making in the face of uncertainty. The risk analysis approach is a set of methods that support “prevention through design,” and involves following a structured process that allows for better characterization (assess) to prevent introduction and then reduction (control) of disease risks within a given system if introduction can’t be prevented. It also allows us to identify areas of greatest uncertainty for research. When designing biosafety protocols it is important to first understand and accept that disease transmission may be adequately controlled and prevented, but the risk is never completely controlled under the best of circumstances (ie. there is no such thing as zero risk).

In the case of emerging infectious diseases, the lack of available information (and resulting high degree of uncertainty) makes disease management confusing and frustrating - especially when compounded by fear. The COVID-19 pandemic, with its vast degree of complexity and uncertainty, is a great example of the need for a risk based approach to support prevention and control efforts. The urgency of this need increased due to:

1. The spread of SARS-CoV-2 from animal to human populations (Please see the study, linked here, for an example of SARS-CoV-2 transmission from human to animal populations, and then spilllover back to humans from infected animals) and
2. The identification of a broad array of potential susceptible/competent mammalian hosts for SARS-CoV-2 greatly increases the number of potential transmission pathways.
3. The current lack of preventative measures and fully validated diagnostics in animals

Using the information shared on SARS CoV-2 and H5-bird flu, outline a risk -based approach to the management of a HPAI outbreak, see the following assignments:

Mapping the risk

Contingency planning for a respiratory disease outbreak

The first step in implementing a risk-based approach is examining the diseases, or "hazards," of greatest concern in the context of the problem. This step is known as "hazard identification" in the jargon of Risk Analysis. In the case of human-nonhuman primate interfaces, we are often concerned with those that can be transmitted between humans (human-human transmission), animals (animal-animal transmission), and humans and animals (zoonotic/anthropozoonotic transmission). This technical page is intended to model the development of a risk-based approach for prevention of an infectious disease, such as COVID-19 or HPAI, in the conservation workplace (wildlife sanctuary or in situ field site), and how to select and implement biosafety practices based on that system. COVID -19 and HPAI are good examples because they are both currently a high priority, and because they are relevant to all the interfaces discussed above. After hazards are identified (ie. COVID-19, HPAI), the next step involves thinking holistically about how disease may enter and spread through the system, as well as the likelihood and consequences of disease entry and spread. Once this "baseline risk" is established, prevention and control strategies for risk management can be discussed and assessed.

Systems Mapping for Biosafety Planning

Good, comprehensive biosafety programs are best designed and implemented by viewing the workplace, whether a physical structure or a forest, as a system. Biosafety tools are then designed to reduce disease transmission risk by blocking the ability of a virus (in this case) to successfully move along a given disease transmission route/pathway. The routes and risks differ between systems, so first the systems in which individuals at risk are working need to be identified and described. For example, a home, shop, school, village, city, wildlife park, or sanctuary can be seen as a system. Many of these can be combined in to a larger dynamic system (eg. a person traveling from home to work and back in a day could be defined as a daily system). Usually, the system is mapped through the creation of a conceptual diagram. The figure 3.4 below provides an example.

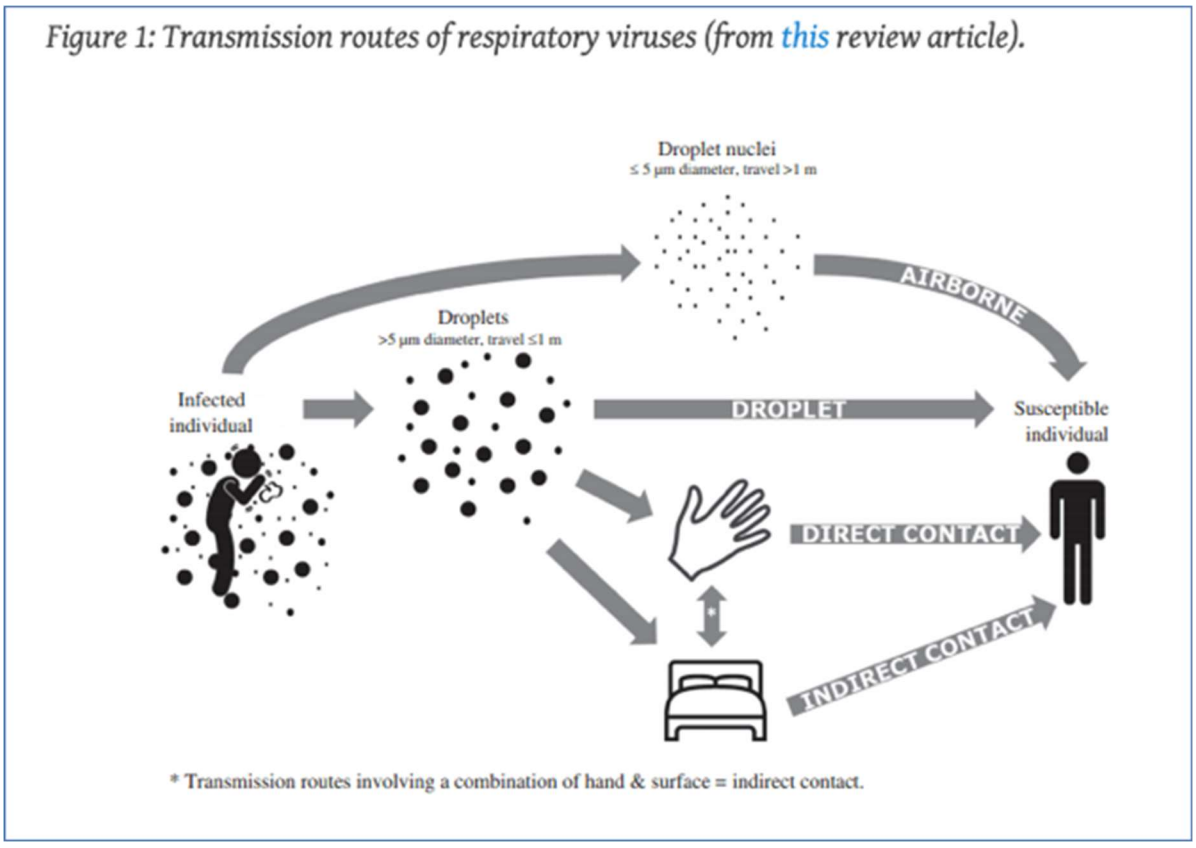

Figure 3.4 Crude systems diagram mapping pathways for human-human transmission of respiratory viruses. Source for this graphic

Once the system in question is defined and mapped, the identification of critical points that enable disease introduction and spread can begin. Identification of these critical disease transmission points allows for the planning and implementation of risk reduction biosafety measures, such as physical distancing or the use of appropriate PPE. It can be useful to refer to the hierarchy of controls again at this stage. After selecting best tools, execution of biosafety measures consists of three parts: basic knowledge (what PPE to use), application (how to properly use PPE), and operationalization (proper and consistent implementation under working conditions). The figure below (Figure 3.5) illustrates how systems mapping can be done to highlight areas of concern for disease transmission, and then how those critical points in the system can be handled to mitigate the spread of disease

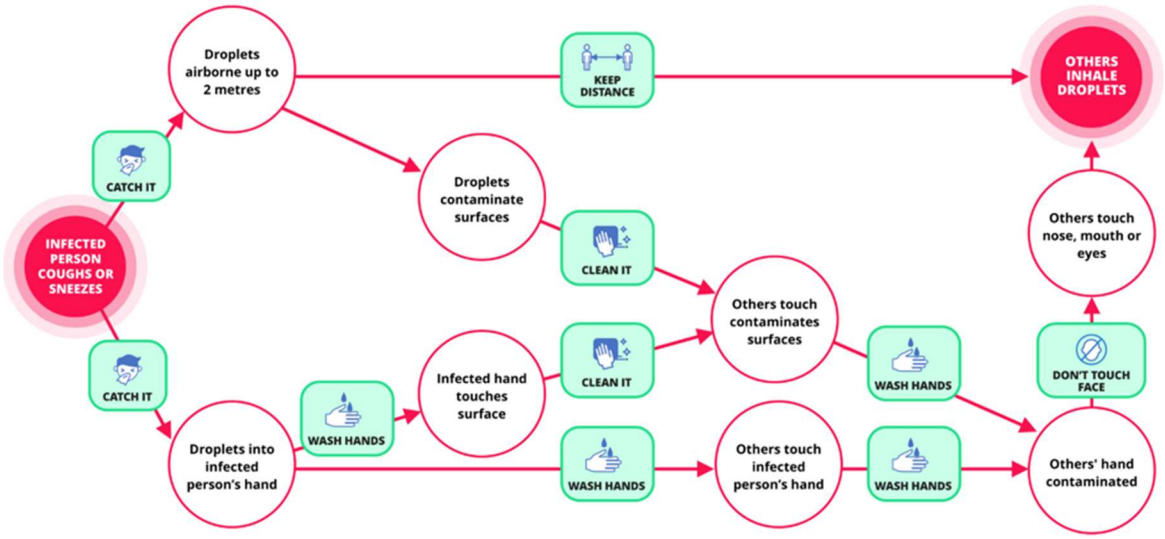

Figure 3.5 Systems diagram of respiratory transmission including control/risk management procedures, highlighting covering coughs and washing hands. (Source Website)

Biosafety failure can occur due to uncontrollable circumstances, such as faulty equipment, but more often it is as a result of inadequate selection, planning, or improper/inconsistent use and implementation. Thus, communication (planning, training or implementation) with all stakeholders is key for both design and implementation planning. Proper stakeholder involvement includes inclusion of all those who influence or are affected by the system such as managers, health professionals, animal care staff, field biologists, local public health authorities. For more information on who/what stakeholders can be and how to best involve them in your organization's activities, please see the following PDF(link) created by the Centers for Disease Control and Prevention.

EXAMPLE: Designing a Risk-based Biosafety Program for Prevention and Control of Covid-19 at the Great Ape-Human Interface (Task for participants: can you adapt to HPAI?)

See the 'Mapping the Risk' assignment: Use this material as background for an assignment for designing a Risk--Based Biosafety Program for Prevention and Control of HPAI at the human-wildlife interface.

A. Systems Mapping for Great Ape Sanctuaries and In Situ Conservation Sites

This resource is concerned with biosafety as it pertains to managing risks associated with transmission of SARS-CoV-2 in great ape sanctuaries or field conservation settings. This setting is uniquely complex since transmission may occur at human-human, animal-animal, or human-animal interfaces (in both directions multiple times). In sanctuary settings, the

risk of transmission between and among the differing human populations (conservation workers, tourists, worker families, other local people) - and NHPs - creates the potential for local wildlife reservoirs and spillback to humans. In in-Situ conservation settings, there are additional dynamics of human interaction both within (researchers, tourists and park workers) and along park boundaries (local inhabitants/villages, extractive industries, agriculture, etc.). The complexity of these systems is represented in figure below (Figure 3.6).

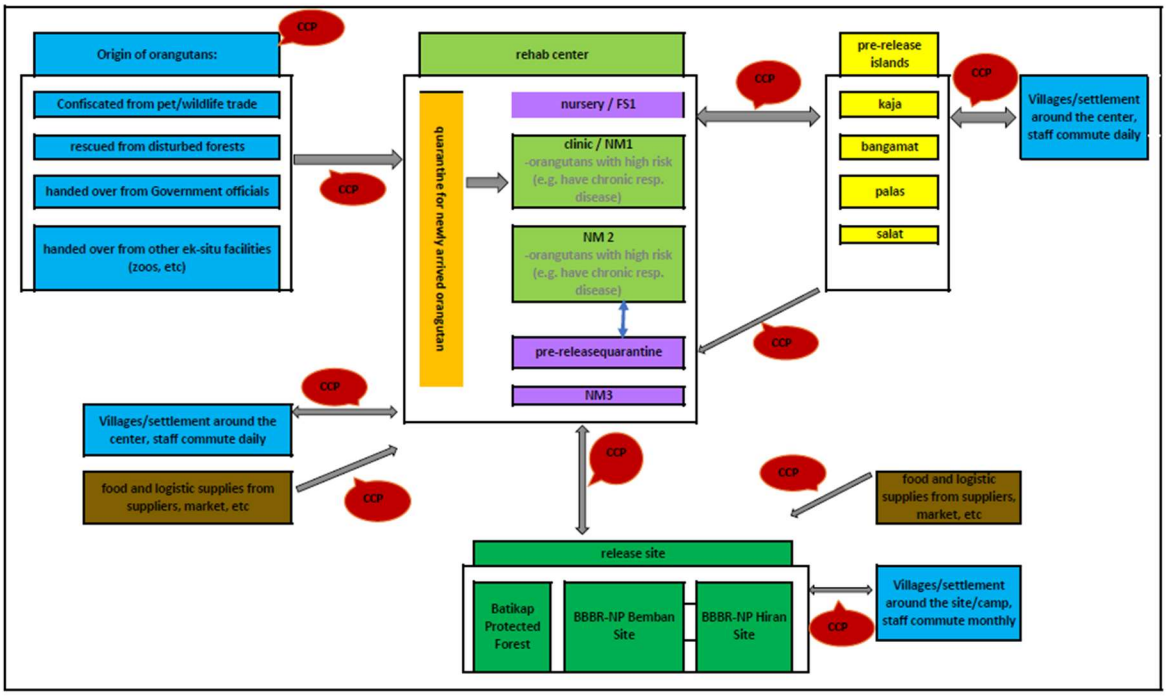

Figure 3.6 A systems diagram of the human-human-great ape interface in a sanctuary setting in response to SARS CoV2 (Author: Sulistyo 2020, Used with permission from BOSF)Nyaru Menteng Orangutan centre, Central Kalimantan

At this point, one should have a) mapped the workflow of the system, b) identified areas of greatest exposure between humans and animals, and c) understand the context of important potential routes of transmission. Figure above is recopied here and is a good example of implementing several types of biosafety controls such as face coverings, physical distancing, disinfection and hand washing.

This model (Figure 3.5) can , and should, be applied to COVID-19 at the sanctuary level to ensure the health and safety of both humans and animals and reduce the risk of SARS-CoV-2 transmission between the two populations. However, it is important to understand that some of the most effective prevention strategies, such as elimination and substitution, may be challenging to implement in places where COVID-19 is already present.

B. Characterizing the Risk of Differing Human Interfaces: What is a risky job?

As shown in the diagrams above, one must consider several different categories of humans when considering the sanctuary or in situ great ape interface. For transmission to occur, many things have to "go right" for the virus:

1. The infectious agent must be present - there must be an infected human who is infectious (regardless of the presence of clinical signs);
2. There must be exposure between infectious and susceptible individuals - the infected human must be in the presence of a susceptible animal (by human transmission standards "competent exposure" means < 2 m for > 15 min);
3. Virus must actually enter the susceptible individual - The virus must be expelled from the host and received on the mucous membranes (directly or indirectly) of the susceptible animal, and
4. The susceptible animal must be infected, which depends upon factors specific to that individual animal (dose, immune status, concurrent disease, etc.)

The alternative pathway includes indirect transmission via contact with a contaminated part of one's environment. The biosafety measures covered in this module assume that points 1, 3 and 4 may happen, and focus on risk reduction measures for point 2 above.

As you can see in the Figures not all human-animal exposure pathways are the same. Exposure differs greatly in spatial proximity (distance), frequency, and length of time under differing conditions. These interfaces are generically described in terms of human-ape transmission potential below (Table 3.1).

Table 3.1 Risk-based approach to ranking the human-NHP interface in terms of exposure risk for sanctuaries and in situ conservation sites.

Human Category	**Proximity**	**Frequency**	**Time**	**Personal Exposure**	**Risk**
Conservation Worker (Park or Sanctuary)	High - intentionally directly engage the human-ape interface	High - daily	Med-High - depends upon job	Local or Regional	High

Tourist	Med-High - intentionally directly engage the human-ape interface	Low - one or few times	~1 hour - time limited	Local, Regional and Global	Medium
Researcher	High - intentionally directly engage the human-ape interface	High - daily	High - usually all day	Local, Regional and Global	Very High
Local Community Member	Low - (indirect exposure)	Low	Low	Local, Regional	Low (unless cohabitate with one of above)
Local worker (extractive industry, day labor, food supply, etc.)	Medium - (likely not intentional, but close exposure does occur, especially through contaminated waste)	Low-Med	Low-Med	Local, Regional and Global	Medium

Human-animal interface exposure pathways can be classified as very high, high, medium, or lower risk. All of these are based upon the idea of "likelihood of exposure" between humans and apes in this example. A "high risk" exposure is currently commonly defined as a) proximity within 2 m for a total of 15 min or more with an infectious individual, or b) having contact with the infectious secretions from a patient with COVID-19 or contaminated patient

care environment, without using proper PPE or not performing appropriate hand hygiene (US Centers for Disease Control and Prevention).

The factors in the model above used to rank risk include:

1. **Proximity** of humans to apes: what is the likelihood that this category of humans will have a high risk exposure to a susceptible ape, or vice versa?
2. **Frequency:** how often will this category of humans have a potentially high risk exposure to a susceptible ape, or vice versa?
3. **Time:** is the exposure of this category of humans to apes (or vice versa) likely to be high risk (>15 min)?
4. **Personal exposure:** what is the cumulative personal exposure of the category of humans potentially exposed to a susceptible ape, or vice versa? This is categorized into local, regional (regardless of international borders), and global definitions.

The above rankings are built from the United States Occupational Safety and Health Administration Occupational Risk Pyramid, which is similar to those implemented by the European Union and WHO. For more information please follow this link: Safety and Health Topics | COVID-19 - Hazard Recognition

It is important to note that risk levels are NOT static, but dynamic, meaning they can change. These categories (or others that fit an individual situation better) will need to be regularly reassessed in order to ensure that you are practicing the best biosafety procedures according to your locally assessed risk level.

C. Transmission and Ecology - SARS-CoV-2

To most effectively prevent and reduce disease transmission, it is necessary to understand the transmission routes of SARS-CoV-2. An overview of disease transmission routes in general can be viewed by following this link to National Geographic Methods of Disease Transmission

It has been confirmed that SARS-CoV-2 may spread by both direct and indirect transmission pathways. The fecal-oral route of transmission for SARS-CoV-2 has not been confirmed, but the virus has been detected in feces from COVID-19 patients (Source: NCBI).

The evidence base for aerosol transmission of SARS-CoV-2 is increasing (World Health Organization updating page on aerosol transmission of SARS-CoV-2: Source: WHO), and may change the current working definition for exposure in high-risk scenarios. The diagram below characterizes high risk transmission routes of zoonotic **coronaviruses**, including SARS-CoV-2, but is limited mostly to the "spillover" story (from wildlife to humans; zoonosis) and unfortunately does not include the larger ecological picture of potential risk of anthropozoonosis. This is highlighted in the figure below (Figure 3.7).

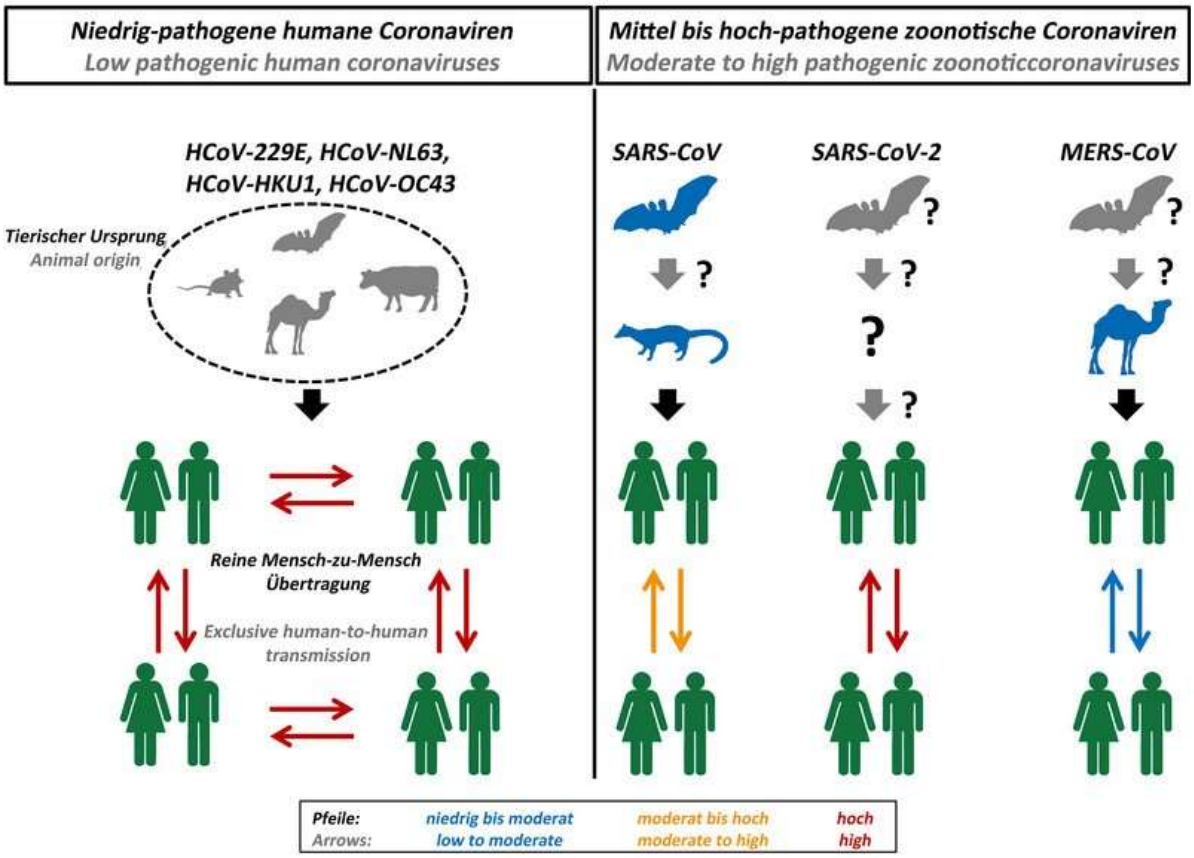

Figure 3.7 Conceptual Diagram of Coronavirus Spillover and Amplification

Source for image

A more comprehensive look at potential transmission expands the story to include anthropozoonotic (spill back or reverse zoonosis) transmission risk. The “spillback” infection of domestic pets and or the large felids from owners or zoo keepers exemplifies this complex potential risk. An evidence based approach based on current molecular epidemiological methods expands the potential ecology of susceptibles (Figure 3.8).

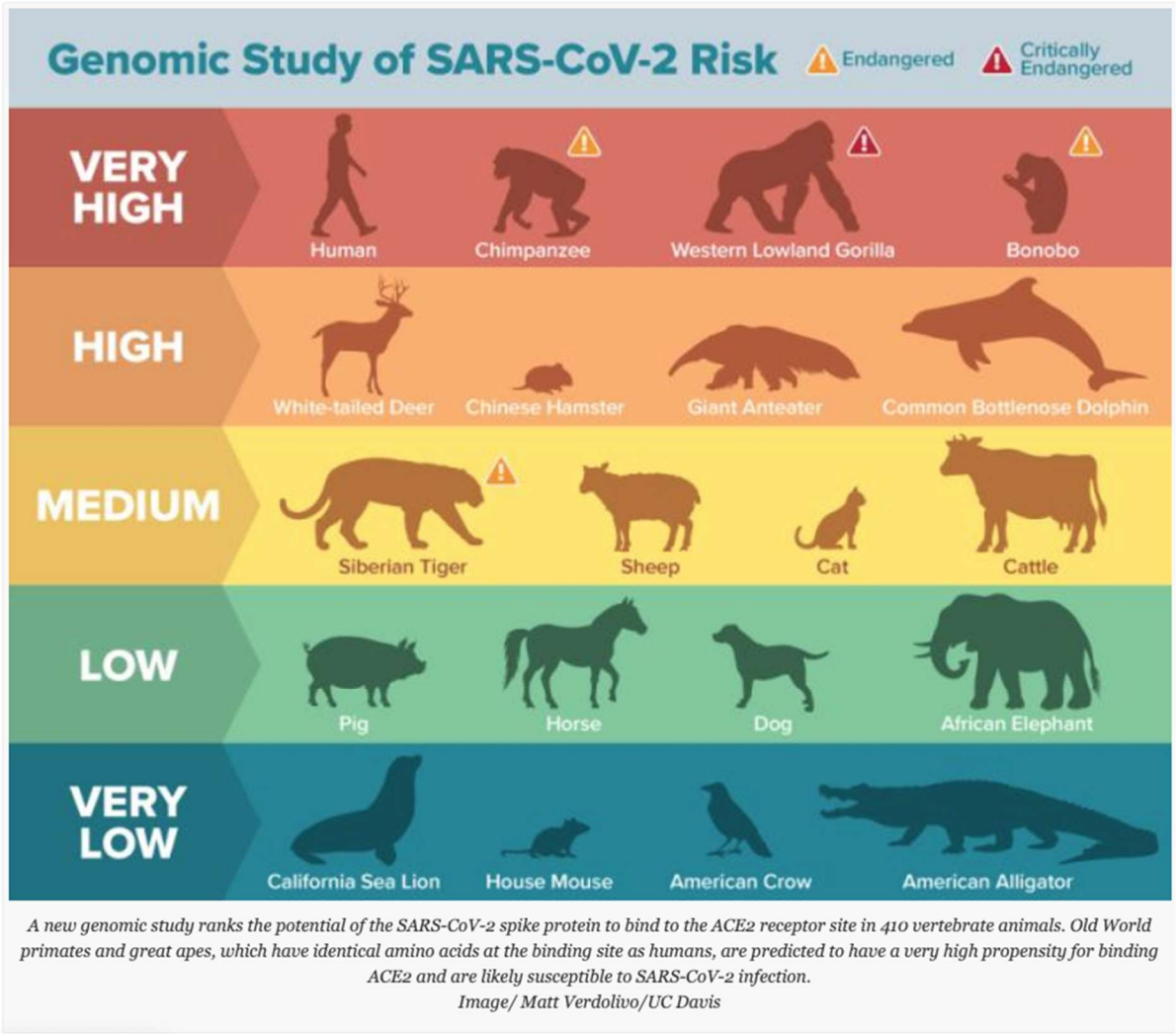

Figure 3.8 Ranking of host susceptibility "risk of being infected" based upon genomic evidence (Damas et al)

Core Biosafety Measures

This section reviews biosafety measures commonly employed using the hierarchy of controls model

A. Elimination

Disinfection: Disinfection is an important process in reducing or preventing the presence of infectious diseases in the environment, especially in high risk workspaces with increased human and animal interactions. A disinfectant is a chemical agent that is used especially on hard surfaces and in water (such as drinking water or wastewater) to destroy, inactivate, or

significantly reduce the concentration of pathogens (Source: Meriam-Webster Dictionary Accessed 11th August 2025).

Disinfectants kill certain bacteria, viruses, mildews, or fungi, and they are used on non-living things, such as stall floors, trailers, buckets, and door handles, to kill the microorganisms living on that inanimate surface. Most disinfectants have a label provided by the manufacturer that communicates the chemical composition of the disinfectant and the known harmful effects of any hazardous substances within the disinfectant. These differ from other cleaning products, such as cleaners or antiseptics. Cleaners do not kill microorganisms, but are important to use with disinfectants because they remove dirt from objects and surfaces. Removing dirt is very important because some disinfectants, such as bleach, are inactivated by organic (dirt) material. Antiseptics are used on living organisms, such as human skin, to kill any microorganisms living on the body's surface.

It is highly important that you read the labels on all cleaning and disinfectant products prior to their use. Never mix ammonia and bleach products, as this combination produces a toxic gas that can be fatal when inhaled.

For guidance on disinfecting or cleaning work or personal spaces, please see this resource by the United States Centers for Disease Control and Prevention: Link to resource

The United States Environmental Protection Agency (EPA) has also compiled a list of approved cleaning and disinfecting products for use against the Influenza A viruses, including Avian influenza here: Link to resource

B. Engineering Controls

Exhibit and Facility Design

Engineering controls are favored over administrative and PPE controls because they are used to remove the hazard (in this case, the SARS-CoV-2 virus) from the environment. These control methods are often more difficult to implement, but are the most effective, long-term protective measures. Exhibit and facility design directly impact workflow, and can be manipulated as an engineering control method to enhance or improve biosafety practices at the human-nonhuman primate interface. Enclosure design and workflow can be assessed using a risk-based analysis that identifies the critical points in the enclosure that allow for human-animal disease transmission. Additionally, critical points of disease transmission also exist and need to be identified in procedures involving enclosure design, such as the cleaning of and maintenance of enclosures, that can be changed as the enclosure itself is altered.

Human-animal interactions at the level of the animal's enclosure poses a risk to both human and animal health. According to the European Association for Zoos and Aquariums, biosafety is key during the cleaning of animal enclosures and equipment within them, to reduce the risk of disease or disease transfer (Source: EAZA Standards for the Welfare, Accomodation and Management of animals in zoos and Aquaria Accessed 11th August 2025).

As previously stated, there is risk for transmission of both SARS-CoV-2 and HPAIas an aerosol or droplet. This mode of transmission is more likely to occur when performing

aerosol/droplet-generating procedures, such as cleaning animal enclosures, and the virus is present in the environment or animal or human bodily fluids/secretions.

However, the exhibit and facility design can be manipulated to decrease the risk of disease transmission. For more detailed information on designing low-risk enclosures and facility settings, please refer to the following sources:

1. World Association of Zoos and Aquariums: Zoolex
2. Biosecurity and Management of wildlife (Wildlife Health Australia webpage linking to various wildlife-focused biosecurity resources)

C. Administrative Controls

Physical/Social Distancing

Physical distancing, also commonly referred to as social distancing, is a biosafety administrative control measure intended to limit your exposure to respiratory infections, in combination with the other risk management strategies described in this section. As previously described, SARS-CoV-2 and HPAI can spread when an infected individual is in close contact with a non-infected individual. Implementing physical distancing is a control measure that can reduce close contact, and therefore reduce the spread of the virus in a population. The United States Centers for Disease Control and Prevention includes both proximity (2 m) and time (>15 min) in the definition of a close contact (inferring high risk exposure) (Source Website, accessed August 11 2025). Designing protective measures must consider both time and proximity when implementing social distancing as a risk management strategy.

Physical distancing can be practiced both at home and at work. Maintaining a distance of at least 6 feet (2m) is extremely important if someone in your household has, or thinks they have, a respiratory infection. Try to avoid crowded or congested areas at work, and maintain a distance of at least 6 feet (2m) between yourself and others when possible, while also practicing proper hand hygiene and wearing the appropriate PPE to keep yourself and others safe.

Recent studies indicate that people who are infected and asymptomatic likely also play a role in the spread of COVID-19 in particular. Since people can spread the virus before they know they are sick, it is important to stay at least 6 feet away from others when possible, even if you—or they—do not have any symptoms. Physical distancing is only successful when it is done in combination with the other techniques mentioned here on this technical page. Please always be conscious of those around you, both at home and in public, and do your part in stopping the spread of COVID-19 and other respiratory infections.

D. Personal Protective Equipment (PPE)

Personal protective equipment, or PPE, **represents the last line of defense in the hierarchy of controls** for reducing or eliminating exposures to occupational hazards. It is the fundamental method of protecting workers from workplace hazards. These can include physical, chemical, biological, and radiation risks. PPE also protects against carrying chemical

or biological contaminants from one individual or site to another, or from the worker to other workers and animals directly. Requirements for wearing PPE are formulated through a risk-based approach. Once these requirements, based on perceived risks, are determined, you can design and implement management practices for proper PPE in the workplace to best reduce occupational risk exposures.

Due to the known transmission routes of SARS-CoV-2 and HPAI, respiratory protection is highly important in protecting individuals from COVID-19 and influenza and reducing disease incidence in the workplace. Facial personal protective equipment includes masks (surgical and other officially validated options), respirators, and face shields. As previously discussed, the factors that influence your PPE selection are anticipated workplace hazards (splash/spray v. aerosol v. touch), the durability of the PPE for the intended tasks, and the fit of the PPE. Availability and conservation of resources for high risk occupations is now an additional factor. PPE shortages will continue to affect nations and communities world-wide from time to time, alternative facial protection has been implemented and heavily debated in terms of efficacy and best practice for use. Cloth masks, or facial covers, **are not PPE**, but are now widely accepted, recommended, and used as a preventative measure to reduce the transmission of SARSCoV-2 (source protection, meaning that they are more likely to prevent release of the virus than prevent the wearer from inhaling the virus from the environment).

First, you need to know the difference between a face mask and respirator, which have differing levels of protection and different uses. **A respirator is** a tight-fitting, fit-tested and disposable filtration barrier that protects the wearer from aerosolized hazards and large droplets. N95 respirators are the PPE most often used to control airborne infection exposures, such as SARS-CoV-2, and their effectiveness is highly dependent upon proper fit and use (Refer to the Fit Test video for N95 masks on this page). N95 respirators are intended to be used once and then properly disposed of and replaced with a new N95 respirator. Respirators must be used in the context of a comprehensive, written respiratory protection program that meets the requirements of OSHA's Respiratory Protection standard. The program should include medical evaluations, training, and fit testing. There are many other types of respirators available for use in a variety of workplace environments. The following infographic is an example of many different types of respirators, other than an N95, and their different agricultural uses.

A (surgical) face mask is a loose-fitting, disposable physical barrier between the wearer and the environment that can protect the wearer from splashes and large droplets, but is primarily used to protect the patient or environment from the wearer's respiratory emissions. Face masks **do not** have filtering capabilities to protect the wearer from airborne contaminants. Face masks are produced and sold as PPE, with validated parameters and guidelines to be categorized as PPE. These are different from face covering (i.e. cloth face covers or homemade face covers), which do not qualify as PPE because they have not been validated as protective according to the same guidelines as face masks. Face covers were generally accepted as a resource to prevent the spread of SARS-CoV-2 within the parameters of the CoVID-19 pandemic, but it is important to recognize that they are **not PPE**. For definitions and detailed safe use of all face coverings, please refer to this page.

When choosing between wearing either a respirator or a surgical mask, you need to consider the hazards you are potentially going to encounter in the workplace. Respirators are intended to be used for respiratory protection against airborne chemical or infectious hazards, unlike surgical masks. Surgical masks do not have the filtering capability that respirators do, and they are designed to protect sterilized or disinfected medical devices, supplies, and patients during surgery from the wearer. So, respirators protect the wearer from incoming threats, and masks generally protect the environment from contamination by the user/wearer.

Face shields are durable forms of PPE that can be disinfected for multiple uses. Face shields are considered a form of PPE most useful for protection from droplets, and not aerosols. Because there is significant risk associated with COVID-19 in particular and droplet transfer, they may serve a critical, protective function to the eyes, ears, nose, and mouth. Face shields should not be used as a substitute for respiratory protection**,** but are most effective when used adjunctly to respiratory PPE. Additionally, face shields provide a protective barrier that prevents the wearer from touching their face and face mask/cover with potentially contaminated gloves or hands. They should always be worn in combination with a face mask/cover.

HPAI Biosecurity & personal protective equipment (PPE) key points

Review and implementation of appropriate biosecurity measures are recommended in veterinary hospitals, rehabilitation settings and in the field. For example, in Australia there are the National Wildlife Biosecurity Guidelines and AVA Guidelines for Veterinary Biosecurity.

• Avian influenza viruses can be transmitted between birds and to other animals via direct contact with respiratory secretions and faecal material, as well as indirect exposure to contaminated environments or objects (e.g. clothing, boots, equipment, etc.).

• Handling of birds or mammals suspected of being infected with HPAI should be conducted with appropriate PPE, including gloves, a facemask and eye protection.

• PPE should be removed properly to avoid self-contamination.

• PPE and other potentially contaminated equipment should be thoroughly cleaned after use, followed by disinfection, or disposed of appropriately.

• Particular attention should be given to hand washing after handling animals, after contact with potentially contaminated materials and after removal of gloves. Hands and arms should be washed with abundant soap and warm water, then dried thoroughly. Hand sanitizer (gel with 60 to 90% ethanol concentration) can be applied to reinforce disinfection but should not replace proper handwashing.

• Further advice on PPE and disinfection, directed to people currently working in locations impacted by HPAI, can be found in the WOAH avian influenza and wildlife risk management recommendations.

Review the following video on the correct field application for PPE. We will be conducting a practical on this during the workshop:

Fit Testing

Fit testing is required for employees wearing tight-fighting respirators, such as disposable or reusable respirators. This procedure helps ensure the user has selected the correct size respirator and that a seal against the face can be made in order to provide the expected protection.

From the manufacture 3M, a resource outlining the Fit Testing procedure (in English)

Review the following video (in Thai) on the Fit Test procedure

Occupational Hygiene and PPE Assignment

This assignment provides you with multiple examples for personal hygiene and basic PPE use when dealing with a respiratory disease.

Its aim is to provide you with the skills to use these skills correctly, AND to be able to impart them to others accurately. The best way is practise.

Following completion of this assignment you should:

- Be able to don and doff multiple mask types correctly
- Wash your hands effectively to minimise pathogen transfer
- Share this knowledge with others

TASKS

1. Review the video and pdf resources shared for this assignment below, and the relevant section of the introductory presentation. Practise the techniques.
2. Share video or report back your skills to the group
3. In 500 words or less, reflect how you can best impart this information to the staff at your facility.

RESOURCES: In Canvas

Technical face cover brief.

This material was originally utilised during the SARS CoV-2 pandemic for those working with Non Human Primates (with the same co-authors as the Covid-19 example above). It is

included here as a case study on PPE recommendations during a respiratory focused zoonotic pandemic (Table 3.2).

In Summary:

Table 3.2 Respiratory Routes of Disease Transmission

Droplet	**Airborne**
An infection that can be spread through close respiratory or mucous membrane contact with respiratory secretions	An infection that can be spread over long distances when suspended in the air
Particles are large and generally travel no more than 1 metre from the patient	Particles are very small (<5um), can also be transported in dust
E.G INFLUENZA, RSV, pertussis, rhinovirus (common cold)	E.G Chickenpox (varicella), measles, Mycobacterium tuberculosis complex
Requires standard precautions plus surgical mask	Requires standard precautions plus N95 mask

Source: www.cdc.gov

The pandemic spread of COVID-19 around the world caused serious and ongoing disruptions to worldwide supply chains for disposable surgical masks and N95 filtering facepiece respirators (FFRs), along with other forms of personal protective equipment (PPE).(Bauchner et al., 2020) Both surgical masks and N95 FFRs are commonly used during the observation and care of wildlife in a wide variety of settings, ranging from their native habitats, conservation or zoo areas, to veterinary and medical contexts worldwide.(Gilardi et., 2015; Grizzle and Fredenburgh, 2001) The standard use of these types of facial PPE serves to protect both the human workers and other species from each other's diseases, including tuberculosis, influenza, HIV, ebola, macacine herpesvirus 1, and COVID-19. Recent studies have shown that multiple species of wildlife including old world primates and wild felids can be experimentally infected with SARS-CoV-2, the virus that causes COVID-19, and show symptoms similar to those seen in humans, including fever and pneumonia.(Lu et al., 2020; Rockx et al., 2020; Shan et al., 2020). For HPAI, review the High Pathogenicity Avian Influenza and Wildlife: Advice for veterinarians and animal health professionals previously shared.

If disposable facial PPE are difficult to obtain during an outbreak, cloth and other alternative face covers may be used in many instances to preserve and supplement current surgical mask and N95 supplies, following the following guidelines.

Different types of facial PPE

Droplets are generally defined as pathogen-containing fluid particles >5 µm in diameter. Droplet size varies greatly based on the force and pressure of emission, environmental variables including temperature, humidity, and airflow, the time spent airborne, turbulence, and the size of the pathogen within a droplet. As droplets evaporate in the air, the remaining dried residue, or droplet nuclei, are often referred to as respiratory aerosols (<5 µm in diameter). While we often talk about droplets and aerosols as separate and different from each other, research shows that they are actually on a spectrum with each other. Droplets at the larger end of the spectrum (>5 µm in diameter) generally settle out of the air relatively quickly and close to their source (<1 m), while smaller aerosols (<5 µm in diameter) evaporate before settling, and the residues may remain airborne and infective for much longer and more variable periods of time.(Bourouiba, 2020) Different types of masks, including disposable surgical masks and N95 FFRs are designed as protection against different types of potential exposures, and serve different purposes, as illustrated in the following graphic from the CDC (Figure 3.9).

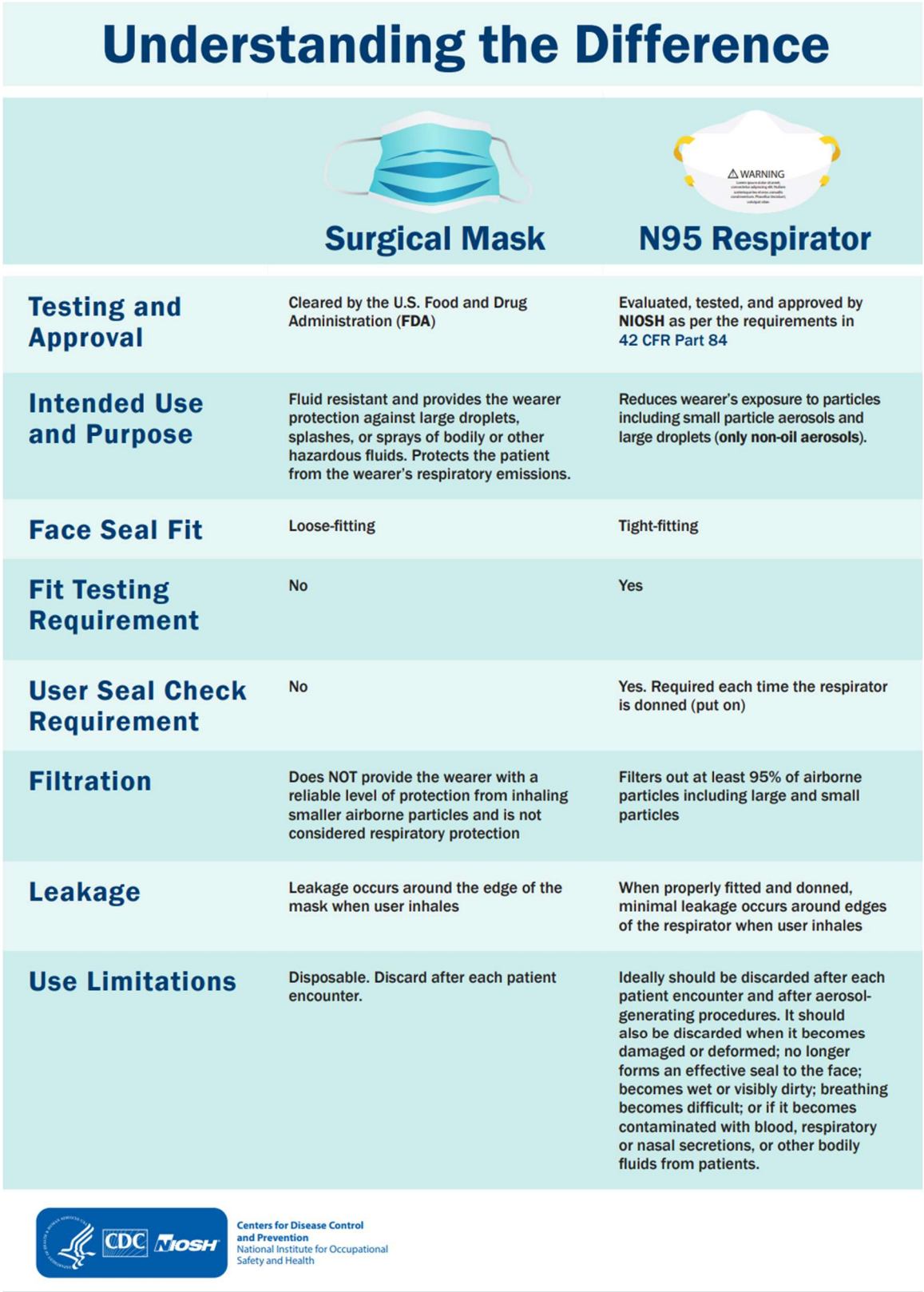

Figure 3.9 Infographic - Understanding the Difference, Surgical Mask, N95 Respirator

Following the CDC's hierarchy of controls (Figure 3.10), the most important steps to preserve existing supplies of facial PPE for use with wildlife, and to protect both wildlife and the people who care for them when a zoonotic disease is suspected or confirmed are to

1. Eliminate non-urgent interactions with wildlife and elective procedures performed on wildlife.
    1. Minimize or cancel tourist visits to conservation sites, sanctuaries, zoos
    2. Cancel or postpone vaccinations, dental cleanings, routine surveillance, etc.
    3. Minimize the people involved who would need to wear facial PPE.
2. Substitution is not really feasible for wildlife, other conservation, or other healthcare settings.
3. Engineering and administrative controls include
    1. Increased remote observation via cameras
    2. Increased distance of at least 10m when monitoring in the wild
    3. Preference to outdoor interactions over indoor interactions
4. Continue to use recommended PPE in remaining necessary interactions with wildlife.

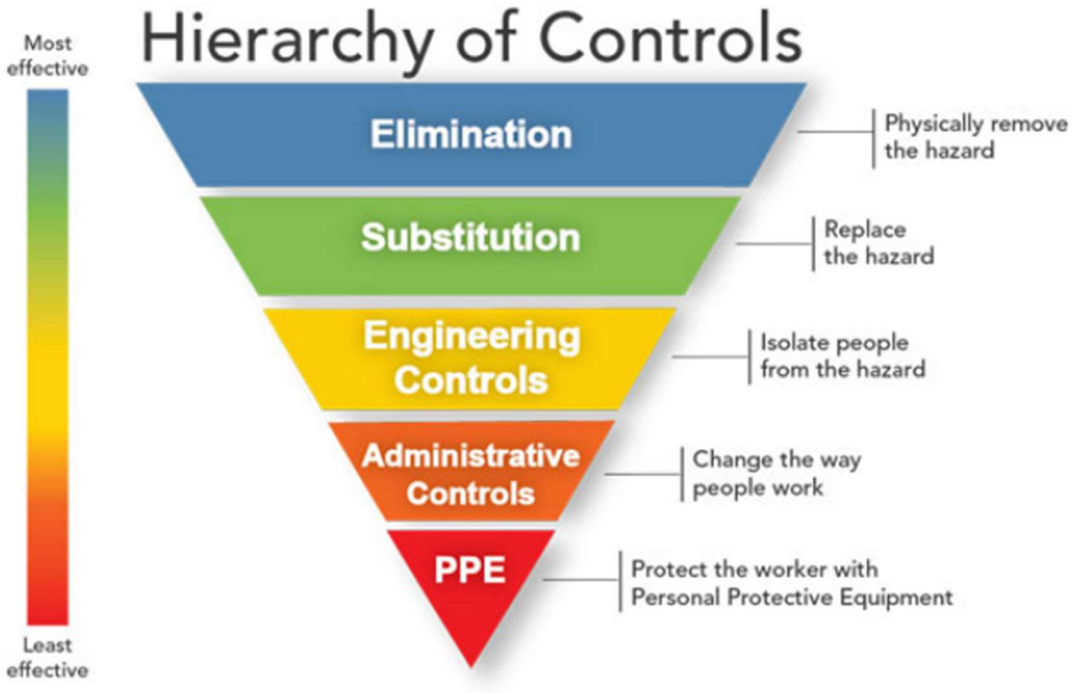

Figure 3.10 Strategies for Optimizing the Supply of N95 Respirators: COVID-19

Use of expired masks or respirators, and the limited reuse of disposable surgical masks and N95 FFRs has also been recommended by the CDC. A single person may reuse a single surgical mask until it becomes soiled or torn, for example. However, a single N95 FFR, for example, may not be shared among multiple individuals without being sterilized between individuals. Unfortunately, liquid sterilization media inactivate the electronegative filter

layers of N95 FFRs. UV irradiation, vaporized hydrogen peroxide, and dry heat at 70°C have all been shown to be effective for a limited number of reuses before masks lose either their filtering function or structural integrity. More information may be found on the CDC's website (CDC, 2020a) Cloth and alternative face covers **are not** PPE. However, used appropriately they both help eliminate virus and other contamination into the environment, and act as an engineering control to better isolate people from hazardous pathogens. Cloth face covers have been shown in studies to significantly decrease transmission of COVID-19 when worn in conjunction with other engineering controls, including social distancing and frequent, thorough handwashing (Cheng et al., 2019; Cowling et al., 2020; Eikenberry et al., 2020; Stutt et al., 2020). Previous studies and historical precedent have similarly indicated that cloth face covers are also effective against other pathogens of concern when working with wildlife. Nevertheless, users must be aware that even the best cloth face covers are not as effective purpose-made surgical masks, and likely cannot replace N95 FFRs (Figure 3.11) (Purens, n.d.).

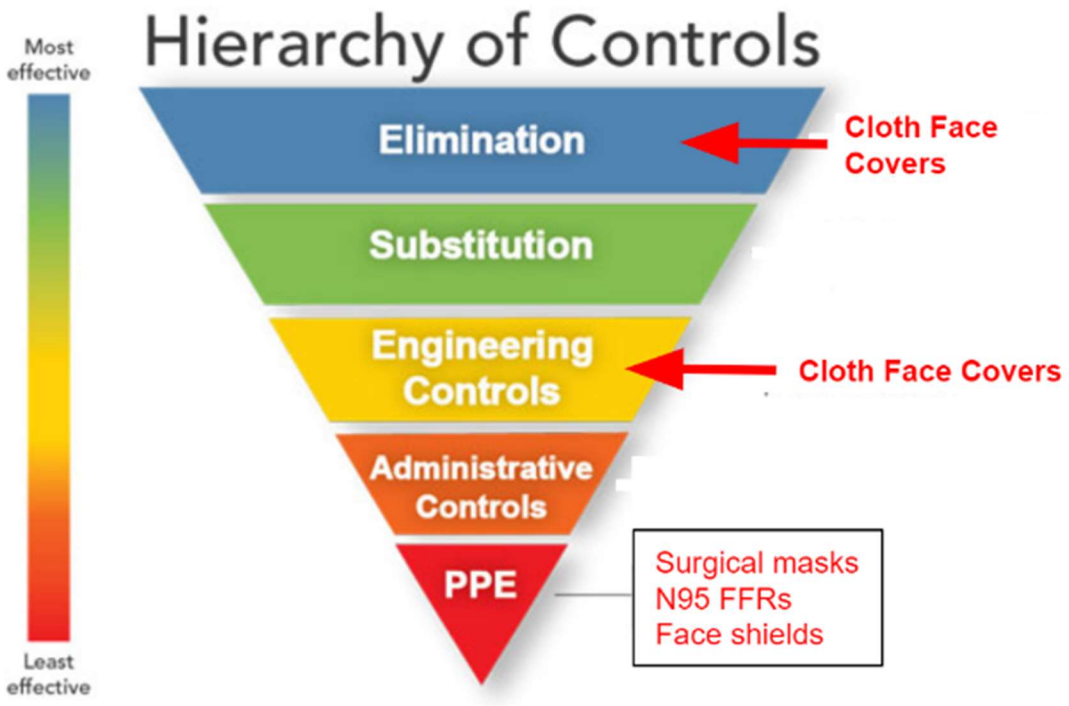

Figure 3.11 Protection applied in Hierarchy of Controls

Alternative Face Cover Design

Multiple studies have shown that the most important considerations for cloth or alternative face cover design are the density of the fabric used, balancing the number of layers of fabric used with face cover breathability, and finding a pattern or design that is both easy to manufacture and comfortable for the wearer (Purens, n.d.).

Many authorities, including the CDC, recommend making face covers out of a minimum of 2 layers of densely woven cotton cloth. A pocket may be made between these 2 layers for an additional, usually disposable filter.(CDC, 2020b) The WHO has put forth a 3 layer recommendation that aims to replicate the 3 layers of most surgical masks. The WHO recommends an outer, mildly hydrophobic woven polyester or poly-cotton blend layer, a middle layer of spunbond polypropylene such as commercially available interfacing, that may have an induced electronegative charge, and an inner hydrophilic densely-woven cotton layer against the face.(WHO, 2020) Interfacing may certainly be used as a filter in the pocket of a 2 layer mask, and so the design recommendations are not necessarily contradictory.

However, the WHO's recommendation may not be as flexible or feasible to varied working conditions as other, less concrete recommendations. The WHO's recommendation cites a recent study done by Zhao et al. in which spunbond polypropylene interfacing was rubbed between latex or nitrile gloved hands to induce the electronegative charge that helps repel very small aerosols, and that paper recommends rubbing the interfacing in this way every few hours to renew the charge.(Zhao et al., 2020) This may be difficult in areas also experiencing glove shortages. Zhao et al. also found that the induced electronegative charge declines over time, and declines more rapidly in high humidity or with similar exposure to water. As many NHP sanctuaries and conservation areas are in tropical climates, relying on the induced electronegative charge for additional filtering capacity becomes problematic.

Nevertheless, interfacing will increase filtration efficiency even without an induced charge.(Zhao et al., 2020; Konda et al., 2020) Other proposed filter materials that have been validated include facial tissue, tissue paper, paper towel, disposable blue shop towels, densely woven silk, and cotton chiffon.(Konda et al., 2020; Amour et al., 2020) However, the disposable paper based options may degrade with moisture over time; more study is needed. Moreover, early studies indicate cloth face covers are effective under study conditions without an additional filter in non-clinical situations where a surgical mask may otherwise be recommended.(Cheng et al., 2019; Cowling et al., 2020; Eikenberry et al., 2020; Stutt et al., 2020) Similarly, many types of "densely-woven" cloths have been successfully tested, including quilting cotton, 600-thread-count cotton sheets, kitenge cloth, and even disposable surgical draping and packing material such as Halyard.(Konda et al., 2020; Kwong, 2020) In particular, using 2 or more layers of Halyard H100, H300, or H600, when properly fitted and sealed to the face, may function as a substitute for an N95 FFR for a limited number of reuses, although one study found that filtration efficiency sharply declined following two autoclave sterilization cycles.(3M MSDS, 2015)

While other mask or filter materials including vacuum cleaner bags, high-MERV (minimum efficiency reporting value, measured on a scale from low to high of 1-20) or HEPA (high-efficiency particulate arrestance, equivalent to MERV (Zhao et al., 2020; Konda et al.,2020; Kwong,2020; Amour et al., 2020) HVAC (heating, ventilation, and air conditioning) filters have been put forth, these materials are not recommended for these uses. Vacuum cleaner bags are rigid and difficult to mold to the face, limiting their ability to function as an appropriate barrier. Moreover, many vacuum cleaner bags and HVAC filters are made with

fiberglass or other potentially harmful materials that may pose a separate health risk to the wearer. The gaining popularity of high-MERV HVAC filters is particularly concerning, as these filters are rated efficient assuming preservation of many layers of material, but many designs only call for the use of a single layer. Similarly, the disassembly of these filters for use exposes one to hazardous materials with risk for injury, and the high proportion of "hot melt adhesive" on 3M's Material Safety Data Sheet (MSDS) for their MERV 12 Filtrete filter may indicate the filter material is intolerant of high heat.(Purens, n.d.; FDA C for D and RH, 2020)

General Design Recommendations

As a general guide to optimizing cloth or alternative face cover design, face covers should have enough surface area to fully cover the lower half of the face, including both the nose and mouth, and extending to the sides of the face beyond the corners of the mouth. Pleating or gathering along the sides may help ensure a good fit across a variety of face types. While more fitted or three dimensional designs are available, they require more customization in cutting and sewing to fit a wearer well and may take longer to make. A piece of jewelry wire, craft wire, or pipe cleaner may be included in the top hem to help fit the face cover over the nose; cloth tape such as Elastikon may also be used to hold the top of a face cover to the face and nose, or to more fully seal face cover to the face. While many patterns currently circulating use elastic over the ears, many who work with NHPs may be more familiar with two sets of ties, which allow for a longer lasting, more customizable fit, and which are more durable in the face of typical laundering and autoclaving practices. Finally, consider making the outside and inside layers of the face cover visibly different so that they are easily distinguishable, to aid in minimizing handling and maintaining face cover hygiene. Cloth or alternative face covers should always be laundered and sterilized, if possible, before use.(Purens, n.d.)

Alternative Face Cover Testing

If the equipment is readily available, consider fit testing all cloth or alternative face covers using an FDA-certified disposable surgical mask and/or an N95 FFR as a control for comparison. While face covers are not FFRs, such testing will allow individuals to assess whether the design they have chosen will meet their barrier and filtration needs in reference to the surgical mask or N95 FFR standard to which they are accustomed. Ideally, fit testing will be over a long enough duration to assess whether absorption of moisture from the wearer's breath is an issue -- perhaps during the course of routine outdoor safety and health monitoring or a surgical procedure. As designs that achieve filtration efficiencies closer to those of N95 FFRs become available, if their use as an FFR is desired, consider testing such masks according to the NIOSH procedures outlined in the FDA Enforcement Policy for Face Masks and Respirators During the Coronavirus Disease (COVID-19) Public Health Emergency.(FDA C for D and RH, 2020)

If fit testing equipment is not available, barrier filtration efficacy may also be tested based on the American Society for Testing and Materials (ASTM) International standard for medical masks, F2101. ASTM recommends nebulizing a culture suspension of *Staphylococcus aureus*, creating droplets similarly sized to respiratory droplets, including those that hold SARS-CoV-

2, and spraying it at the material of interest. A vacuum is used to pull the spray through the material. Agar plates are placed downstream of the material, and cultured for 48 hours, then compared to a control collected using the same apparatus without any filter material or fabric (American Society for Testing and Materials, 2019). One variation used by Amour et al. used a spray bottle to mist a face cover with *Staphylococcus aureus* and *Escherichia coli*. Sterile swabs from the unsprayed side of the face cover were then used to transfer to culture media, and the colony forming units formed at regular time intervals up to 48 hours were used as a metric of barrier efficacy (Amour et al., 2020). However, be aware that ASTM testing and similar variations do not measure the breathability of any potential face cover material, and therefore further testing may be necessary to assess the actual utility of a potential face cover material.

Face Cover Hygiene

Enough face covers should be available that covers may be changed as frequently during a normal day as they would using disposable surgical masks or N95 FFRs, as the continuous use of a cloth or alternative face cover has at least the same potential for the face cover acting as a fomite that a surgical mask carries (Seale et al., 2012). Cloth face covers should be laundered and sterilized with other linens. Finally, these recommendations are in addition to and not instead of already existing recommendations for when surgical masks and/or N95 FFRs should be used when interacting with NHPs. Cloth and alternative face covers should only be utilized as a last resort, and after careful analysis of the situation in which they will be used. These face covers should only be used in conjunction with other available PPE as recommended, along with rigorous hand hygiene, distancing from other humans, and maintaining recommended distances from NHP groups under observation. Consider using the above guidelines for extended use, reuse, and resterilization to extend the current supply of masks, or even the use of a single layer cloth face cover over a surgical mask to help keep the surgical mask clean and further extend its useful life.

Managing Risk

- Species-specific risk profiles
- Environmental risk (e.g., seabird colonies)
- Co-infection risks and seasonal flu vaccination

Applies Infectious Disease Risk Analysis to prioritize actions.

Veterinarian Tasks

- Evaluate individual animal risk (species, clinical signs)
- Assess co-infection potential; advise seasonal flu vaccination
- Recommend antiviral prophylaxis per CDC interim guidance

Ranger Tasks

- Map colony density and human-animal overlap

- Report environmental contamination (e.g., carcass sites)
- Coordinate with (for example) Government response teams, land owners and managers etc.

People working with, or responsible for wildlife should take all reasonable and practical measures to prevent or minimise the risk pathogens entering, emerging, establishing or spreading. Review the following resources and slides and consider you you would manage the risk of a zoonotic infectious respiratory disease outbreak sunch as HPAI or SARS CoV-2 in your area (Figure 3.12).

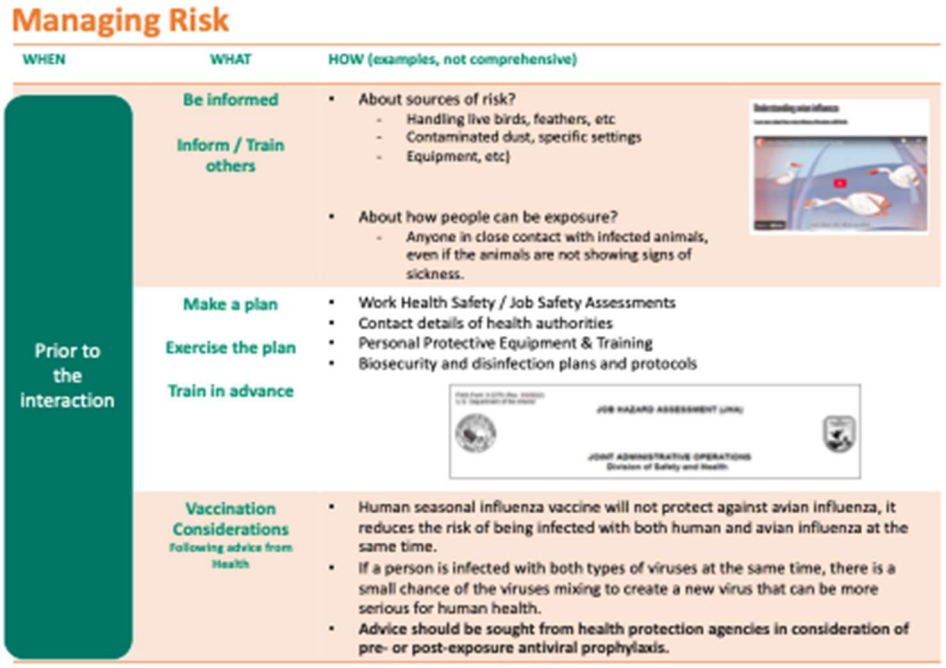

Figure 3.12 Managing Risk Prior to Interaction

Be aware and assess the risk (this requires EDUCATION & TRAINING)

- Species

- Clinical signs (may not have signs and still be a risk)
- Environments (seabird bird colonies likely present higher virus higher exposure risk)
- Review details here.

Risks to human health mean that contingency and emergency outbreak plans should **contain information and contact details about health protection agencies** so that advice and decisions on **practicalities relating to human health can be made quickly.** To reiterate, **planning before the outbreak happens is required (Figure 3.12).**

Make sure appropriate infection prevention and control and biosecurity measures are in place, accessible, and well understood in your workplace to reduce the risk of disease such as avian influenza to your employees, contractors, volunteers, visitors and animals

Vaccination: For example, personnel should consider receiving seasonal human flu vaccination, not as protection against avian influenza viruses, but to reduce chances of co-infection and subsequent re-assortment of circulating human infection and HPAI viruses.

Additionally, advice should be sought from health protection agencies in consideration of pre- or post-exposure antiviral prophylaxis.

Figure 3.13 Managing Risk Prior to Interaction

Despite the relatively low zoonotic potential of the current circulating HPAI viruses, strict health and safety measures should be employed for those handling infected birds, mammals and materials.

This should include use of PPE, including face coverings, and personnel should familiarise themselves with protocols for putting on and removing PPE and cleansing and disinfecting in a safe manner (see pages one and four).

Regular and proper washing of hands and clothing and footwear is indicated and this should always be done after handling birds or other animals.

There should also be good understanding of the need to monitor personal health and personnel should be familiar with symptoms of potential infection which may develop up to two weeks following exposure (Figure 3.13).

Medical attention should be sought immediately if any symptoms of fever are noted after contact with wild birds or other animals.

Managing Risk Example: Infectious disease biosafety in an African willdife sanctuary setting

Mapping the risk ASSIGNMENT

In your group, we want you to produce a map of the HPAI system you have each been assigned with or choose. We recommend you use MURAL for this task, or you can draw onto a flip chart.

Examples of systems maps from other sources in line with the instructions (Figure 3.14–3.16):

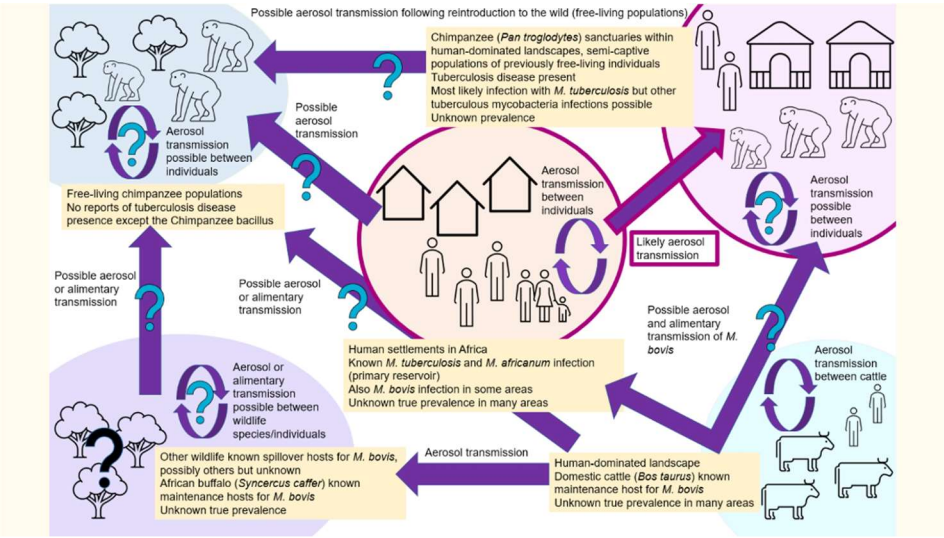

Figure 3.14 Mapping the system to help describe the problem of *Mycobacterium tuberculosis* complex transmission in an African setting, highlighting data gaps (Quinlan et al 2022 Unpublished)

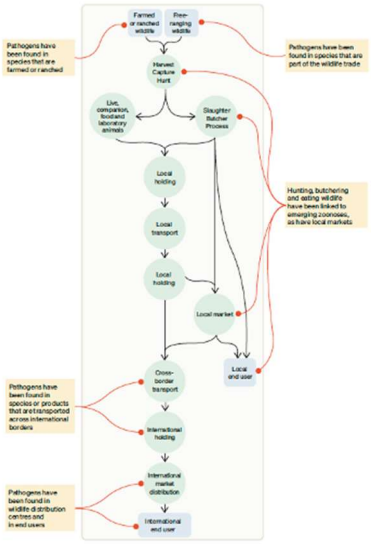

Figure 3.15 Wildlife trade generic supply chain from [Guidelines on Addressing Disease Risks in the Wildlife Trade](#) (2024).

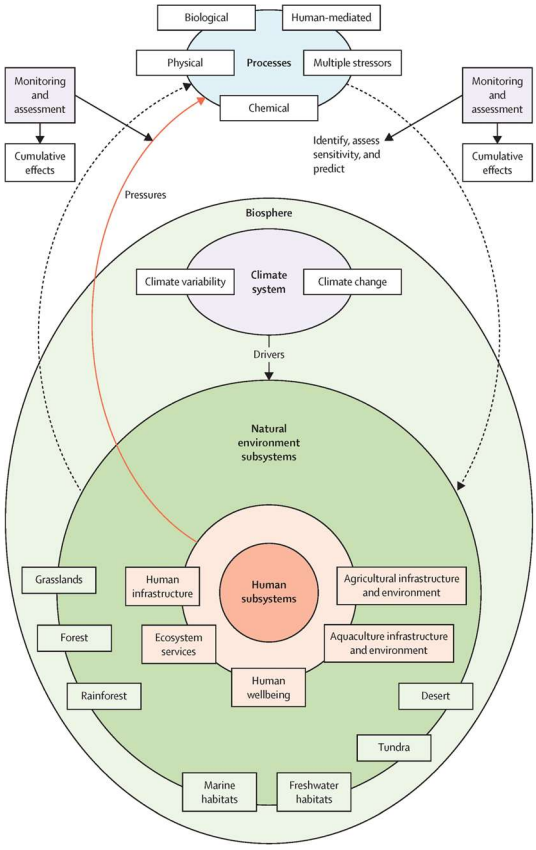

Figure 3.16 An overarching view of biological systems to assist participants on considering all aspects of their system (From the paper '[The need for One Health systems-thinking approaches to understand multiscale dissemination of antimicrobial resistance](#))

Assignment step 2. Develop a systems map for your chosen human-wildlife interface situation

An EXAMPLE for inspiration: Systems Mapping for Great Ape Sanctuaries and In Situ Conservation Sites

This resource is concerned with biosafety as it pertains to managing risks associated with transmission of SARS-CoV-2 in great ape sanctuaries or field conservation settings. This setting is uniquely complex since transmission may occur at human-human, animal-animal, or human-animal interfaces (in both directions multiple times). In sanctuary settings, the risk of transmission between and among the differing human populations (conservation workers, tourists, worker families, other local people) - and NHPs - creates the potential for local wildlife reservoirs and spillback to humans. In in-Situ conservation settings, there are additional dynamics of human interaction both within (researchers, tourists and park workers) and along park boundaries (local inhabitants/villages, extractive industries, agriculture, etc.). **The complexity of these systems is represented in Figure 3.6.**

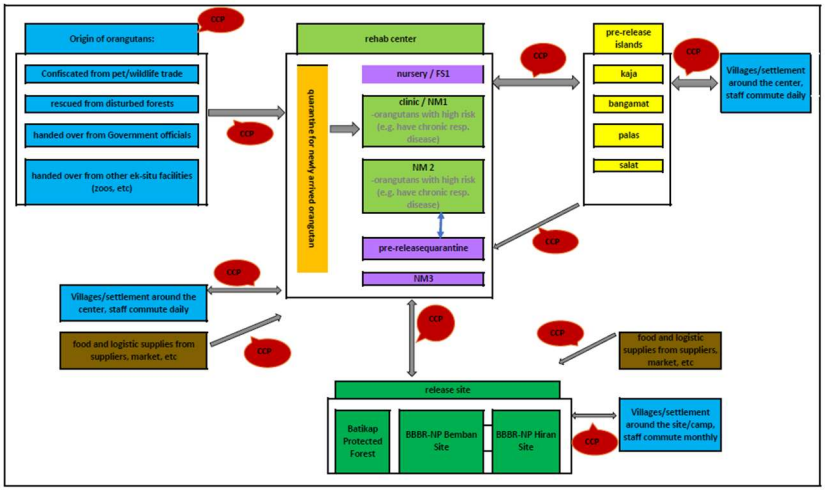

Figure 3.6 A systems diagram of the human-human-great ape interface in a sanctuary setting in response to SARS CoV2 (Author: Sulistyo 2020, Used with permission from BOSF)

Nyaru Menteng Orangutan centre, Central Kalimantan

At this point, one should have a) mapped the workflow of the system, b) identified areas of greatest exposure between humans and animals, and c) understand the context of important potential routes of transmission. Figure 3.6 above is recopied here and is a good example of implementing several types of biosafety controls such as face coverings, physical distancing, disinfection and hand washing.

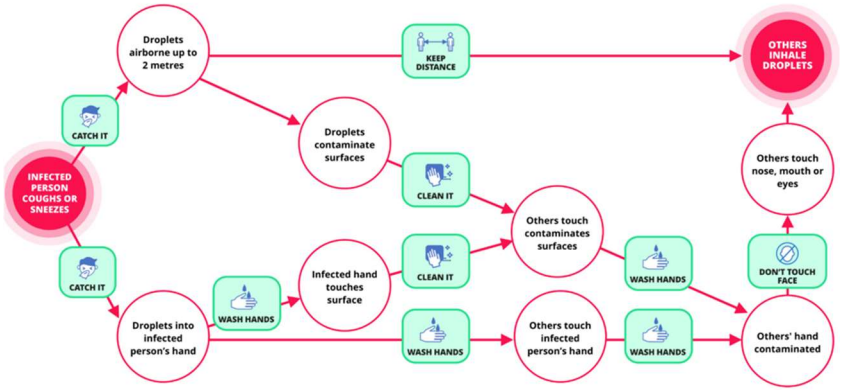

Figure 3.5 Systems diagram of respiratory transmission including control/risk management procedures, highlighting covering coughs and washing hands. (Source Website)

This model (Figure 3.5) can, and should, be applied to COVID-19 at the sanctuary level to ensure the health and safety of both humans and animals and reduce the risk of SARS-CoV-2 transmission between the two populations. However, it is important to understand that some of the most effective prevention strategies, such as elimination and substitution, may be challenging to implement in places where COVID-19 is already present.

Assignment step 3. Characterizing the Risk of Differing Human Interfaces: What is a risky job?

An EXAMPLE for inspiration. Characterizing the Risk of Differing Human Interfaces: What is a risky job?

As shown in the diagrams above, one must consider several different categories of humans when considering the sanctuary or in situ great ape interface. For transmission to occur, many things have to "go right" for the virus:

1. The infectious agent must be present - there must be an infected human who is infectious (regardless of the presence of clinical signs);
2. There must be exposure between infectious and susceptible individuals - the infected human must be in the presence of a susceptible animal (by human transmission standards "competent exposure" means < 2 m for > 15 min);
3. Virus must actually enter the susceptible individual - The virus must be expelled from the host and received on the mucous membranes (directly or indirectly) of the susceptible animal, and
4. The susceptible animal must be infected, which depends upon factors specific to that individual animal (dose, immune status, concurrent disease, etc.)

The alternative pathway includes indirect transmission via contact with a contaminated part of one's environment. The biosafety measures covered in this module assume that points 1, 3 and 4 may happen, and focus on risk reduction measures for point 2 above.

As you can see in the Figures, not all human-animal exposure pathways are the same. Exposure differs greatly in spatial proximity (distance), frequency, and length of time under differing conditions. These interfaces are generically described in terms of human-ape transmission potential below (Table 3.1).

Table 3.1 Risk-based approach to ranking the human-NHP interface in terms of exposure risk for sanctuaries and in situ conservation sites.

Human Category	**Proximity**	**Frequency**	**Time**	**Personal Exposure**	**Risk**
Conservation Worker (Park or Sanctuary)	High - intentionally directly engage the human-ape interface	High - daily	Med-High - depends upon job	Local or Regional	High
Tourist	Med-High - intentionally directly engage the human-ape interface	Low - one or few times	~1 hour - time limited	Local, Regional and Global	Medium
Researcher	High - intentionally directly engage the human-ape interface	High - daily	High - usually all day	Local, Regional and Global	Very High

Local Community Member	Low - (indirect exposure)	Low	Low	Local, Regional	Low (unless cohabitate with one of above)
Local worker (extractive industry, day labor, food supply, etc.)	Medium - (likely not intentional, but close exposure does occur, especially through contaminated waste)	Low-Med	Low-Med	Local, Regional and Global	Medium

TASK: Update Table 1 for HPAI in your chosen system.

Human-animal interface exposure pathways can be classified as very high, high, medium, or lower risk. All of these are based upon the idea of "likelihood of exposure" between humans and apes in this example. A "high risk" exposure is currently commonly defined as a) proximity within 2 m for a total of 15 min or more with an infectious individual, or b) having contact with the infectious secretions from a patient with COVID-19 or contaminated patient care environment, without using proper PPE or not performing appropriate hand hygiene (US Centers for Disease Control and Prevention).

The factors in the model above used to rank risk include:

1. Proximity of humans to apes: what is the likelihood that this category of humans will have a high risk exposure to a susceptible ape, or vice versa?
2. Frequency: how often will this category of humans have a potentially high risk exposure to a susceptible ape, or vice versa?
3. Time: is the exposure of this category of humans to apes (or vice versa) likely to be high risk (>15 min)?
4. Personal exposure: what is the cumulative personal exposure of the category of humans potentially exposed to a susceptible ape, or vice versa? This is categorized into local, regional (regardless of international borders), and global definitions.

The above rankings are built from the United States Occupational Safety and Health Administration Occupational Risk Pyramid, which is similar to those implemented by the

European Union and WHO. For more information please follow this link: Safety and Health Topics | COVID-19 - Hazard Recognition

It is important to note that risk levels are NOT static, but dynamic, meaning they can change. These categories (or others that fit an individual situation better) will need to be regularly reassessed in order to ensure that you are practicing the best biosafety procedures according to your locally assessed risk level.

Assignment Step 4. Review the transmission and ecology of HPAI in wildlife.

Utilise the materials shared in this module as well as other sources to produce a similar summary as this shown in the example below.

An EXAMPLE for inspiration - Transmission and Ecology - SARS-CoV-2

To most effectively prevent and reduce disease transmission, it is necessary to understand the transmission routes of SARS-CoV-2. An overview of disease transmission routes in general can be viewed by following this link to National Geographic Methods of Disease Transmission

It has been confirmed that SARS-CoV-2 may spread by both direct and indirect transmission pathways. The fecal-oral route of transmission for SARS-CoV-2 has not been confirmed, but the virus has been detected in feces from COVID-19 patients (Source: NCBI accessed August 24 2020). The evidence base for aerosol transmission of SARS-CoV-2 is increasing (World Health Organization updating page on aerosol transmission of SARS-CoV-2: Source: WHO), and may change the current working definition for exposure in high-risk scenarios. The diagram below characterizes high risk transmission routes of zoonotic **coronaviruses**, including SARS-CoV-2, but is limited mostly to the "spillover" story (from wildlife to humans; zoonosis) and unfortunately does not include the larger ecological picture of potential risk of anthropozoonosis. This is highlighted in the figure (Figure 3.7).

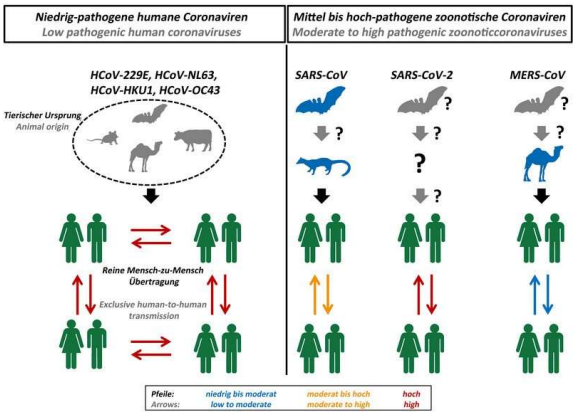

Figure 3.7 Conceptual Diagram of Coronavirus Spillover and Amplification

Source for image

A more comprehensive look at potential transmission expands the story to include anthropozoonotic (spill back or reverse zoonosis) transmission risk. The "spillback" infection of domestic pets and or the large felids from owners or zoo keepers exemplifies this complex potential risk (Figure 3.8). An evidence based approach based on current molecular epidemiological methods expands the potential ecology of susceptibles

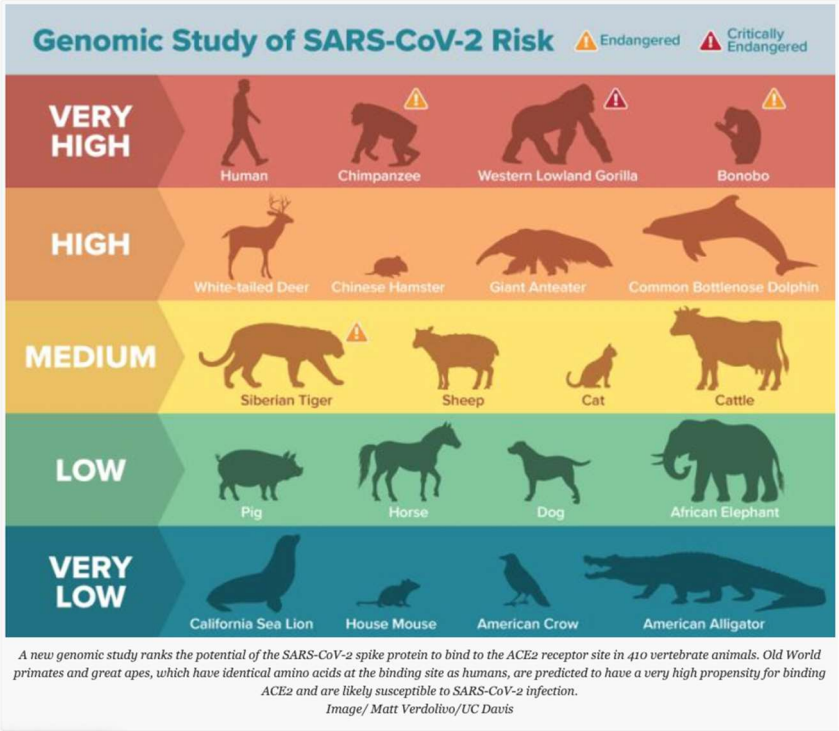

Figure 3.8 Ranking of host susceptibility "risk of being infected" based upon genomic evidence (Damas et al)

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Resources:

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Guidelines for working with albatrosses and petrels during the high pathogenicity avian influenza (HPAI) H5Nx panzootic.

.Scientific task force on Avian influenza and wild birds.

Chapter IV: Wildlife Sample Collection

Pre-course Materials

- Wildlife welfare and sampling permissions
- Sample collection techniques for live wildlife
- Sample collection techniques from wildlife carcasses categorized by species groups infected with avian influenza virus

Skills and Knowledge to be Acquired

- Learning outcomes:
    - Identify appropriate sample types along with appropriate preservation and storage methods for different wildlife species and surveillance objectives.
    - Safely and effectively collect samples from wildlife for surveillance purposes.
- Contents:
    - Necropsy demonstration by instructors: Marine mammal; small mammal; avian spp.
        - Review of lesions associated with infectious diseases of concern
    - Small group practical:
        - PPE/Biosafety for necropsy
        - Sample collection on live animals and avian carcass
        - Avian necropsy

Evaluation

- Practical: Skills checklist (observer-based):
    - Sample collection & avian necropsy
- Quiz: Field techniques, practical knowledge, etc. (See at the end of this chapter)

Welfare and permission to collect samples

Wildlife sample collection for avian influenza and other high-risk pathogens requires careful consideration of legal regulations, ethical approval for animal use, and strict biosafety practices. Compliance with relevant laws ensures accountability and alignment with national and international standards. Ethical approval, guided by the 3Rs principle (Replacement, Reduction, Refinement), guarantees that animal research is conducted humanely and is scientifically justified. Efficient biosafety procedures protect human and animal health and prevent disease transmission that link on the interconnected relationships of humans, wildlife, and ecosystems within the One Health concept.

National laws and regulations regarding wildlife sample collection may vary between countries or regions. However, the essential principles to be observed include maintaining public health, minimizing risks to humans and animals, and protecting animal welfare. These principles are guided by widely recognized regulatory frameworks and standards, including:

1. Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES): Establishes restrictions on the possession, import, export, and study of wildlife species that are protected or listed as endangered.

2. World Organisation for Animal Health (WOAH, formerly OIE) Standards: Provides international guidelines on animal health and welfare, including the prevention, control, and management of animal diseases.

3. The CDC (Centers for Disease Control and Prevention): A key aspect of its mission involves zoonotic diseases. It provides extensive resources related to animals and public health, especially concerning diseases that can spread between animals and humans.

4. The World Health Organization (WHO) Biosafety Guidelines: An international reference that focuses primarily on human health, providing detailed guidance on laboratory and field biosafety practices, including the handling of infectious agents, risk assessment, and measures to prevent the transmission of pathogens, including those originating from animals.

5. Local Animal Welfare Acts and Wildlife Protection Laws: Many countries require additional rules that regulate the use of animals, sample collecting, and research.

The welfare and permission to collect samples are based on three key elements

1. Legal Permissions

Before any sample collection begins, researchers must obtain formal authorization from relevant authorities. These permissions serve as the legal foundation for all wildlife research activities and ensure compliance with both national and international obligations.

Key elements include:

• National Wildlife Permits: Issued by government agencies responsible for conservation or natural resources. These specify the species, number of animals, locations, and duration of sampling.

• Institutional Approvals: Typically granted by university or research institute review boards, confirming that the research is scientifically justified and compliant with institutional policies.

• International Conventions:

- Convention on Biological Diversity (CBD): Ensures that the use of biological resources supports conservation and sustainable use.
- CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora): Regulates cross-border movement of samples from listed species, requiring import and export permits.

• Transport Regulations: The International Air Transport Association (IATA) Dangerous Goods Regulations govern packaging, labeling, and shipping of biological specimens internationally.

Legal compliance ensures transparency, prevents illegal exploitation of wildlife, and aligns research with broader conservation and public health objectives.

2. Animal Ethical Approval

Legal compliance does not guarantee responsible practice. Research also requires ethical approval for animals, which certifies that sample collection procedures respect the humane treatment and welfare of the animals. Institutional Animal Care and Use Committees (IACUCs) or corresponding ethics committees frequently monitor proposals by reviewing them to ensure that the use of animals is scientifically justified, proportionate, and humane, in accordance with the widely acknowledged 3Rs principle of animal ethics.

Principle	Definition	Practical Application in Avian Influenza Surveillance
Replacement	Employing methods that do not require capturing or handling live animals whenever possible.	Wherever possible, use other procedures instead of direct animal sampling. Environmental DNA (eDNA/eRNA) testing, fecal sample, or water analysis are examples of avian influenza surveillance methods that reduce or eliminate the need for live capture.

Reduction	Limiting the number of animals used for surveillance to the minimum required for statistically robust results.	Minimize the number of animals sampled by applying robust epidemiological design and statistical methods. Surveillance should be carefully planned so that the fewest possible animals are used to achieve reliable outcomes. Coordination with other programs prevents duplication and over-sampling.
Refinement	Implementing procedures to minimize potential pain, suffering, and distress in animals that must be handled.	Improve techniques to reduce suffering, stress, and suffering. This involves using species-appropriate trapping and handling protocols, reducing handling time, providing immediate veterinary care when needed, and ensuring only well-trained personnel conduct fieldwork.

3. Biosafety Approval

Wildlife sample collection for avian influenza carries inherent biosafety risks due to the zoonotic potential of pathogens. To protect both personnel and communities, strict biosafety risk assessment and control measures in sample collection must be followed.

The research involving infectious agents (especially those classified as Risk Group 2 or higher) to be reviewed and approved by an Institutional Biosafety Committee (IBC). The IBC is responsible for ensuring that all proposed research activities comply with national and institutional biosafety guidelines, assessing the containment level required for lab and field operations, and verifying the training and competence of the research staff.

In field operations, the following are the main biological control principles used to reduce the risk of pathogen exposure:

- Personal Protective Equipment (PPE): Proper use of gloves, masks, protective suits, and goggles to prevent pathogen exposure.

- Field Team Zoning: Establishing “clean” and “contaminated” areas to minimize cross-contamination during sample collection.
- Decontamination: Applying approved disinfectants to tools, equipment, vehicles, and protective gear; managing biological waste appropriately.
- Emergency Protocols: Ensuring rapid response to accidental exposures, injuries, or animal escapes.
- Transport of Infectious Substances: Following the triple packaging system (primary, secondary, and outer containers) and labeling according to IATA UN 3373 for Biological Substances, Category B.
- Occupational Health and Safety: Ensuring field staff are medically fit, appropriately vaccinated, and trained in biosafety practices.

By implementing biosafety into fieldwork, research teams reduce the risk of disease spread, protect sample integrity, and contribute to enhanced surveillance

Bird handling for sample collection

- Small bird - banders' grip or ringer’s hold technique

  Small birds, like a passerine or a shorebird, can be held by one person. The bird should be held by wrapping the index and middle fingers of a non-dominant hand around either side of the bird’s neck while cradling its body with the palm and other fingers. The other hand is then free to perform simple activities, such as swabbing or taking body measurements.When handling long-legged birds like shorebirds, cranes, and herons, it's important to be gentle. Ensure their legs are left free for activities such as banding, and never force them into an unnatural position by folding them against the bird's body (Figure 4.1).

Figure 4.1 Handling of wild birds

- Medium bird - two-handed grip

A medium-sized bird, such as a waterfowl or gull, should be restrained by one person using two hands. Firmly but gently grasp the bird with the hands placed either side of the bird. The thumbs should be placed on the bird's backbone at the level of the scapulae or shoulder and the fingers curled around the breast and abdomen, with the legs tucked up against the underside of the bird (Figure 4.2).

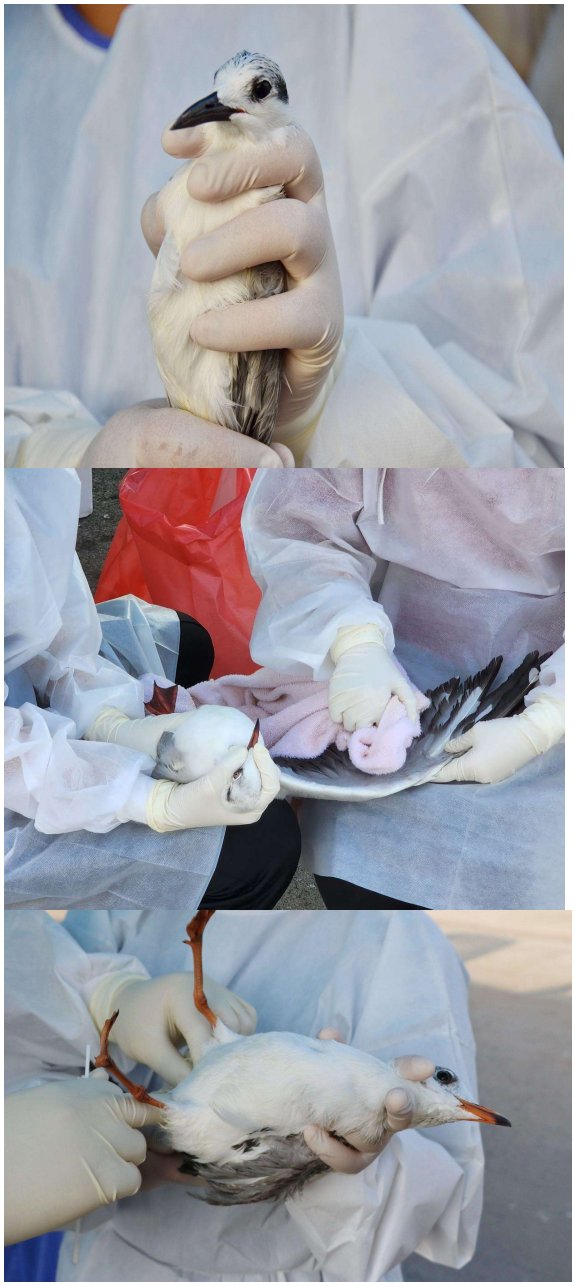


Figure 4.2 Handling procedures for medium-sized wild birds prior to blood and cloacal sample collection

- Large bird - underarm hold

Openbills and egrets captured by hand netting, should be restrained by at least two handlers; one to hold the body and wings (underarm hold) and another to restrain the head and legs. Cloth towel and bag could be used to cover the bird's head to reduce visual stimuli.

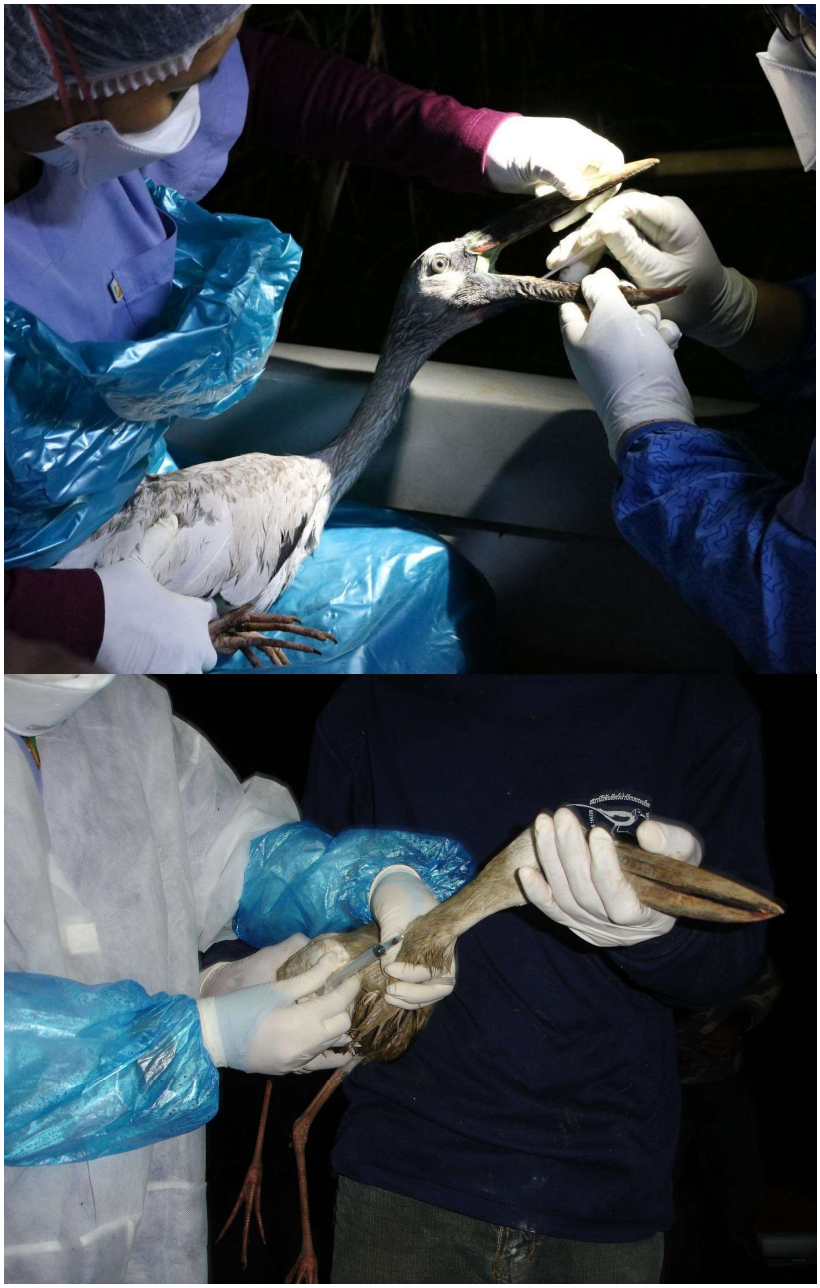


Figure 4.3 Handling procedures for large-sized wild birds for tracheal swab

Sample collection from live animals

Viral isolation and Real-time RT-PCR are the recommended methods for detecting and confirming Avian Influenza Virus in clinical cases. Viral isolation is the reference method but requires considerable effort and time, so it is used primarily for the diagnosis of the first clinical case in an outbreak and to obtain virus isolates for further laboratory analysis. The real-time RT-PCR method, however, is the first choice for the detection of Influenza A because of its rapid, direct, and highly sensitive detection of viral RNA.

Samples from live birds should include both oropharyngeal

(choanal or tracheal) and cloacal swabs. While non-pathogenic Avian influenza virus replicates primarily in the intestinal tract, the H5N1 subtype replicates to higher levels and for longer periods in the respiratory tract.

Specific swab types, including Dacron or rayon-tipped swabs, should be used. Avoid using cotton-tipped or wooden-stemmed swabs, as their inherent RNase activity or wood cellulose may inhibit genetic detection or viral growth. Swabbing small-sized birds requires the use of specially sized, extra-small swabs (such as those commercially available for human pediatrics), or researchers may decide to collect fresh feces instead. Similar swab samples can be pooled from the same anatomical site, and most commonly, pooling of 3-5 (or occasionally more) is acceptable, provided it is appropriately validated to ensure it does not reduce the sensitivity of detection.

Swabbing Procedures

Tracheal and cloacal swabbing procedures generally utilize similar equipment and techniques, though the sampling sites differ. For small birds (such as passerines) that have narrow tracheal openings, a tracheal swab may not be possible; in these cases, an oropharyngeal swab must be performed instead.

Crucially, always use a swab size that is appropriate for the bird. The procedure begins by opening the swab package from the stem end, taking care not to touch the swab tip with anything either before sampling to maintain sterility or after sampling to prevent sample contamination.

For a tracheal swab, the sample is collected from the air passage at the back of the bird's mouth. It is often helpful to gently pull the tongue forward to expose the opening of the trachea. Wait until the bird takes a breath and the tracheal cartilage is open before inserting the swab to gently touch the sides and back of the trachea. An oropharyngeal swab is performed by gently rolling the swab tip around the inside of the bird’s mouth, behind the tongue, and in the choanal cleft (Figure 4.3).

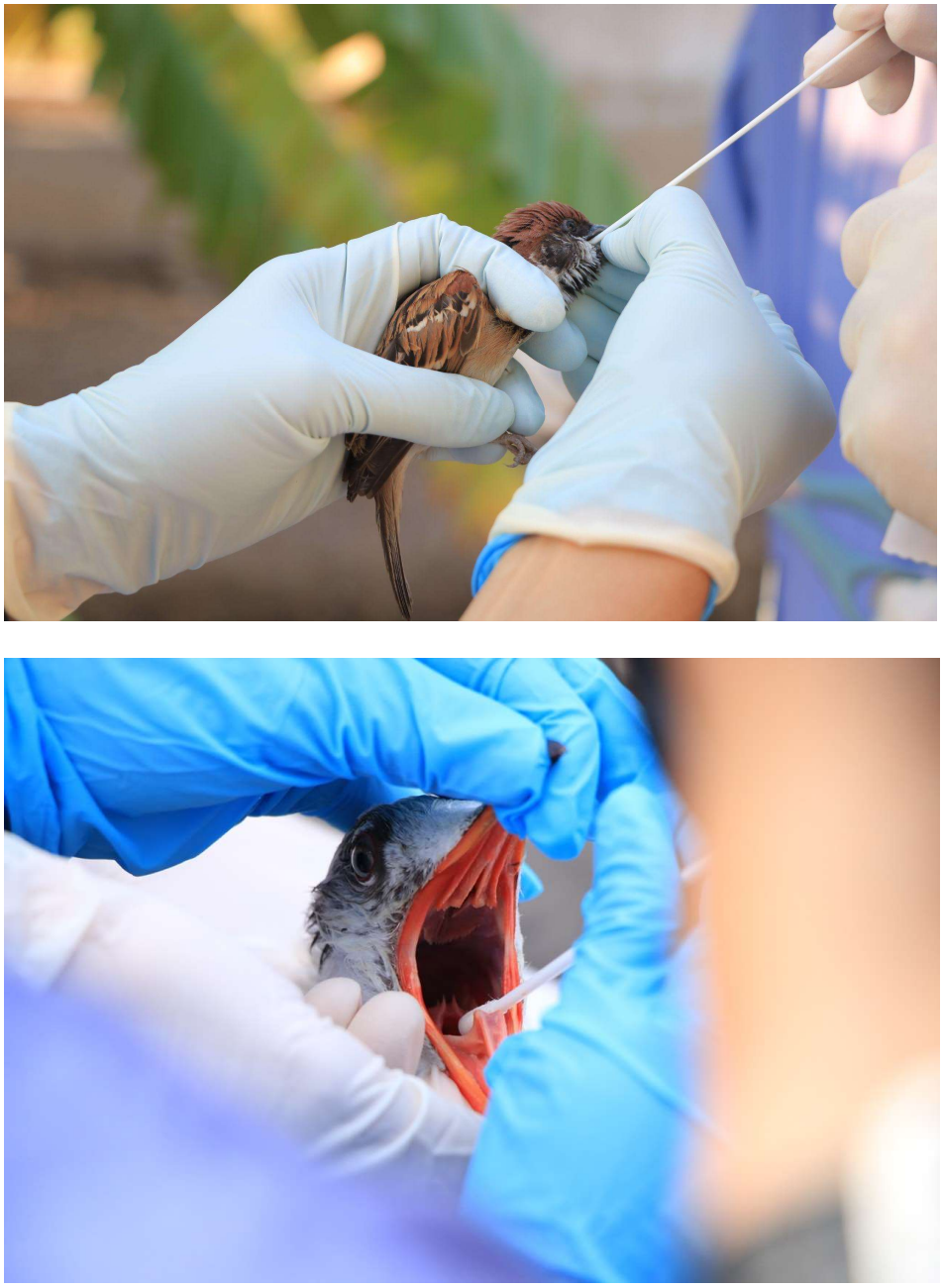


Figure 4.3 Sample collection from tracheal swab in wild bird

Cloacal swabs are collected by inserting the entire tip of the swab into the cloaca and applying around 10 times, gentle circular motions against the mucosal surfaces. Before placing the swab in the tube, gently shake off any large fecal residues. Once the sample is collected, carefully remove the swab and immediately place it into a tube containing media (Figure 4.4). Cut or break the stem, ensuring the swab tip remains inside the vial. Label each vial clearly, then store the samples in a temperature-controlled container for transportation to the laboratory.

Figure 4.4 Sample collection from cloacal swab in wild bird

Blood sample collection

For blood collection, the amount of blood drawn at one time from a healthy animal should not exceed 1 percent of its body weight. Each bird should be measured for body length and weight before sample collection. Blood collection should not be performed if a bird weighs less than 25 g. The needle size will be suitable for the animal size: a 26-G needle will be used with passerine birds, while a 23-G or 24-G needle will be used with openbills and egrets.

The venipuncture site varies by species, with larger birds offering easier access, though handler experience ultimately improves outcomes across all sizes. After collecting the blood, firm pressure must be applied to the site with gauze or a cotton ball for 30 seconds to prevent the formation of a painful hematoma that could affect the movement of the wing or leg (Figure 4.5).

To minimize the risk of hemolysis, carefully transfer the blood by removing the needle from the syringe and gently expelling the blood against the inner wall of the blood tube.

Figure 4.5 Blood sample collection from wild birds

Sample collection from dead animals or wildlife Carcasses

Introduction

Avian influenza (AI) surveillance in wildlife depends on systematic sample collection from carcasses of wild birds and other susceptible animals to support virus detection, pathogenesis studies, and epidemiological tracing. All necropsy procedures must follow biosafety precautions (minimum BSL-2) and use proper PPE, including gloves, N95 masks, face shields, disposable gowns or coveralls, and rubber boots (Figure 4.6). A Class II Biosafety Cabinet (BSC) is required for avian influenza animal necropsies to protect personnel, the environment, and the sample. The cabinet's design provides a negative pressure barrier, filters both the downflow

and exhaust air with HEPA filters, and uses an inward airflow to contain aerosols. Field personnel should be vaccinated against seasonal influenza and trained in safe carcass handling and disinfection procedures.

Figure 4.6 Necropsy for Avian Influenza should be performed in Biosafety Cabinet Class II (BSC II) with a proper PPE

A standardized necropsy should be performed in a well-ventilated or designated area to minimize aerosols. Samples should represent key organs targeted by influenza virus replication (respiratory and nervous systems). Both external and internal examinations are required, including record species, body condition, lesions, and parasites, then carefully examine the thoracic and abdominal organs (lungs, trachea, brain, liver, spleen, intestines). Collect fresh tissues for molecular testing and formalin-fixed tissues for histopathology. Even when the cause of death seems clear, a complete and systematic sampling, especially from key organs such as the brain, lungs, tracheobronchial lymph nodes, liver, kidneys, spleen, and heart, ensures accurate diagnosis and understanding of disease processes.

Fresh tissues should be placed in sterile screw-cap tubes, kept at 2–8 °C, and frozen at –80 °C when possible. For histology, use 10% neutral buffered formalin at a 10:1 fixative-to-tissue ratio, slicing samples to ≤0.5 cm thickness for proper fixation. All samples must be clearly labeled (species, case ID, tissue type, date, and location) and recorded on a submission form.

For AI virus testing (RT-PCR or virus isolation), swabs and affected organs must remain chilled or frozen to preserve RNA integrity. Maintain the cold chain using refrigerators (4 °C), freezers (–20 °C to –80 °C), or liquid nitrogen, with backup power and trained staff for handling dry ice or liquid nitrogen. Avoid repeated freeze-thaw cycles and ensure regular temperature and documentation checks to maintain sample quality for accurate and reliable avian influenza surveillance.

Avian Species

Avian carcasses are the primary focus for AI surveillance since wild waterfowl and shorebirds are major natural reservoirs of avian influenza viruses (Figure 4.7).

Figure 4.7 Wild migratory bird carcasses

Recommended samples (Figure 4.8):

- Trachea and lungs; primary sites of viral replication.
- Brain; for birds showing neurological signs or suspected highly pathogenic strains.
- Cloacal and oropharyngeal swabs; for detection of virus shedding.
- Liver, spleen, and intestine; for systemic infection and secondary lesions.
- Air sacs and heart; when hemorrhages or exudates are present.

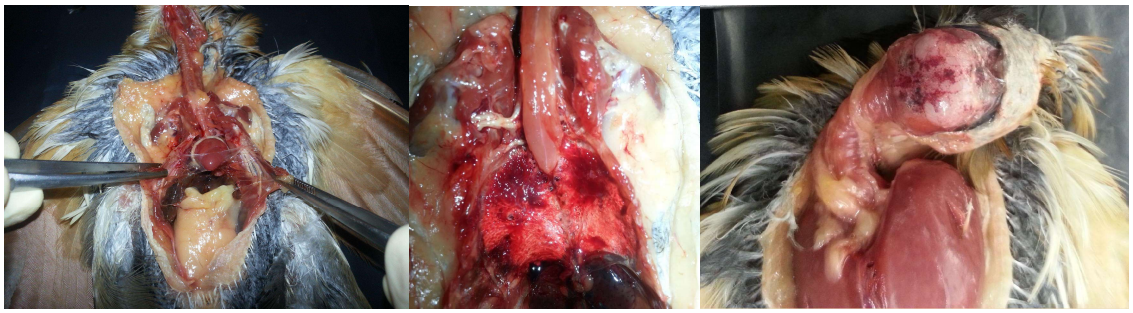


Figure 4.8 Airsac, lung, and brain of wild bird carcasses

Sampling notes:

- Use sterile scissors and forceps for each tissue to prevent cross-contamination.
- Place swabs in viral transport medium (VTM) or phosphate-buffered saline with antibiotics.
- Optional: Collect tissue in separate containers, such as sterilized eppendorf tubes, for virus isolation and real-time RT-PCR (Figure 4.9).
- Avoid contamination with intestinal content.
- Collect tissue in 10% buffered formalin for histopathological examination.

- Record observed lesions such as hemorrhages in the trachea, pancreas, or proventriculus (suggestive of HPAI).

Figure 4.9 Fresh tissues were placed in sterile eppendorf and kept at 2–8 °C for laboratory testing, and fixation of tissue samples in 10% buffered formalin for histopathological examination.

Small Mammals

Small mammals, such as rodents, bats, and insectivores, are occasionally collected in AI investigations when found near bird mortality events (Figure 4.10). Although infection is rare, these species may serve as spillover indicators.

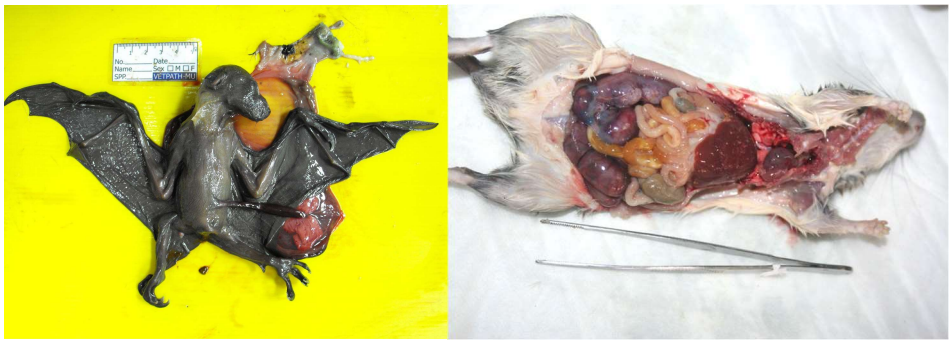


Figure 4.10 Small mammal carcasses

Recommended samples:

- Lung and trachea; for viral detection.
- Brain; when neurological symptoms or proximity to infected birds are suspected.
- Liver and spleen; for systemic infection.
- Nasal and rectal swabs; for molecular screening.

All tissues must be handled under biosafety precautions to prevent zoonotic exposure and cross-species contamination.

Large Mammals

Large mammals (e.g., carnivores such as foxes, civets, or cats) may die following spillover infections from infected birds or poultry. H5N1 outbreaks have been confirmed in wild carnivores through ingestion of infected carcasses (Figure 4.11).

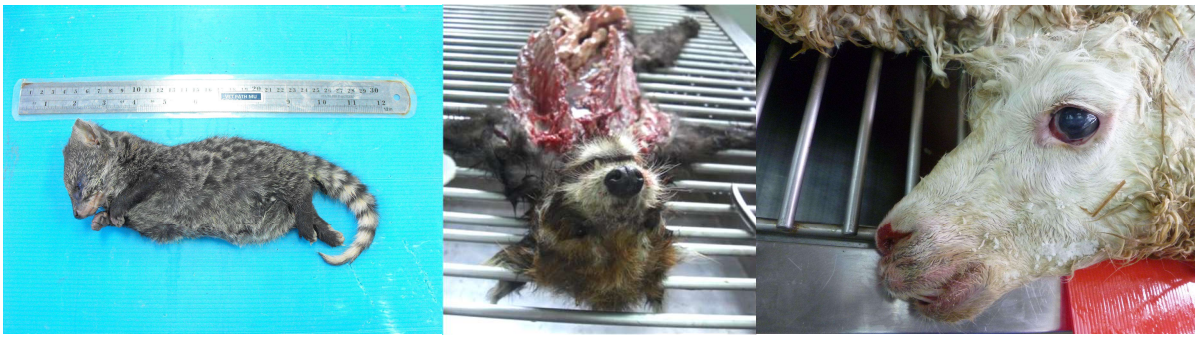


Figure 4.11 Large mammal carcasses

Recommended samples:

- Brain; for detection of neurotropic strains.
- Lung and trachea; for respiratory infection.
- Liver, spleen, and intestine; for systemic spread.
- Nasopharyngeal and rectal swabs; for viral detection.

Perform necropsy in a contained area or at a diagnostic facility whenever possible. All carcasses should be double-bagged, labeled as suspected HPAI, and transported under a cold chain system with appropriate permits.

Marine mammals

Occasionally, avian influenza viruses have been detected in marine mammals such as seals or sea lions, often linked to exposure to infected seabirds. These cases are important for cross-species surveillance.

Recommended samples:

- Lung and trachea; for respiratory infection.
- Brain; when neurological signs are reported.
- Liver, spleen, and lymph nodes; for systemic detection.
- Skin lesions or blubber; for additional microbiological or toxicological testing.

Use waterproof PPE, avoid aerosol generation, and decontaminate equipment with 10 % bleach or appropriate disinfectants after each necropsy.

Necropsy for Avian Influenza Surveillance

Necropsy provides essential information for detecting avian influenza virus (AIV) in suspected animal cases. Through systematic post-mortem examination, veterinarians can confirm infection, identify the distribution of viral lesions across organs, and collect high-quality tissue samples for laboratory testing, such as real-time reverse transcription-polymerase chain reaction (rRT-PCR) and virus isolation. These findings help link clinical signs to pathological changes, revealing how the virus affects different host species and whether infection is localized to the respiratory or gastrointestinal tract, or systemic, thus helping to clarify potential pathways of transmission.

In outbreaks involving domestic poultry, die-offs in wild birds, or wild mammals, necropsy findings allow epidemiologists to trace the source of infection, delineate the extent of disease spread, and differentiate AIV from other causes of mortality, thereby enhancing the accuracy of event-based and syndromic surveillance.

Furthermore, coupling necropsy results with host and environmental data supports risk factor analysis, helps identify silent carriers or reservoir species, and informs biosecurity, movement restrictions, and vaccination strategies. By providing evidence that integrates field observations, laboratory confirmation, and spatial-temporal mapping, necropsy strengthens early warning systems. It supports timely, targeted interventions to reduce the impact of AIV on animal and public health.

Target species for AIV surveillance include both domestic and wild animals that play key roles in the virus's ecology and spread. Among wildlife, migratory waterfowl, such as ducks, geese, and swans, along with migratory shorebirds or waders, like sandpipers and plovers, are recognized as the primary natural reservoirs that can transport and disseminate AIV across regions during seasonal movements. Other wild birds, including cormorants, gulls, terns, raptors, and corvids, have also been reported as susceptible and may act as bridge hosts between wetlands and terrestrial habitats. In addition, wild mammals, for example, foxes, otters, bears, leopards, lions, and marine mammals such as seals and sea lions, can become infected through scavenging or close contact with infected birds, highlighting the importance of better monitoring wildlife carcasses in outbreak areas to understand cross-species transmission risks and potential public health implications.

Necropsy Basics

Preparation is arguably the most important step of a post-mortem examination. Necropsy of wild animals requires strict personal protective equipment (PPE) and biosecurity measures, based on a risk assessment that considers regional diseases and species involved. Core PPE includes gloves, masks or powered air-purifying respirators (e.g., N95 or PAPR), eye protection,

waterproof gowns or coveralls, aprons, boots, and head covers. Extra protection is needed when using aerosol-generating tools, such as saws or high-pressure hoses. Essential preparations also include 1) documentation tools (necropsy forms, labels, waterproof pens, camera), 2) disinfection supplies (bleach, alcohol, brushes), 3) cutting instruments (scalpels, knives, bone shears, saws), 4) tissue-handling containers (sterile vials, standard sterilized closure bags, cryovials), 5) sample preservation supplies (10% neutral buffered formalin, RNA/DNA stabilization reagents, viral transport media), 6) morphometric tools (scales, rulers), and 7) a reliable cold chain for specimen preservation (dry ice? liquid nitrogen with appropriate transport containers?). Additional field equipment, including ropes, tarps, lighting, first-aid kits, communication devices, and generators, supports safe and efficient examinations of small birds to large mammals. Potential laboratory tests should also be considered during the preparation phase, as these will affect the sample collection approach and storage methods.

Additional necropsy equipment varies depending on the size of the carcass. For tiny specimens (<5 g), tools such as a dissecting microscope, magnification headband or surgical loupe, and fine micro-dissection forceps and scissors are needed for precise work. For megavertebrates like whales, heavy-duty instruments are required, such as flensing knives, chain saws, large hammers or mallets with chisels, sharpeners, and large meat or gaff hooks, together with long measuring tapes (≥20 m), hoists or cranes for moving carcasses, thick ropes or chains, sealable containers for bulk tissues, and appropriate labeling materials. Field teams must also plan for safe site access, crowd control, environmental hazards, vaccination of personnel against zoonoses, clear assignment of roles (lead prosector, sample/data manager, cleanup/disposal), and compliance with local wildlife and public-health regulations, especially during mass-mortality events or when working in challenging field conditions.

Necropsy Examination

Before conducting a post-mortem examination, it is essential to acknowledge that the investigator's own skills, judgment, and attention to detail are the most valuable resources in the process. The accuracy of any mortality investigation depends on the quality of observations, records, and samples collected by the team. Good intentions or memory cannot replace thorough written notes and photographic documentation. Because entire carcasses are rarely preserved for future study, the necropsy often represents the first and sometimes the only chance to secure circumstantial evidence, obtain diagnostic material, and permanently document key findings that explain the death and establish baseline data. Each participant must act responsibly and remain fully engaged, using all senses, including sight, smell, touch, and hearing, to achieve reliable results.

The choice of equipment should match the size, number, and condition of the carcasses, ranging from dissecting microscopes for very small specimens to hoists, cranes, or power tools for large animals. Regardless of whether the investigation involves a single animal or a mass-mortality event, the process follows the same basic structure: an evidence-based diagnostic plan applied against case definitions and differential diagnoses to determine the cause of death. Besides carcass findings, investigators must gather and record relevant basic, clinical

history, and environmental information, and epidemiological details, such as species, sex, age, health background, contact species data, geographic location, weather, site conditions, number of deaths, and spatial/temporal events, which can be critical for understanding the circumstances of death or for forensic purposes.

Before performing a necropsy, it is essential to understand the normal anatomy, biology, and life history of the species being examined, including its typical diet, fecal appearance, body coloration, sexual differences, and common or zoonotic diseases. This knowledge is especially critical when working with unfamiliar species, as it guides proper use of PPE and helps assess potential risks. Consulting species-specific chapters, other reference books, online databases, and colleagues with relevant expertise can be highly valuable. Although every species has unique anatomical traits, familiarity with well-studied domestic or farmed animals often provides a helpful reference for most wild mammals and birds.

Even with digital photography, written descriptions of visual observations, including the animal and its surroundings, continue to be a crucial part of necropsy records. Two key rules are: (1) describe only what you see, and (2) avoid interpretation or diagnosis in the description. Notes should include details such as location, size, shape, number, color, distribution (e.g., focal or diffuse), consistency, and texture of lesions. For example, “dozens of pinhead-sized, firm, pale nodules were scattered in the liver.” Organ weights and measurements in three dimensions (length × width × thickness) help provide baseline data, and all descriptions must be detailed enough that someone who was not present can accurately picture the findings. Written records should always be supported by photographs, morphometric data, and clear animal identification (ID number, date). Use rulers and scales in photos without covering the lesion. All collected samples and notes should be entered into logs or databases to ensure they are preserved for rapid data retrieval, mortality investigations, and future research.

Necropsy ideally should be performed on fresh carcasses, as freezing alters tissue color, damages cells, and can reduce histology quality despite killing pathogens. If immediate examination is not possible, carcasses should be refrigerated, and freezing should be used only as a last resort. A systematic necropsy must follow standardized procedures to produce consistent organ-by-organ assessments, especially important in mass mortality events. Examiners must also record carcass condition, including fresh, mildly to severely autolyzed, or mummified, as the degree of decomposition affects the value of diagnostic results. In vertebrates, the external examination is usually performed with the animal in lateral or ventral recumbency. It involves documenting both normal and abnormal findings in visible structures such as the eyes, ears, nares, as well as the bill, beak, feathers, hair, hooves, skin, glands, reproductive organs, fat stores, muscles, bones, joints, and nervous system. All lesions should be measured, photographed with rulers or scales, and supported by written descriptions that avoid interpretation or diagnosis, focusing only on what is observed. Samples of lesions for histopathology should include both normal and abnormal tissue (typically the margin of a lesion is best), measure no more than 1cm in thickness, and the volume of tissue:formalin should be 1:10 (in order for fixative to adequately penetrate the tissue).

The internal examination begins with a ventral midline incision from the jaw to the groin, reflecting the skin and limbs, followed by opening the abdominal cavity either below the xiphoid process or by cutting the diaphragm to check thoracic pressure. Ribs may be removed as needed to access the thoracic cavity, and the pericardial sac is opened to examine the heart. All organs and systems should be assessed for overall body condition, fat stores, and organ size. Representative tissue samples should be collected for histology in 10% neutral buffered formalin (NBF) or in sterile containers for diagnostic testing.

Careful handling of body cavities helps minimize contamination of sterile samples and preserves tissue quality for archiving. For detailed thoracic and abdominal dissections, there are three main approaches: (1) removing both cavities together (en bloc), (2) removing tissues by organ or system, or (3) a combination of both. The choice depends on the species, the condition of the carcass, suspected diseases, and the examiner's preference. When handling and sampling tissues, it is also good practice to move from “clean” to “dirty” to minimize the chance of contamination (i.e., heart and lung from thoracic cavity [“pluck”], followed by spleen, kidneys, reproductive tract, liver, and lastly, the gastrointestinal tract) (Table 4.1).

Table 4.1 Lists key organs and tissues typically examined and sampled during necropsy

Vertebrate: carnivore

	skin		gallbladder
	umbilicus (for neonates)		pancreas
	skeletal muscle		spleen
	peripheral nerves (e.g., sciatic)		adrenal glands
	bone or bone marrow (femur, rib)		kidneys (cortex, medulla, pelvis)
	salivary		ureters
	lymph nodes		urinary bladder
	tongue		urethra
	thyroid glands (both)		accessory glands
	parathyroid glands		testes/epididymis (bisected)
	trachea		penis
	esophagus		ovaries
	heart (walls, septum, valves)		uterus
	lungs (each lobe and bronchi)		cervix
	thymus		vagina
	diaphragm		tonsils
	stomach (cardia, fundus, pylorus)		brain (whole or midline cut)
	small (with/without pancreas)		spinal cord
	large intestines		eyes
	liver (with/without gallbladder)		

Necropsy Approach by Animal Group

Carnivores

For carnivores, necropsy is typically performed with the animal in dorsal or lateral recumbency. After a thorough external inspection, internal organs are removed systematically for detailed examination and sample collection.

In the thoracic cavity, the “pluck”, which includes the tongue, oropharynx, esophagus, thyroid and parathyroid glands, larynx, trachea, lungs, thymus (if present), and heart with its great vessels and pericardial sac, is carefully extracted. Small structures, such as the tongue, thyroid, parathyroid glands, thymus, and lymph nodes, should be examined closely, as they are easily overlooked. The esophagus and trachea are opened along their length to check the mucosal lining for discoloration, erosions, ulcers, proliferations, and intraluminal contents. The lungs are palpated for firmness, nodules, or abnormal fluids; their buoyancy in formalin is noted, and sinking often indicates abnormality. The heart chambers and major vessels (aorta, vena cava, pulmonary artery) are opened to assess valves, walls, thrombi, or defects, and samples of myocardium, valves, and vessels are collected.

In the abdominal cavity, examination includes the stomach, intestines, hematopoietic tissues (spleen, lymph nodes), endocrine organs (adrenal glands), liver, gallbladder, pancreas, urinary tract, and reproductive organs. Paired organs such as the kidneys, ovaries, and adrenal glands should always be examined on both sides for comparison. Organs may be removed en bloc (thoracic and abdominal organs together) or dissected by system (e.g., digestive, endocrine, urogenital). For better visibility, the intestines can be linearized (opened along their length) before other organs are removed. Solid or sac-like organs (e.g., pancreas, liver, spleen, urinary bladder, uterus) should be cut longitudinally or in parallel slices to expose their internal surfaces for complete assessment.

For brain and pituitary examination, two primary methods are commonly used. The first is midline bisection, where the head is disarticulated at the atlanto-occipital joint, the skin and soft tissues are removed, and the head is then split longitudinally along the midline to expose the brain. The second method, often preferred for preserving the brain’s structure, is intact brain removal. In this approach, a rhomboid-shaped skull cap is created by making paired parasagittal cuts starting from the occipital condyles and connecting them with a coronal cut across the parietal bone. The connective tissue overlying the sella turcica is carefully removed

to expose and collect the pituitary gland, allowing for a more intact retrieval of both the brain and pituitary structures.

A systematic, consistent approach ensures representative samples for diagnostics, histopathology, and archiving.

Ruminants and Other Herbivores

Ruminant necropsies are often performed in left lateral recumbency to facilitate access to abdominal organs, such as the liver and spleen. The rest of the procedure is similar to that for carnivores.

A key concern in ruminants is anthrax (*Bacillus anthracis*), a highly infectious zoonotic disease that can be fatal in humans. If suspected, indicated by swollen tissue or dark, bloody discharge that doesn't clot, the carcass should not be opened to prevent the release of bacterial spores. Instead, samples such as blood from the ear, coronary band, or aspirates from the thoracic cavity or lymph nodes should be collected and stained (e.g., McFadyen reaction or Giemsa stain) for rapid cytology. If positive, the carcass should be buried or burned without further necropsy, and authorities should be notified immediately.

Marine Mammals

Anatomy

While the anatomy of marine mammals, including cetaceans (whales, dolphins and porpoises), pinnipeds (which literally means "fin- or flipper-footed," and includes seals, sea lions and walruses), and sirenians (manatees and dugongs) is generally similar to that of terrestrial mammals, there are some notable differences, for the most part associated with the requirement of surviving in an aquatic environment. Since mammals generally maintain relatively high core temperatures, and water conducts heat 25 times faster than air, marine mammals have developed physiologic and anatomic adaptations (such as elevated metabolic rates, countercurrent vascular heat exchange, and blubber), to minimize heat loss. Along with flexible ribs, the terminal airways of all marine mammals are reinforced with cartilage or smooth muscle to prevent collapse, and some species exhibit dilation of the aortic arch ("aortic bulb") which helps maintain diastolic pressure during deep diving. Skeletal muscle appears dark red, due to a relatively high myoglobin concentration. In many marine mammals, particularly in otariids, elephant seals, and sperm whales, adult males are larger than females. Conversely, adult female baleen whales are larger than males (Rommel et al., 2018).

Cetaceans have two suborders, *Mysticetes* ("mustache" or baleen whales) and *Odontocetes* (toothed whales). Among *Odontocetes*, dolphins have conical, pointed teeth, while porpoises have flattened spade-shaped teeth, and lack a "bottle-shaped" nose, as the lower cranial melon extends all the way to the upper jaw (beak). In contrast, the baleen of *Mysticetes* consists of two keratin plates with a hard fibrous fringe, meant to filter plankton. Sea lion teeth often appear to be stained dark brown or black; this is not due to dental calculus, but rather oral chromogenic bacteria coating the teeth (1,2).

Among pinnipeds, *Otariidae* (fur seals and sea lions) are also called eared seals because they have external ear canals with distinct pinnae. They have robust vibrissae (whiskers) and a relatively thin layer of blubber with a thick haircoat for streamlining and insulation. *Phocidae* are true earless seals (i.e., harbor seals) lacking pinnae (*Odobenidae* [walruses] also lack pinnae). Phocids have a lobulated "scale-like" epidermal pattern that helps reduce drag and flippers with long curved nails and a thick keratin layer on weight bearing surfaces. Pinnipeds and sirenians are haired (with widely variably hair density), thus their skin is more similar to domestic mammals than that of cetaceans. The cetacean integument generally lacks hair follicles (with the exception of a few on the snouts of some species), sebaceous and apocrine glands. The extensive fatty blubber layer serves thermoregulatory, energy storage and structural functions. Larger whale species often require flensing knives or saws to remove skin and thick blubber layers. There is no external ear in cetaceans, just a rudimentary canal. Pinnipeds and sirenians are haired (with widely variably hair density), with skin more similar to that of domestic mammals than cetaceans (1,3).

The cardiac silhouette of most marine mammals is flattened dorsoventrally with a rounded apex, and many species possess reniculate (multilobulated) kidneys (Figure 4.12), possibly associated with large body size (vs. aquatic environment). Seals and sea lions have a muscular caval sphincter at the cranial aspect of the diaphragm to help regulate the flow of oxygenated blood to the heart during dives. In most species, the diaphragm and transverse septum are confluent, attaching ventrally to the sternum and separating the pleural cavity from the peritoneum; however, in manatees these are separate. The transverse septum is found in the area typically occupied by the diaphragm, and two separate hemidiaphragms extend the length of the body cavity along a horizontal plane. This position is thought to contribute to buoyancy control; it also results in two separate pleural cavities (Rommel et al., 2018).

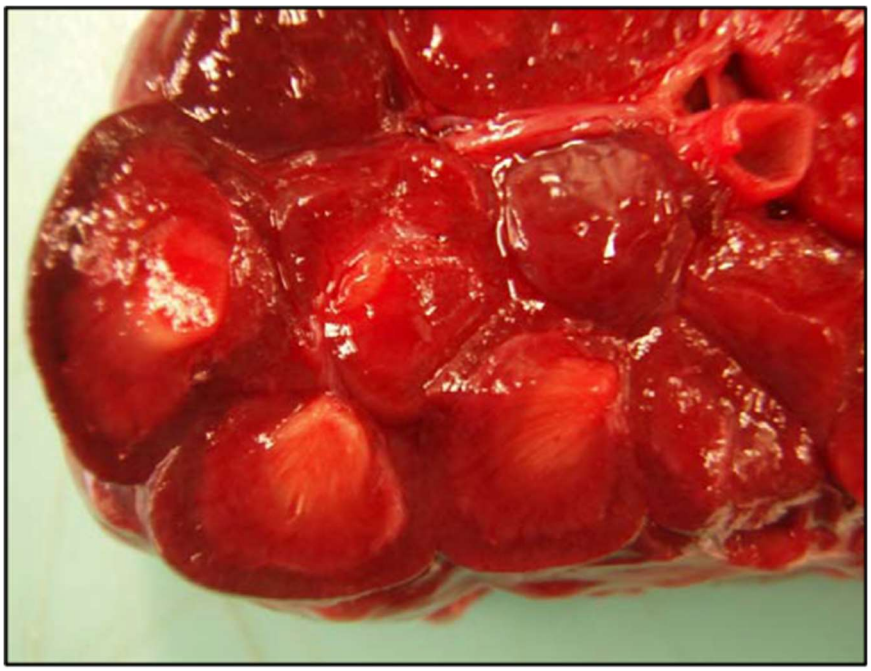


Figure 4.12 Reniculate cetacean kidney (Pugliares et al., 2007).

Cetaceans have a blowhole (the proximal end of the respiratory tract), an elongated larynx with a modified portion ("goosebeak") that extends through small opening in the roof of the pharynx and is important for production of sound, cartilage that extends from the trachea to the distal bronchioles to prevent collapse during deep dives, and unlobed, grossly smooth lungs (vs. pinnipeds with lobulated lung lobes). In otariids, the tracheal bifurcation occurs at the thoracic inlet (vs. the hilus in phocids) (Rommel et al., 2018). Large tidal volume/air exchange rates combined with a lack of turbinates allows particulates and pathogens to penetrate deep into the cetacean respiratory tract; thus respiratory disease can be a significant cause of morbidity/mortality in these species (St. Leger, et al., 2018). Manatee lungs are unlobed, located dorsal to the heart, and, like the hemidiaphragms, extend the length of the body cavity (Rommel et al., 2018).

In cetaceans and sirenians, the female urogenital opening tends to be relatively caudal (vs. the opening for the penis). Cetacean and sirenian gonads, uterine horns, and epididymides are all intra-abdominal ("testicond") and, in cetaceans, can be identified immediately upon opening the caudal abdominal cavity, as they are attached to the lateral abdominal wall. The marine mammal uterus is typically bicornuate (Rommel et al., 2018). The penile opening of male pinnipeds is located on the ventral midline, caudal to the umbilical scar. Lactating females have horizontally spaced, off center teats caudal to the umbilicus; these are less prominent (and may only look like small areas lacking hair) in non-lactating animals. Female pinnipeds have two openings in the peri-anal region (anus and vagina), while males have just one (anal) (Figures

4.12A-B) (Pugliares et al., 2007). The testes of phocids lay within the inguinal canal, while sea lions have a shallow scrotum that allows the testes to ascend or descend based on temperature. The marine mammal penis is retractable, typically found within the body wall. It is fibroelastic with a sigmoid flexure, similar to ruminants, in cetaceans. In contrast, the pinniped penis has a baculum (os penis) similar to dogs. The prostate gland is the only male accessory sex gland present in cetaceans and pinnipeds. Cetaceans and some phocids have a single pair of nipples, while otariids have two pairs. Cetaceans' single pair of nipples lay within mammary slits lateral to the urogenital opening, which provides another relatively easy way to determine sex (Figures 4.13A-4.13B); however, in some cetacean species, males also have distinct mammary slits. Manatees have axillary mammary glands (vs. lateral in other marine mammals) (Rommel et al., 2018).

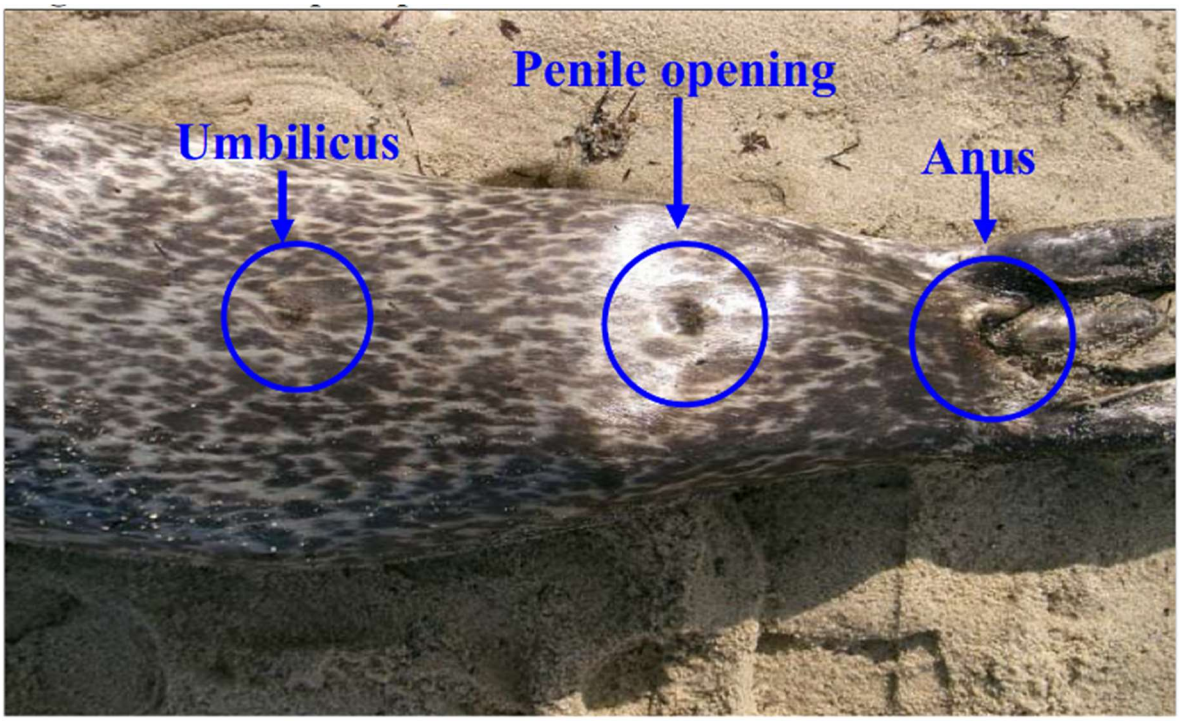


Figure 4.12A Male pinniped (Pugliares et al., 2007).

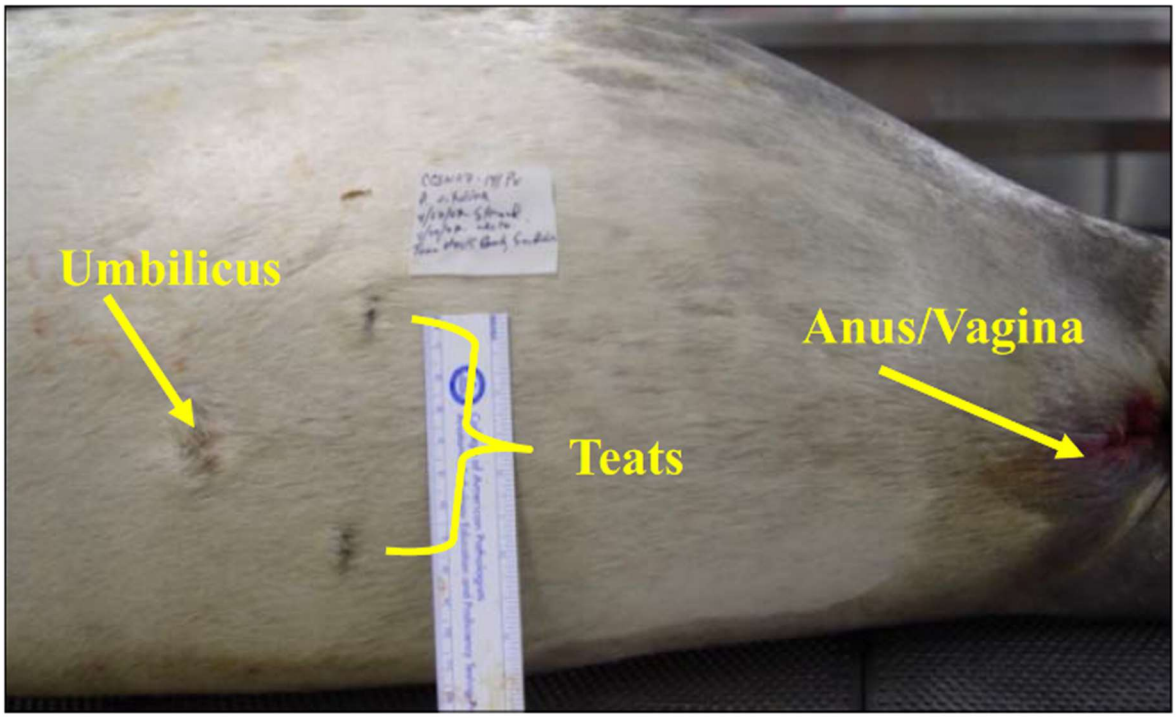


Figure 4.12B Female pinniped (Pugliares et al., 2007).

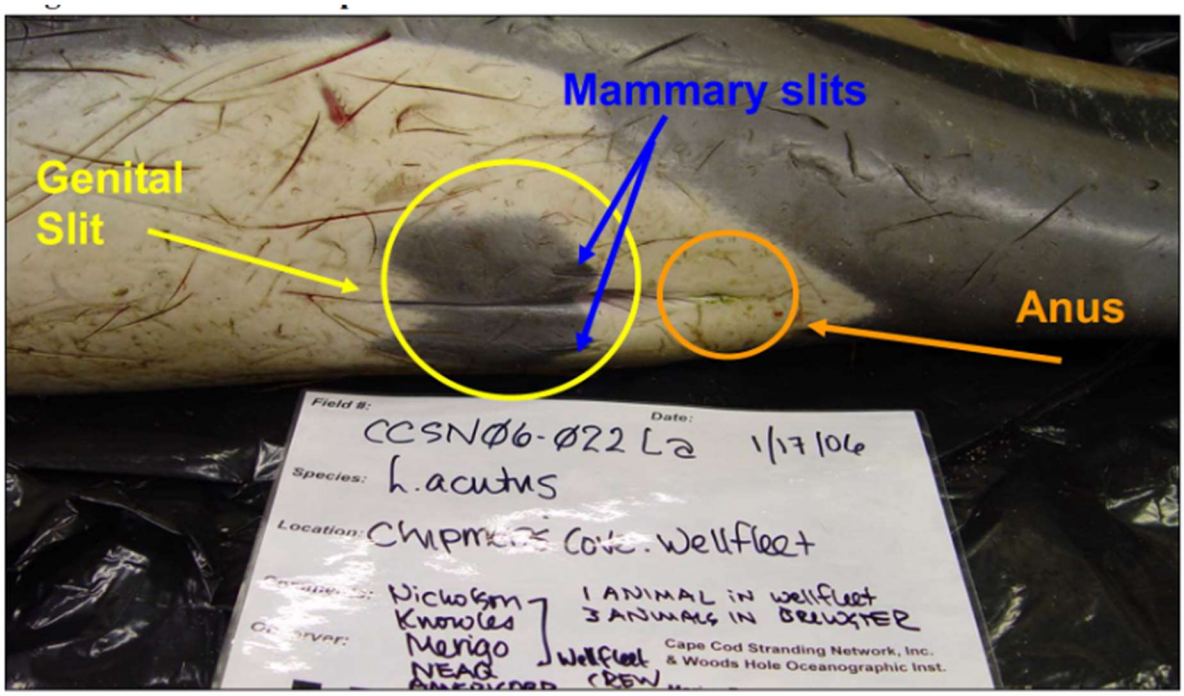


Figure 4.13A Female dephinid (Pugliares et al., 2007).

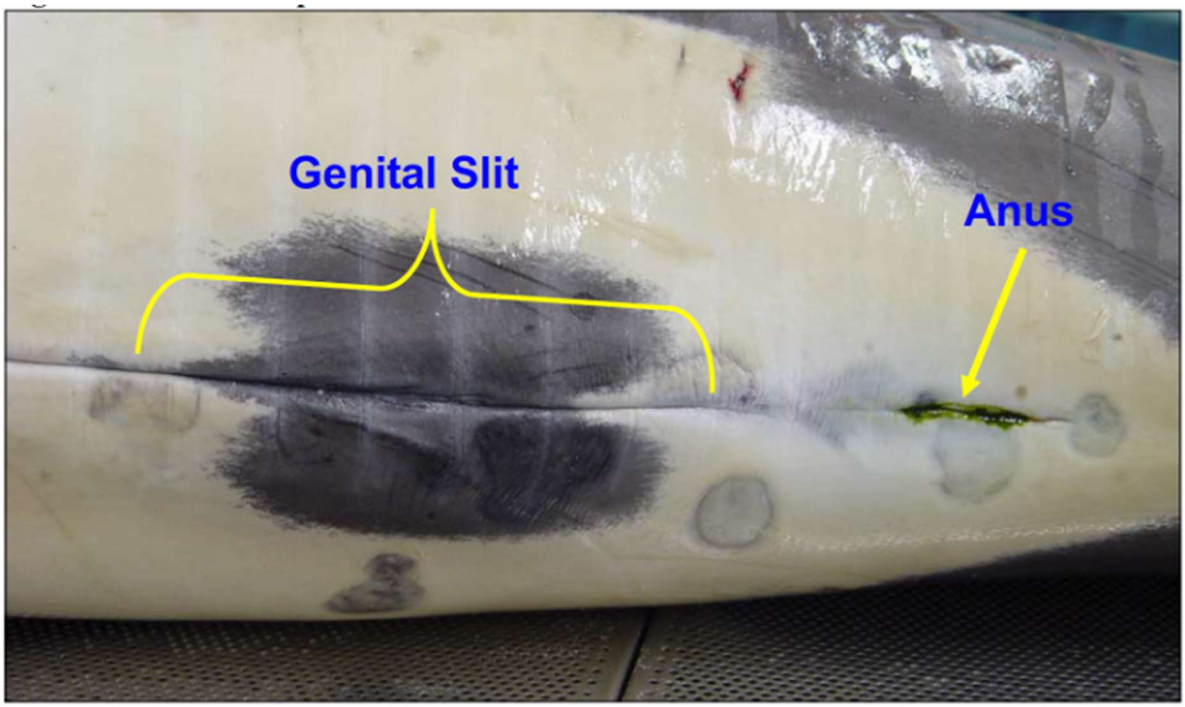


Figure 4.13B Male delphinid (Pugliares et al., 2007).

The cetacean stomach is comprised of three compartments: 1) a distensible forestomach (essentially an enlargement of the esophagus that acts as a receiving chamber similar to a bird's crop); 2) a glandular compartment separated from the non-glandular forestomach by the Margo plicatus (secretes digestive enzymes and hydrochloric acid); and 3) a U-shaped third compartment with a strong pyloric sphincter (Figure 4.14). Gastric foreign bodies occur most commonly in the cetacean forestomach (Rommel et al., 2018). The proximal duodenum has a small, sac-like ampulla; otherwise, gross distinction between the cetacean small and large intestine can be difficult. In contrast, sirenians are herbivorous hindgut digesters (similar to horses) with a single-chambered stomach and a prominent large intestine with a cecum comprised of two blind pouches (cecal horns) and a large, well-developed colon for fermentation. The GI tract and its contents can account for up to 20% of the manatee's body weight (Rommel et al., 2018).

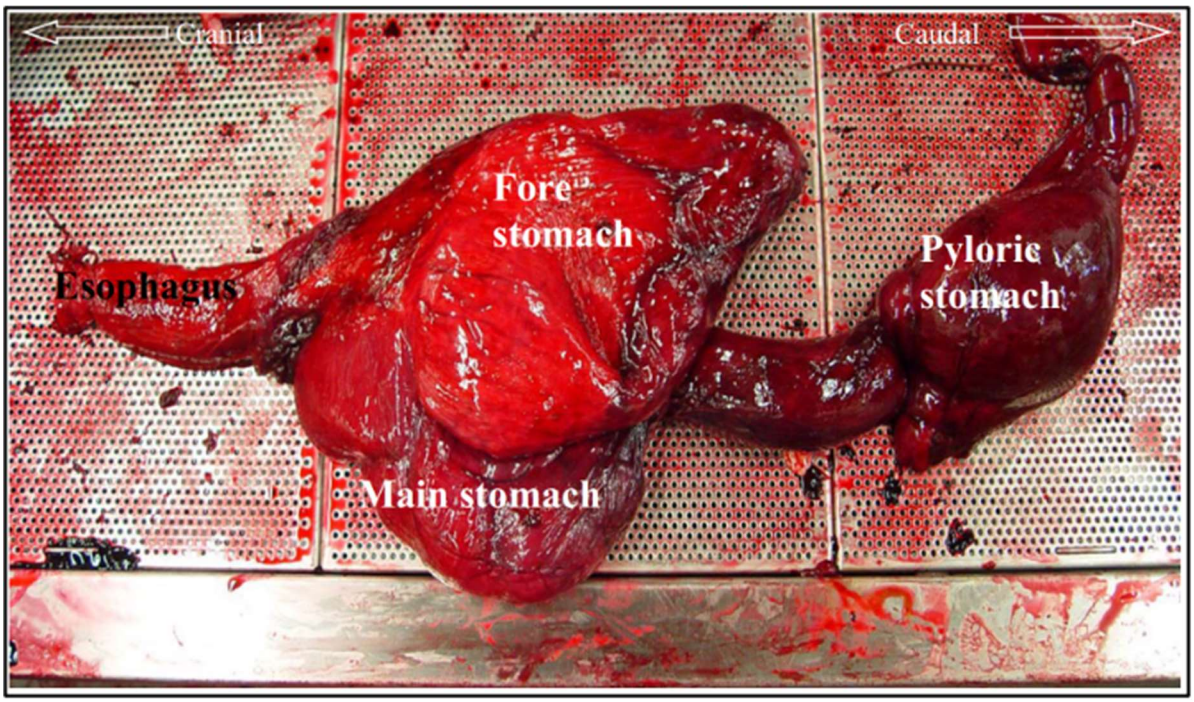


Figure 4.14 Three-compartment cetacean stomach removed from the carcass (Pugliares et al., 2007).

In cetaceans, the liver is bilobed and there is no gall bladder (vs. pinnipeds). The cetacean pancreas, typically adjacent to the duodenum, decomposes quickly and is somewhat dispersed, making it difficult to identify visually. A lymphoid "anal-" or "colonic-tonsil" is typically present in the distal colon of cetaceans. In deep-diving pinnipeds the spleen is very large with a deep fissure ("lingulate") and can temporarily store red blood cells (Rommel et al., 2018). Additionally, many cetaceans have accessory spleens in addition to "normal" spleen (St. Leger, et al., 2018). Pinniped lymph nodes have a thick capsule and trabeculae, often with abundant hilar/medullary connective tissue; this fibrosis appears to increase with age and may be secondary chronic drainage reactions. The thymus in bottlenose dolphins extends into the neck (Rommel et al., 2018).

Cetaceans have reduced bone density. As the long bones of the forelimb do not have a medullary cavity, bone marrow samples (ideally impression smears) should be obtained from the vertebrae or ribs. Most manatee bones are also amedullary, so the vertebrae are generally used for bone marrow impression smears (Rommel et al., 2018).

In marine mammals, the dorsal cranial area generally provides access to the brain. The melon is important in cetacean echolocation- returning sound is focused toward the mandibular fat body "pan" (St. Leger, et al., 2018). Similar to higher primates, the temporal lobes of the cetacean brain are well-developed, which can make brain removal challenging. Other than a larger cerebellum, the brain of pinnipeds is similar to the canine brain. Manatee brains, on the

other hand, are characterized by pronounced lissencephaly and large lateral ventricles. The pituitary gland is relatively large in both cetaceans and pinnipeds; in pinnipeds, it is often inadvertently sheared off during brain removal. Though seasonally variable, the pinniped pineal gland, which can remain attached to the falx cerebri when the calvarium is removed at necropsy, is even more sizeable (up to 1.5 cm in diameter), particularly in neonates.In both cetaceans and sirenians, the cervical and thoracic spinal cord is surrounded by epidural arterio-venous retia (rete mirabilia), an adaptation thought to help regulate blood pressure during deep diving; this makes it difficult to obtain CSF. Manatee eyes are small and lack upper/lower eyelids- instead they close with a sphincter (Rommel et al., 2018).

Marine Mammal Necropsy and Sampling

Marine mammals present a number of unique challenges when it comes to sampling and post-mortem examination. In some countries, IACUC approval is required for sampling from live marine mammals. In Thailand, marine mammal tissue collection falls under the regulation of the Department of Fisheries (DOF), with oversight from the Ministry of Agriculture and Cooperatives and its National Fisheries Policy Committee (Press Release, 2025), which should be consulted prior to tissue collection, as permits may be required. Additionally, all cetaceans are protected under CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora), thus permits are required for international import or export.

In recent years, there have been significant advances in both diagnostic and research technologies (including molecular and immunologic assays, toxin detection capabilities and sample archiving techniques), which not only facilitate the detection of infectious diseases and toxins, but also allow for the preservation of these precious samples indefinitely (‘omics). However, given the heightened sensitivity of many of these contemporary assays, preservation of biological sample integrity requires an increased emphasis on sterile sampling and processing techniques (particularly next generation sequencing [NGS] and culture), avoiding contamination with exogenous DNA/RNA, microbes, or environmental contaminants, and appropriate sample preservation and storage procedures (Van Cise et al,, 2024).

Downstream diagnostic assays inform sample collection approach and storage methods and must be considered during the preparation phase of live animal or carcass sampling. In general, it is recommended to collect 2–3 replicate samples of each tissue type- enough for expected downstream assays and to reduce the number of freeze-thaw cycles on each archived sample. Minimizing the time from tissue collection to preservation (either with a stabilizing buffer or via flash-freezing fresh tissue) is important both for preserving RNA integrity and for capturing an accurate transcriptomic profile. RNA is temperature sensitive and tends to undergo rapid degradation following sampling, while DNA is relatively tissue-stable, even without immediate

preservation in a stabilizing buffer. The sample preservation gold standard for most downstream analytical approaches is ultracold storage (≤-80°C) or cryopreservation (flash freezing in liquid nitrogen), often with a stabilizing buffer (e.g. RNAlater, RNA/DNA Shield, etc.). Tissues that are flash-frozen at the time of collection (with or without stabilization buffer) and stored at -80°C until subsequent analyses have been shown to result in the highest quality RNA; however, this is not always feasible in field conditions, where dry ice or liquid nitrogen (along with appropriate storage and shipping containers) may not be readily available. In these cases, stabilizing buffers provide adequate preservation, and some studies suggest that this type of preservation prior to long-term ultracold freezing may even yield higher RNA quantities. When utilizing liquid stabilizers, the sample-to-preservative ratio should be at least five times the volume of liquid to tissue. It is also useful to additionally collect and store a negative control of the liquid stabilizer, in order to identify potential contamination from the reagent itself (if necessary) in the future. For parasite collection (i.e., nematodes) rinsing gently with saline followed by storage in 95% ethanol is recommended (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025).

Prior to initiating biologic sample collection on live or dead animals, metadata should be recorded, including date, time, description of field conditions and location of sample collection, latitude/longitude (or GPS coordinates), species, sex (if known), age class (if known), anatomical sample site, collection method, time from collection to preservation, field processing techniques, storage method/ duration, archiving details (i.e., location and method), number of freeze–thaw cycles a sample has undergone after archiving (Vigil et al., 2024).

Samples to collect from live marine mammals (using asceptic technique where possible):

Blood: EDTA tube, plain/serum tube, LiH/plasma tube

- Cetaceans: In dolphins (Figure 4.15), blood is usually collected from the periarterial venous rete on the ventral fluke blades (CRC ch35). In cetaceans, voluntary samples are primarily obtained from the main superficial vessel of the fluke (dorsally or preferably ventrally). The dorsal fin veins, pectoral flipper veins, and ventral aspect of the peduncle are less commonly used (Lacave, 2018)
    - Collecting cetacean blood can be challenging due to vascular adaptations such the arteriovenous anastomoses that occur throughout the circulatory system, including the peripheral vasculature; these anastomoses can also cause mixing of arterial and venous blood, confounding some bloodwork results (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025)

- Pinnipeds:
    - In phocids and walruses (Figure 4.16), blood is typically collected from the epidural intravertebral vein/sinus or the plantar interdigital veins of the pelvic flippers. Sampling from the latter location yields smaller volumes and is more likely to result in an arterial/venous mixture; in the event of post-sampling hemorrhage, apply firm pressure (Barbieri, 2018).
    - In otariids (Figure 4.17), the caudal gluteal vein, interdigital vessels of the hind flippers, and ventral brachial veins of the front flippers are the most common venipuncture sites (Barbieri, 2018).
    - The jugular vein, which runs from the jaw through the thoracic inlet, can also be used for a venipuncture site in these species; blood can be collected from the angle of the jaw, the mid-cervical region, or at the base of the neck. However, it can be difficult to locate so either ultrasound guided sampling or anesthesia is generally recommended (Barbieri, 2018).
- Sirenians: Sirenian blood is usually collected from the brachial vascular bundle (Figure 4.18) of the pectoral flipper (which is not visible and is located using anatomic landmarks) via either a medial or lateral approach (Figure 4.19); a medial approach is more common in the field, as it facilitates faster blood collection and easier needle redirection (Walsh et al., 2018; Davis and Walsh et al., 2018).
- Allow blood in plain/serum tubes to clot at room temperature
- EDTA blood: Make 3 smears, chill remaining blood
- Centrifuge remaining blood samples (except EDTA) at 3000 rpm for 10 minutes pipette off serum/plasma, divide/aliquot/freeze/store as necessary based on diagnostic assays planned.

Urine (can be useful for toxins [e.g., domoic acid] and pathogens [e.g., Leptospira]):

- Cetaceans: Prior to urine collection, the genital slit is flushed with sterile water, then the surrounding skin is cleaned gently with alcohol. Don sterile gloves, using the glove wrapping as a drape. An assistant may be useful to gently open the urogenital slit with the animal in lateral recumbency; dorsiflexion of the caudal

peduncle and flukes may also help improve visualization of the urethral opening (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025).

- For male dolphins, insert the tip (coated with sterile lubricant) of a sterile catheter (red rubber 8 French x 60cm or multipurpose 8.5 French x 60cm catheter), into the penile urethra and on to the bladder (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025).

- Similar techniques are used for female dolphins, using a multipurpose drainage catheter (10.2 French x >40 cm), a Foley catheter (10 French x >40 cm), or a human "self-catheter" (10 or 12 French x 40 cm) (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025)

· Pinnipeds:

  - Cystocentesis (anesthetized animals; similar technique to dogs)

    - Insert a sterile 20-gauge, 3 in. needle cranial to the pelvis and advance it ventrally (Barbieri, 2018)

  - Abdominal compression (reported in anesthetized sea lions) (Barbieri, 2018)

  - Urinary catheterization (also similar technique to dogs) (Barbieri, 2018)

· Manatees/Dugongs:

  - Once restrained, rolled on its side and sexed, rinse the urogenital area (deionized water), dry, and place a Frisbee to catch any urine released during sampling. Similar to sea lions, abdominal compression (applied cranial to the vulva in females, caudal to the genital opening in males) can help stimulate urination (Davis and Walsh et al., 2018).

  - Urine collection via urethral catheterization is not routinely performed due to the anatomical configuration of sirenians (Davis and Walsh et al., 2018)

Feces: Divided- 10% NBF and frozen

- If culture is indicated or no fresh fecal sample is available, it is possible to collect uncontaminated feces by

- Cetaceans: Inserting a 16-French Levin-type stomach tube (or similar open-ended tube with side ports) into the rectum and gently advancing it into the colon. The semi-liquid fecal sample should flow passively into the tube. Then the tube is clamped off and withdrawn (Nollens, et al., 2018)

- Pinnipeds: A similar method to cetaceans is employed; however, it may yield less sample, these species tend to have harder stools (Nollens, et al., 2018)

- Sirenians: Passing a small, flexible, lubricated tube into the rectum (Davis and Walsh et al., 2018)

Blowhole:

- Swab of (wiped) blowhole (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025)

- To capture exhaled particles “blow” from the blowhole, a petri dish or other sampling medium is held above the blowhole (CRC ch

    - Dolphins and other marine mammals can be trained to exhale strongly (“chuff”) to supply blow (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025; Lacave, 2018)

    - Recently, drones have been employed to collect “blow” samples from large whales (O'Mahony et al., 2024)

Skin/muscle (sedation or local anesthetic may be necessary) (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025):

- Cetaceans: 2x “cheese grater scrapes” 1.5-3mm deep in 10x volume of saturated salt solution (+/- 10% DMSO) or in 10x volume 80% ethanol

- Pinnipeds: Not always collected, but common skin and blubber biopsy locations are cranial and lateral to the pelvic girdle

    - Flipper samples can be used if only skin is needed (often paired with tagging to minimize stress/impacts to the animal)

- Pinnipeds: Muscle biopsies can be obtained from the longissimus dorsi, pectoralis, or gluteal muscles

Skin lesions (if present) (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025):

- Biopsy: 5mm wedge or punch biopsy in 10% neutral buffered formalin (NBF); or

- Cytology: Fine needle aspirate or swab smeared on glass slide

Best practice for serum, feces and blubber (portion not intended for histopathology) samples is to flash-freeze immediately via dry ice or liquid nitrogen. If this is not feasible in the field, they can be stored for up to 8 hours if kept below 0^{o}C (e.g., in coolers with ice packs). One exception is for culture- these specimens should be immediately placed in tissue culture and stored (in a refrigerator) at 4^{o}C. If zoonotic pathogens are suspected potential exposure should be minimized by conducting sample processing in a biosafety level 2 cabinet in a laboratory setting. Cholesterol-derived steroid hormones (i.e., sex steroids and corticosteroids) are assessed in the blubber, but can also be measured in feces, skin, respiratory blow, claws, vibrissae, baleen and earplugs. Stress hormones (i.e., cortisol) can be altered due to sampling activities, so samples should be collected quickly (within 60 minutes of sampling initiation) (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025).

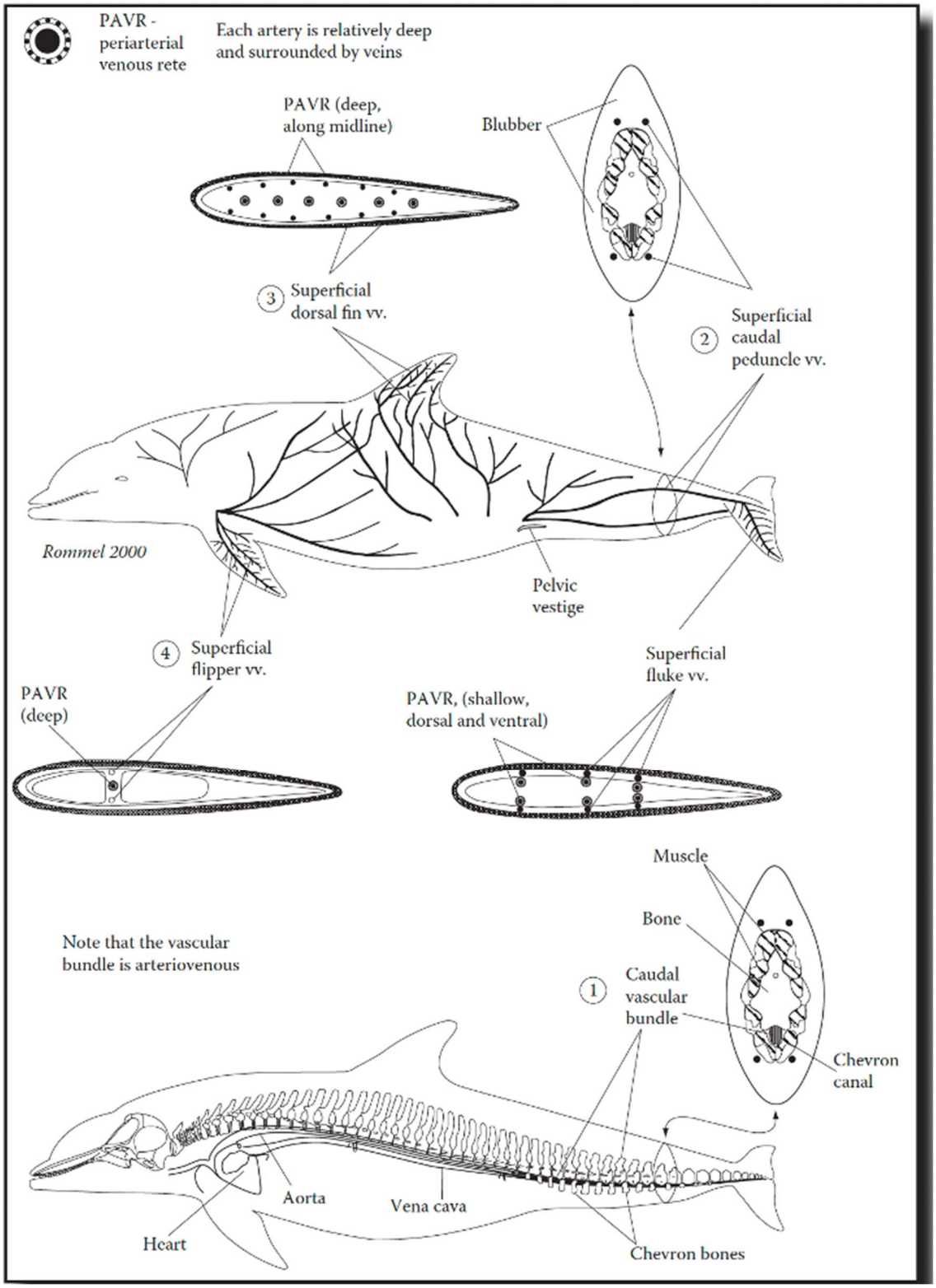


Figure 4.15 Veins used for blood sample collection from bottlenose dolphins (Townsend et al., 2018).

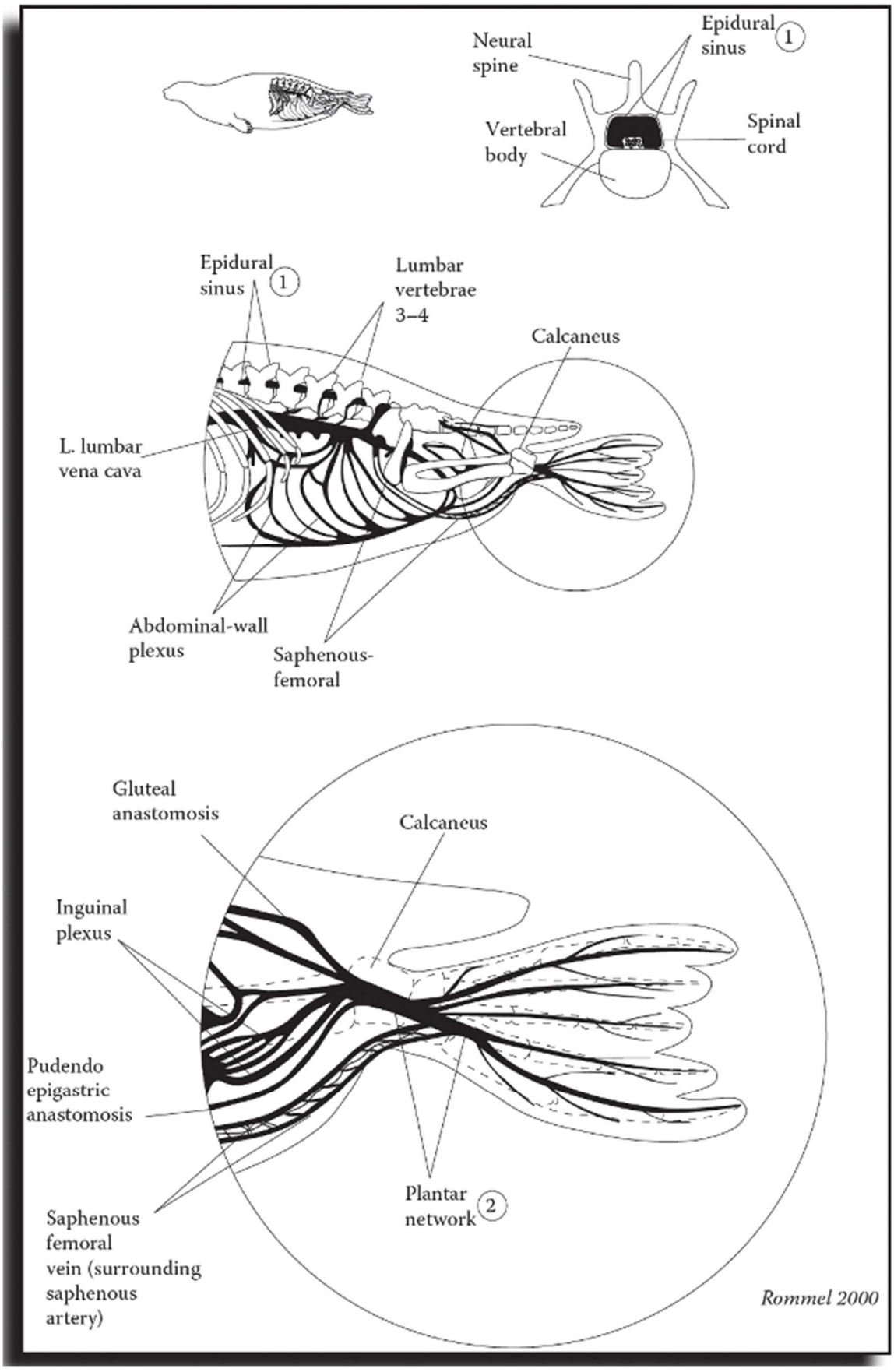

Neural spine
Epidural sinus 1
Vertebral body
Spinal cord
Epidural sinus 1
Lumbar vertebrae 3–4
Calcaneus
L. lumbar vena cava
Abdominal-wall plexus
Saphenous-femoral
Gluteal anastomosis
Calcaneus
Inguinal plexus
Pudendo epigastric anastomosis
Plantar network 2
Saphenous femoral vein (surrounding saphenous artery)
Rommel 2000

Figure 4.16. Veins used for blood sample collection from phocids (and walruses). To access the epidural intravertebral vein, a needle is inserted between the two vertebral bodies of the third and fourth lumbar vertebrae, identified by their dorsal spinous processes, after palpating from the iliac crest cranially along the midline. For pups/juveniles, a 20-gauge, 1 inch needle is usually sufficient, while an 18-gauge, 3-3.5 inch spinal needle with stylette is likely necessary for large adult phocids. To collect blood from the plantar interdigital vein, insert an 18- or 20-gauge, 1.5 in. needle at a 10º-20º angle to the skin, either immediately over the second digit, or medial to the fourth digit, at the edge of the interdigital webbing (Barbieri, 2018).

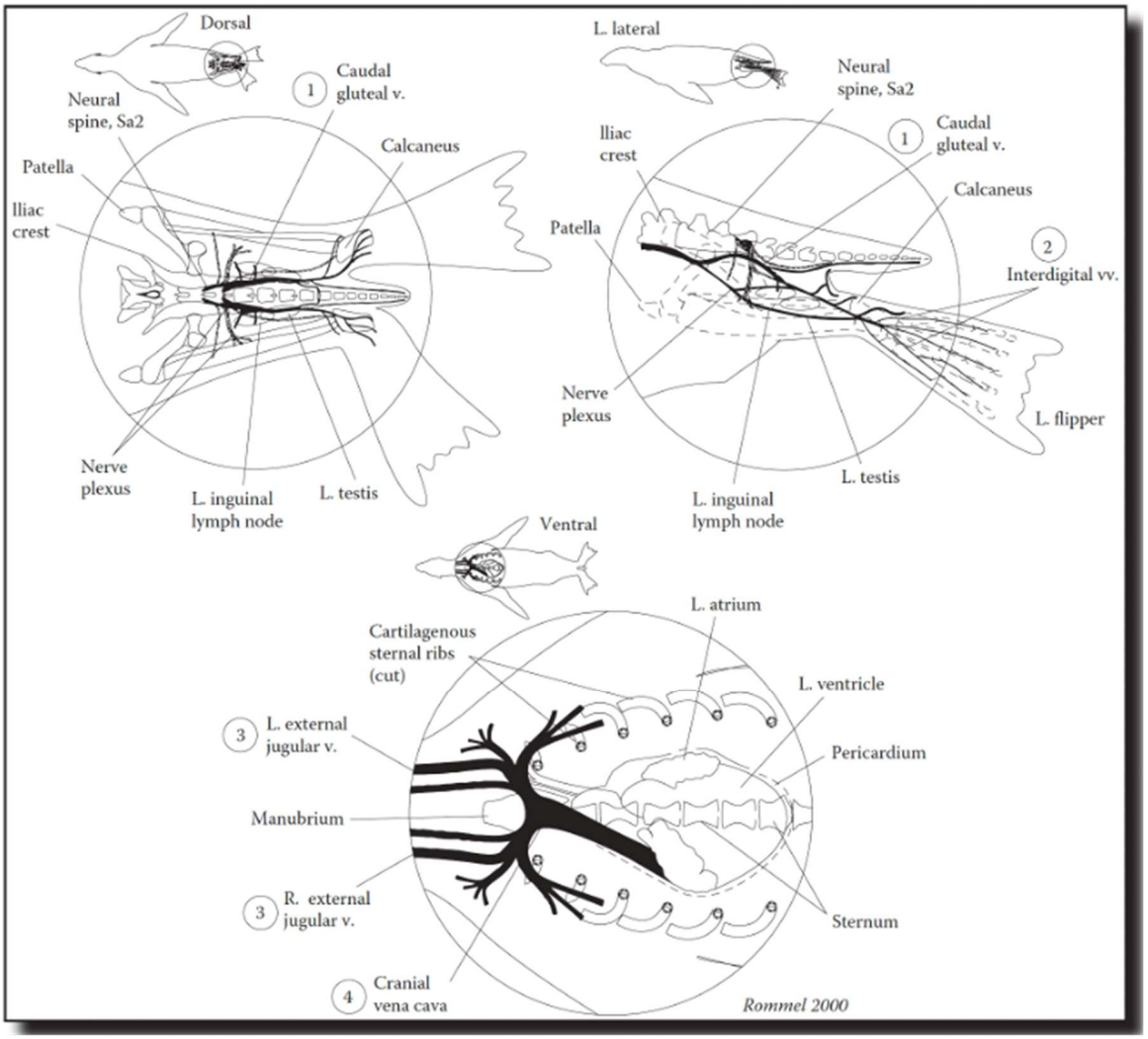
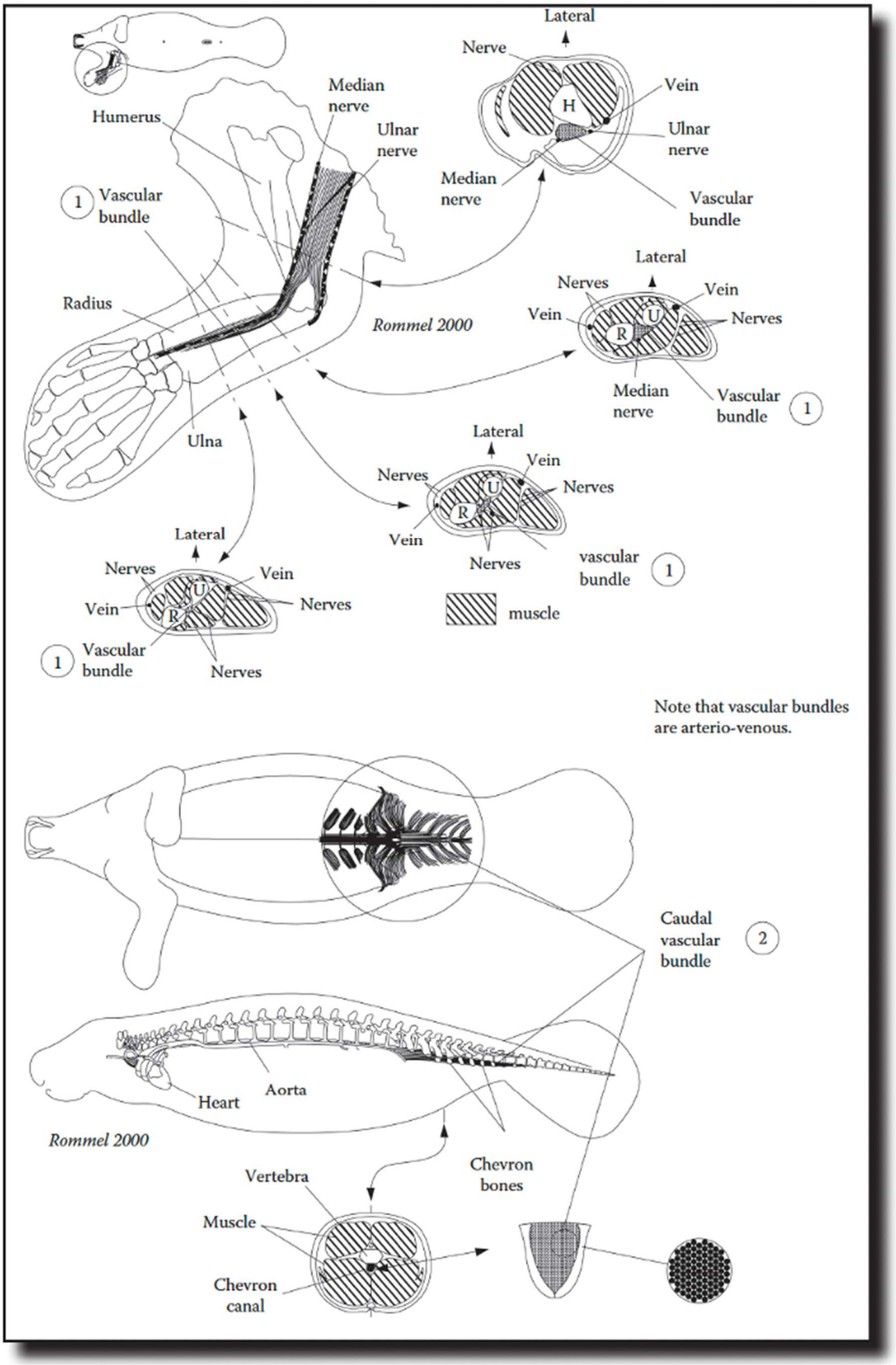


Figure 4.17 Veins used for blood sample collection from otariids. The caudal gluteal vein is easily accessed with the animal restrained in ventral recumbency. It is lateral to the sacral

vertebrae, located approximately one-third of the distance between the femoral trochanters and the tail base. The size of the interdigital vessels is variable between species; however, they can usually be visually identified and palpated. Applying pressure (e.g.tarsal tourniquet) and/or local warming the flipper can help open the vessel, facilitating venous access. Similar techniques may also enable brachial venous access (Barbieri, 2018).


Humerus
Median nerve
Ulnar nerve
1 Vascular bundle
Radius
Ulna
Rommel 2000
Lateral
Nerve
Vein
H
Ulnar nerve
Median nerve
Vascular bundle
Lateral
Nerves
Vein
Vein
U
R
Nerves
Median nerve
Vascular bundle
1
Lateral
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Nerves
U
R
Nerves
Vein
Nerves
vascular bundle
1
muscle
Lateral
Nerves
Vein
U
R
Vein
Nerves
1
Vascular bundle
Nerves
Note that vascular bundles are arterio-venous.
Caudal vascular bundle
2
Heart
Aorta
Rommel 2000
Chevron bones
Vertebra
Muscle
Chevron canal

Figure 4.18 Veins used for blood collection in the manatee. To access the brachial vascular bundle, the flipper is restrained, and, upon identification of the elbow and carpal joint and palpation of radius and ulna, the interosseous space with its vascular bundle can be located. Blood is collected using an 18-gauge 1.5 inch needle; a 20-gauge 1 inch needle may be used in animals less than 150 kg (11,12).

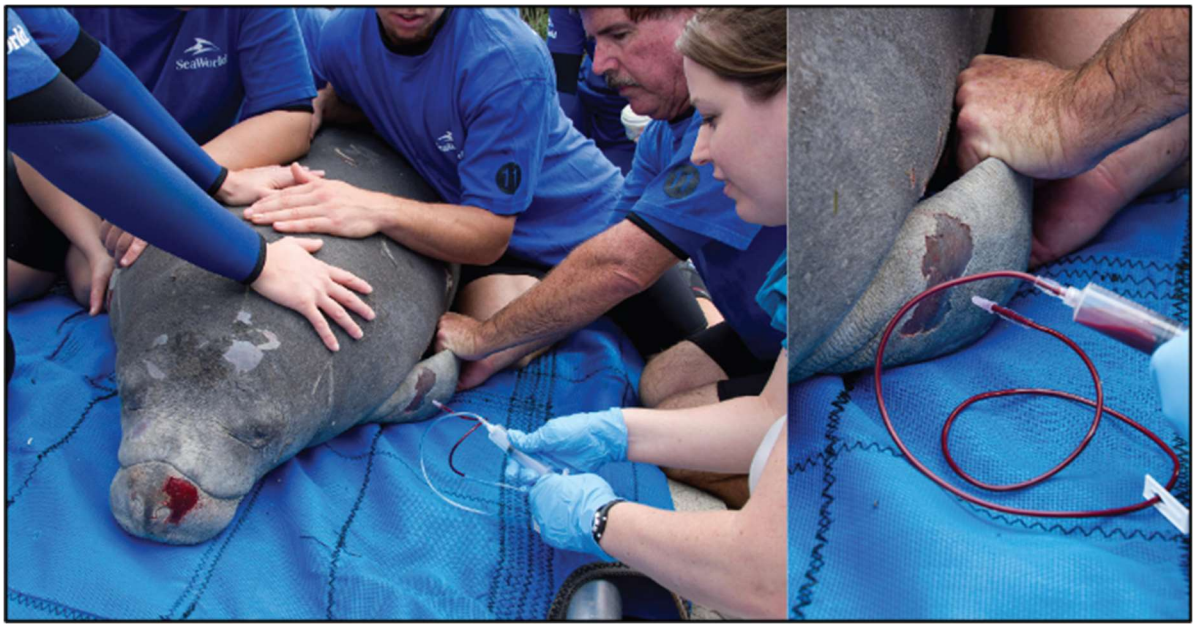


Figure 4.19 Blood collection from the interosseous space between the radius and ulna in a Florida manatee. (Courtesy of SeaWorld Parks & Entertainment, Inc.) (12).

Performing a Marine Mammal Necropsy

For a detailed step by step necropsy guide for cetaceans and pinnipeds, see: Woods Hole Inst. Technical Report (Pugliares et al., 2007).

In the event a large marine mammal species needs to be moved, injecting compressed air into the abdomen can help refloat the animal. Ideally, carcasses should be photographed in situ and transported to a diagnostic or research facility for post-mortem examination; however, in some cases, field necropsy is the only suitable option, so maintaining a prepacked necropsy kit (Table 4.2) is highly recommended to facilitate rapid response when environmental conditions (i.e., tide, weather) are suitable. Either way, PPE is an important consideration, as marine mammals are known hosts to a number of zoonotic pathogens of public health concern (Raverty et al., 2018).

Table 4.2 Example Necropsy and Sampling Equipment Checklist (Raverty et al., 2018).

NECROPSY AND SAMPLING EQUIPMENT CHECKLIST*

- ☐ Morphometric data sheets, gross necropsy forms, human interaction forms, and sample collection checklists
- ☐ Dissecting instruments, scalpel handles and blades, scissors, forceps, knives
- ☐ Sharpening steel and oil stones
- ☐ Flensing knives with retraction hooks, chain, reciprocating saw or hacksaw, hammers, chisels
- ☐ Retractors and come-alongs with lengths of rope (up to 20 m)
- ☐ Sterile instruments, propane torch/gas burner, and searing spatula for sterile culture collection
- ☐ Flood lamps, gas generator, flashlights, and/or headlamps with extra batteries and light bulbs
- ☐ 10% neutral buffered formalin (1–10 L)
- ☐ 4% buffered glutaraldehyde or suitable EM fixative (10–20 ml split in multiple small vials)
- ☐ 20% DMSO/saturated saline solution for genetic analysis (5 ml)
- ☐ Isopropyl alcohol for flaming instruments
- ☐ RNAlater (Thermo Fisher Scientific) for samples for future molecular analysis (5–20 ml split in multiple small vials)
- ☐ Sample collection containers with lids, including ice chest, dry ice, and liquid nitrogen (if possible)
- ☐ Bacterial and viral culture swabs with transport media
- ☐ Red top serum tubes for fluid, blood, and urine collection
- ☐ Aluminum foil, Teflon bags, and plastic bags/Whirl-Paks for freezing tissues
- ☐ Paper for notes, labels, and waterproof (Sharpie) marking pens
- ☐ Tape measure (metric), at least 20 m long, and small 12–15 cm plastic rulers
- ☐ Personal protective equipment (PPE): coveralls, aprons, boots, gloves, caps, masks, protective eye and head gear
- ☐ Digital camera, extra batteries, with additional memory cards
- ☐ Labels to identify digital images
- ☐ First aid kit
- ☐ Plastic tarps, 10 m in length
- ☐ Plastic tape and pylons to cordon off necropsy site
- ☐ Ice chest or cooler with ice to hold fresh samples
- ☐ Garbage bags, dish soap, disinfectant, scrub brushes, paper towels for cleanup

*Note: This equipment checklist represents an ideal situation. Postmortem exams can be completed with less equipment.

Recommended Personal Protection Equipment (PPE) for marine mammal sampling/necropsy (field or laboratory) (https://mmapl.ucsc.edu/normal-anatomy/cetaceans/harbor-porpoise) (University of California, Santa Cruz, Marine Mammal Pathology Lab, n.d.):

- Gloves (double-glove during necropsy)
- Rubber boots (or footwear that can be disinfected)
- Face shields +/- N95 mask (especially if unknown zoonotic disease status)
- Tyvek suits, slickers, other coverall type clothing, waterproof aprons, and/or full wetsuits/skins as appropriate for conditions (temperature and location)

For Procedures that create aerosols (collection of respiratory blow samples from live animals; stryker saw/high pressure hose cleaning)

- Above + respiratory protection such as NIOSH-certified N95 (or greater) filtering facepiece respirator or equivalent is recommended. All respirator users should be fit-tested before use and respirators should be used within the context of a complete respiratory program.

Utilizing preparatory teams (i.e., to lay out/organize instruments, pre-label sample containers, organize paperwork, prepare disinfectants, establish a knife-sharpening area, and set up a tissue dissection station) prior to beginning the necropsy can help streamline the process significantly, particularly for large marine mammal species. Samples for histopathology should be no thicker than 1 cm. The ratio of tissue to fixative (typically 10% buffered formalin) volume should be 1:9. Lesions should be sampled at the junction with normal tissue. It is important to collect samples from a full range of tissues, even if they appear grossly normal, because significant microscopic changes may still be present (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025).

In large whales, postmortem gas may cause extrusion of the tongue, rectum, umbilicus, penis, vagina, and/or uterus; it is recommended to make a small incision to gently release this gas. The degree of postmortem decomposition is also important to note (Table 4.3), as this will inform the extent of tissue sampling possible as well as the interpretation of diagnostic findings. Teeth should be collected, as marine mammal age is typically estimated from growth layers of teeth and bone. For pinnipeds, growth layers are counted in both dentine (hard, yellowish, calcified material that makes up most of the tooth) and cement (softer, thinner, bone-like material that covers the tooth root). In most small cetacean species, dentine (from the canine teeth) is primarily used, except in very old animals whose pulp cavity is occluded; then the cement of post-canine teeth is used instead. Teeth cannot be used to estimate age in baleen whales and manatees. Baleen whales lack teeth, while manatees have a variable number of molar teeth that are repeatedly lost and replaced throughout life. However, annual growth layers are present in the auditory bones, so baleen whales and manatees can be aged by this method. (Raverty et al., 2018).

Table 4.3 Valuable sample analysis according to decomposition code (Pugliares et al., 2007).

Code 2 Fresh Carcass	Histology, Cytology, Virology (tissue), Microbiology (swabs or tissue for culture, tissue for PCR), Parasitology, Contaminants, Biotoxins, Life History, Genetics
Code 3 Moderate Decomposition	Histology (limited), Virology (PCR), Microbiology (PCR), Parasitology, Contaminants, Biotoxins, Life History, Genetics
Code 4 Advanced Decomposition	Histology (limited) Virology (PCR), Life History, Genetics
Code 5 Mummified/Skeletal Remains	Life History, Genetics

Pinnipeds should be placed in dorsal recumbency to flatten the spine, while small cetaceans are generally kept in lateral recumbency. Locate the mammary glands and genital slit to help identify the sex of the animal. Next, flense the carcass. Transverse, full thickness parallel incisions in the blubber (from the anus cranially, spaced 0.5–1.0 m apart) can be connected via horizontal cuts high up on the flank, making a checkerboard-like pattern (Figure 4.20); this facilitates reflection and removal of the blubber layer. Examine the subcutis and underlying muscle for abnormalities (such as edema, hemorrhage, gas bubbles), keeping in mind that bruising can be difficult to detect in cetaceans due to the dark red color of their muscle. Blubber thickness should be measured (locations specified in: "Standardised Protocols for the collection of biological samples from stranded cetaceans" (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025) and examined for parasitic cysts. Collect full thickness blocks of skin, blubber (~20cm x 20cm) and underlying muscle (~6cm x 6cm x 6cm). Cut off the flipper and scapula to locate and collect the axillary lymph node. Tissues should be kept chilled if there will be a delay between sample collection and sub-sectioning.

Figure 4.20 Pinniped in dorsal recumbency with numerous full blubber thickness incisions (Pugliares et al., 2007).

Collect one or both eyes (first obtaining vitreous humor, which is a thick fluid located in front of the lens that can be useful for detecting toxins and analyzing blood metabolites), then remove the head (intact) by cutting behind the blowhole down to the joint between the skull and cervical vertebrae. Avoid using a chainsaw. If indicated, cerebrospinal fluid (CSF) can be collected (via syringe/needle inserted at the atlanto-occipital joint) prior to removing the head. Examine the oral cavity for abnormalities. Collect cetacean teeth from the center of the lower left mandible by making an incision between teeth, inserting a tooth extractor or a flat head screwdriver, and working it down toward the base of the mandible until each tooth is loosened

enough for extraction. In pinnipeds, the whole lower left jaw is typically removed (Figure 4.21) (Pugliares et al., 2007).

Figure 4.21 Removal of a lower left tooth in a small cetacean (Pugliares et al., 2007).

The brain is very delicate and easily damaged. To remove the brain intact, remove skin, blubber, muscle and connective tissue from the skull. Use a Stryker saw or hacksaw to make cuts. In pinnipeds, cuts should be made behind the base of the skull, down between the occipital condyles and first vertebra (Figure 4.22). In cetaceans, cuts are made through the middle of each occipital condyle, up each side of the skull, and, finally, across the dorsum (posterior to the marked transverse ridge at the skull's apex) (Figure 4.23). It is very easy to penetrate all the way through the skull and damage the brain, so work carefully. Use a chisel to gently crack the remaining bone, pulling evenly, until the back of the skull comes away in one piece, trying not to let the tentorium cerebellae (bony shelf) damage the brain. Use fingers to gently pull the meninges away from the skull and sever cranial nerves so that the brain falls out upon inversion of the skull (Pugliares et al., 2007).

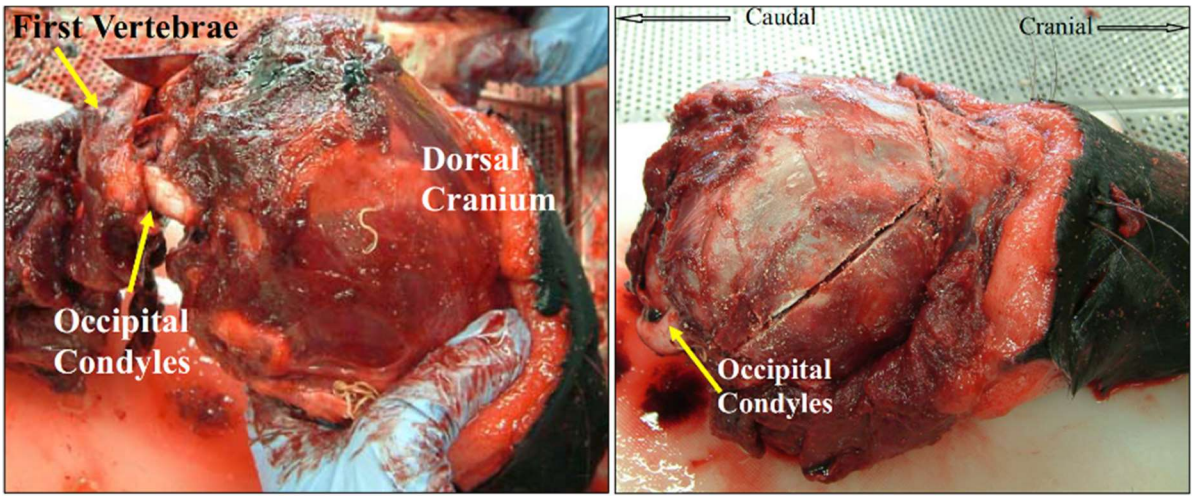


Figure 4.22 Skull landmarks for pinniped brain removal (Pugliares et al., 2007).

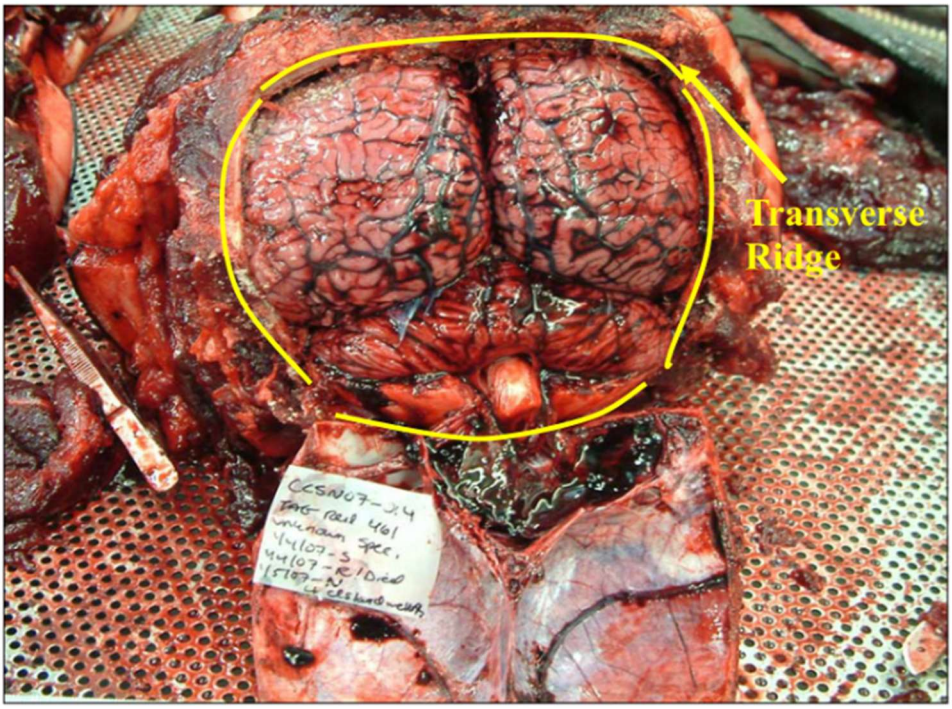


Figure 4.23 Brain with dorsal skull removed. The meninges exhibit vascular congestion (Pugliares et al., 2007).

Dissecting out the ears for microscopic/ultrastructural evaluation (+/- age estimates) can be useful, as acoustic factors may contribute to strandings in cetaceans and pinnipeds; however, cochlear hair cells are sensitive to autolysis, so these tissues should be collected as soon as possible after death (ideally within 5 hours). In order to collect the tympanoperiotic complex: 1) separate periotic from tympanic bone; 2) remove the stapes

with tissue forceps; 3) use a needle to superficially perforate the round and oval windows; and 4) perfuse fixative through the opening (Figure 4.24) (Raverty et al., 2018).

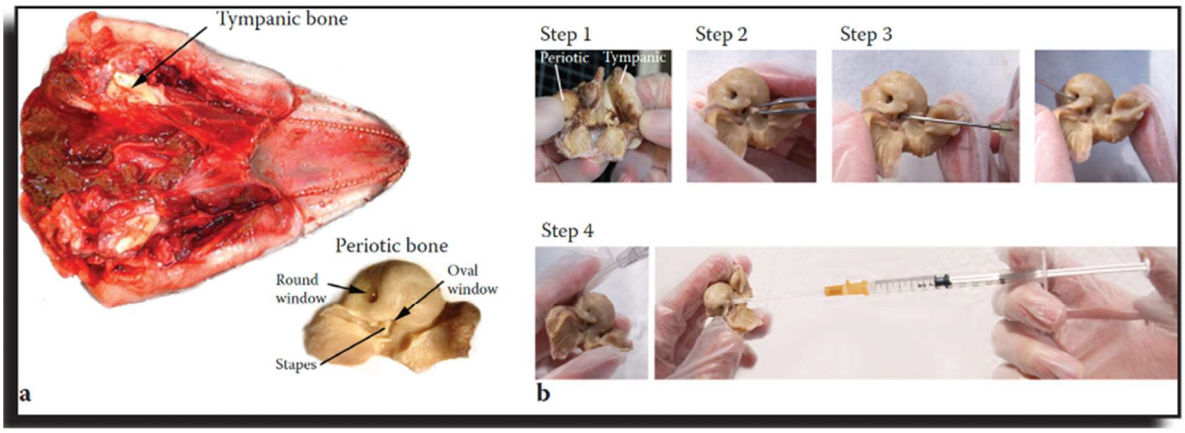


Figure 4.24 (a) Removal of the mandible in a harbor porpoise reveals the tympanoperiotic complex (TPC), from which the periotic bone can be extracted as shown; (b) Illustration of the perfusion steps on a periotic bone of a bottlenose dolphin.

To expose the abdominal organs, the abdominal wall is typically incised along the costal arch and dorsally to the level of rectum. Before cutting the ribs, puncture the diaphragm to check for negative pressure (deflation); if this does not occur, it is possible that the animal has pneumothorax or severe pneumonia. At the cetacean thoracic cavity, the area of articulation between each rib and vertebrae, as well as costochondral junctions, are transected (Figure 4.25A) and intercostal muscles are incised, releasing individual ribs. Cetacean cranial ribs are double-headed along the vertebrae (Figure 4.25B) and are removed by severing the transecting the first articulation, then sliding the scalpel along the second head to its spinal articulation. In pinnipeds, trim intercostal muscles at their attachment to ribs, then cut through each thoracic rib mid-articulation, at the cartilaginous flex point which can be palpated. Visual examination of the internal surface of the rib cage also reveals articulation lines (Figure 4.26) (Pugliares et al., 2007). Chain saws should be deployed sparingly, and then only by experienced personnel with appropriate PPE and safety equipment. In some species, the diaphragm can be excised to avoid having to transect ribs. A window can be created through the intercostal muscles to access the heart/lung of larger animals, and multiple ~1x3m windows can be made into the thorax and cranial/caudal abdomen for sampling larger whale species- this is a useful, expedited method for tissue collection when there are complicating factors such as potential infectious disease and/or harmful algal blooms, or when access or time is limited (Raverty et al., 2018). Always examine and photograph pleural and abdominal cavities/organs in-situ prior to sampling or dissection (Figure 4.27). If there is excessive, cloudy fluid, fibrin strands or adhesions in the thorax or abdomen, collect the fluid in a sterile syringe/appropriate container.

If swabs will be collected for culture, this should also be performed prior to further dissection, in order to prevent contamination (Pugliares et al., 2007).

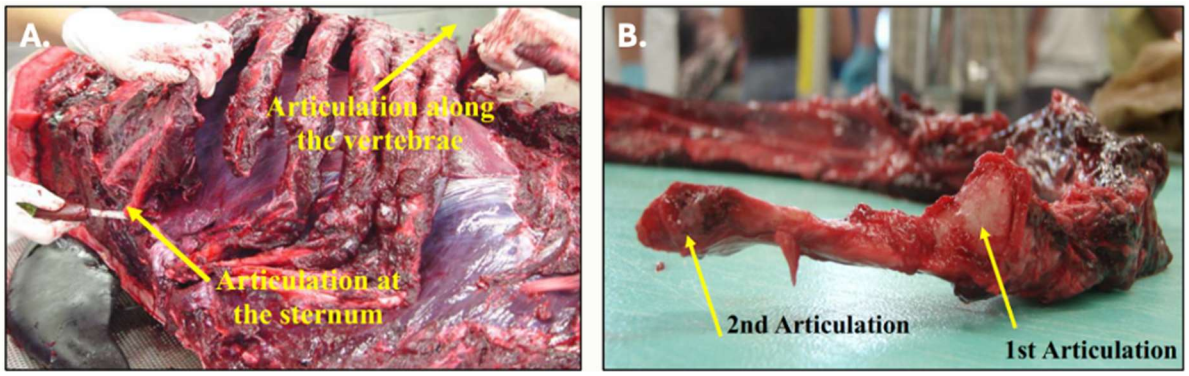


Figure 4.25 Cetacean thorax. A) Removal of ribs at sternal and vertebral articulations; B) Double articulation of cranial ribs along the vertebrae (Pugliares et al., 2007).

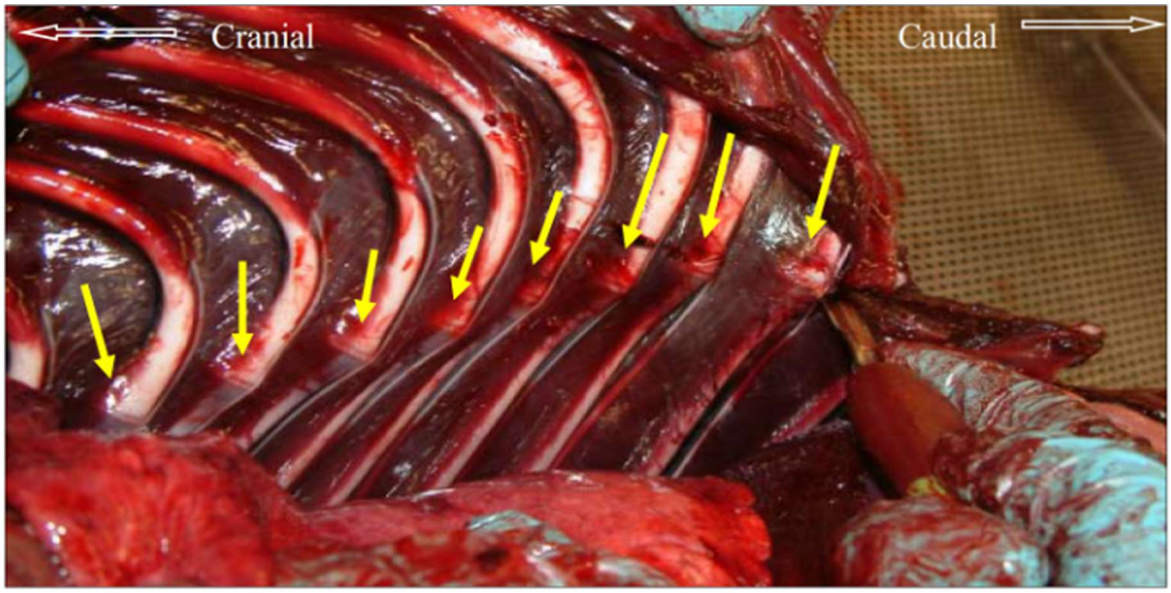


Figure 4.26 Pinniped lateral rib cage articulation (Pugliares et al., 2007).

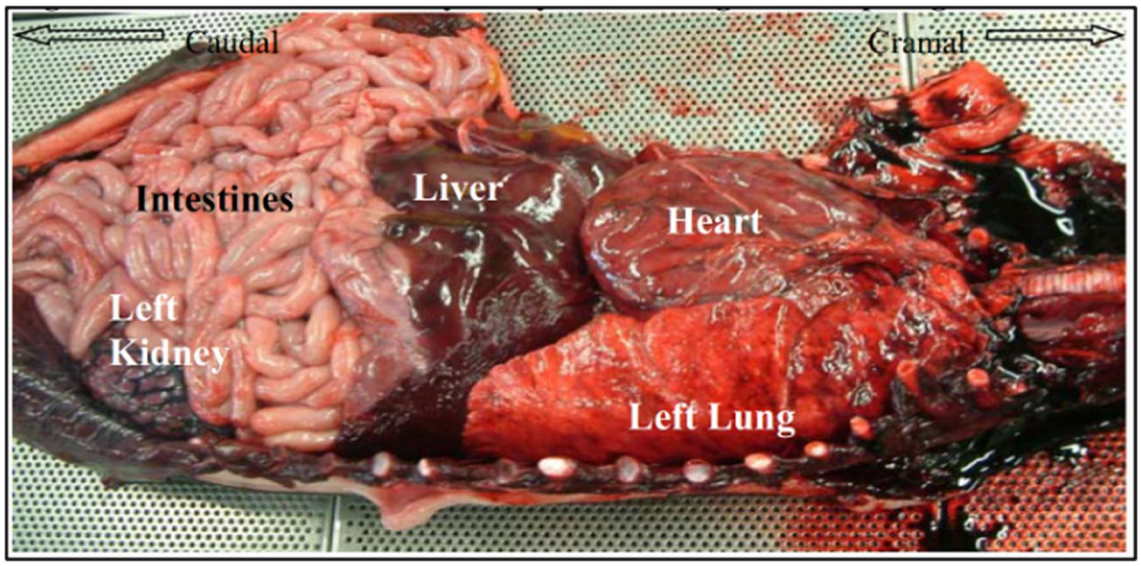


Figure 4.27 Pinniped thoracic and abdominal organs in situ (Pugliares et al., 2007).

Remember to try to sample tissues from “clean” to “dirty.” Tie off the esophagus at its entry to the stomach and cut above the tie. In the pleural cavity, examine the pericardium (and collect any fluid, if present), then aspirate blood from the heart with a sterile syringe and place in a plain or serum separator tube; if it hasn’t clotted collect a few ml in an EDTA tube too. Locate/collect the bi-lobed thyroid (at the ventral base of the trachea in cetaceans). In young animals, the thymus may be present between the heart, sternum and thyroid gland. If possible, remove the entire respiratory tract, heart and esophagus (“pluck”) by cutting out the tongue, transecting the hyoid bones and dissecting down through the chest cavity. Ideally in a separate (clean) area designated for sub-sectioning, collect samples from the respiratory tract and heart. Open the trachea and follow airways as far as possible, looking for abnormalities and collecting samples, including the tracheobronchial lymph node (Figure 4.28). Lung samples should be collected from multiple locations, including the periphery, the center, and including some airway. Lung should float when placed in water or formalin. Pleural lymph nodes, typically located on the caudal border of the lung, should also be collected if possible. Open the major vessels and heart, trying to follow the blood flow from vena cava to right atrium, through right AV valve to right ventricle, then up through the pulmonic valve to the pulmonary artery, through pulmonary vein into the left atrium, out left AV valve to the left ventricle and then up through the aortic valve into the muscular aorta (Figure 4.29). Collect samples from the left and right ventricular walls, septum and AV valves (Standardised Protocols for the collection of biological samples from stranded cetaceans, 2025).

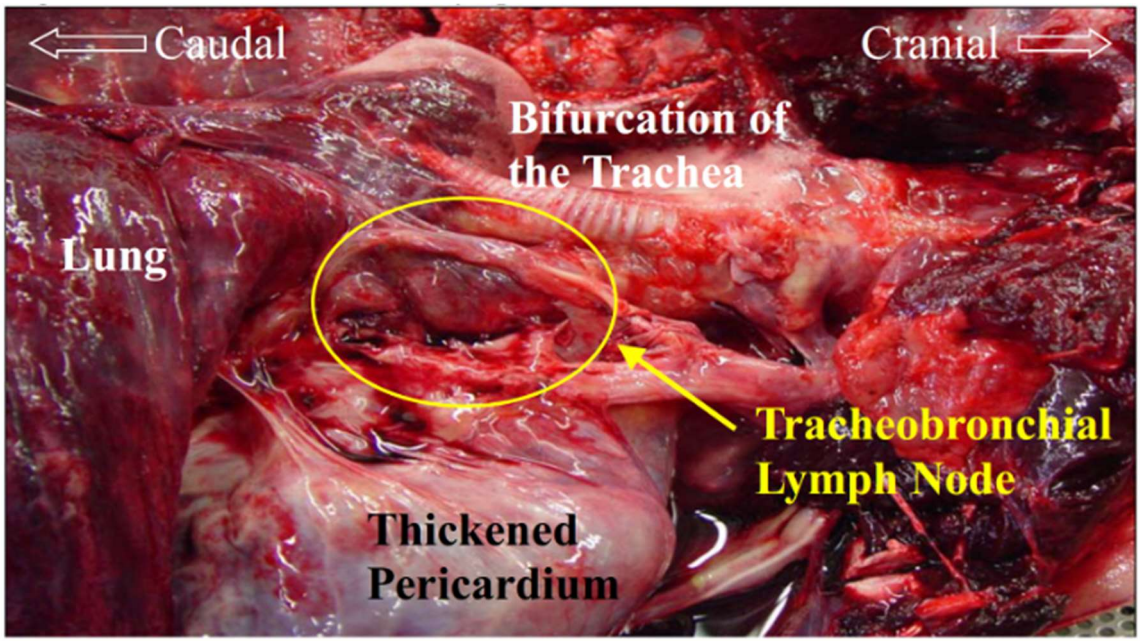


Figure 4.28 Location of the cetacean tracheobronchial lymph node in situ (Pugliares et al., 2007).

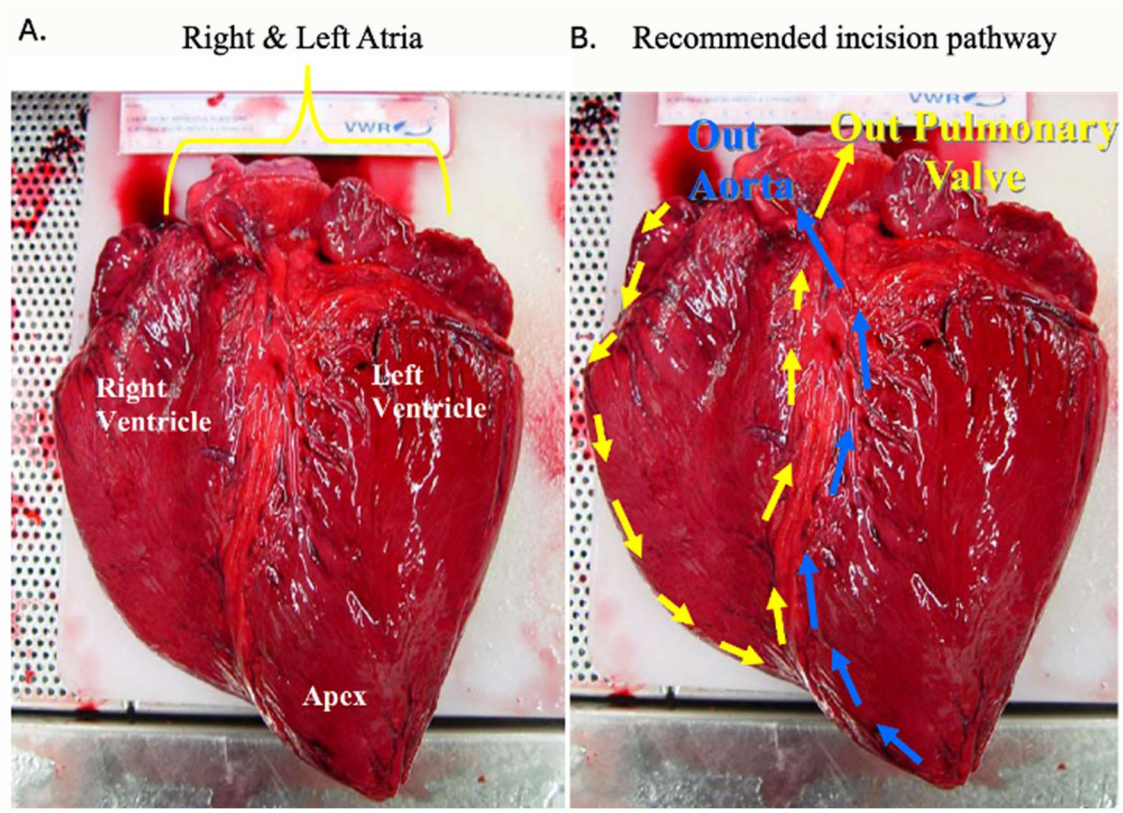


Figure 4.29 A) External structures of the heart; B) Recommended incision pathway to open the heart (Pugliares et al., 2007).

Identify liver, gall bladder (not present in cetaceans) (Figures 4.30 and 4.31), and male/female reproductive organs (cetacean: Figure 4.32; pinniped Figure 4.33). Lift the kidney from the abdominal wall and follow the ureters to the bladder (Figure 4.32). The adrenals (which can indicate recent stress) are located craniomedial to the kidneys under the (sometimes thick) peritoneal membrane (Figure 4.34). If indicated, aspirate urine and/or bile in a sterile syringe and chill. If the entire reproductive tract is too large collect en block, collect the gonads. Dissect out and remove these organs for further sectioning- examine for abnormalities and collect samples. Be sure to open bile ducts to check for parasites. Examine the mesentery and collect mesenteric lymph node (Figure 4.35) (Pugliares et al., 2007).

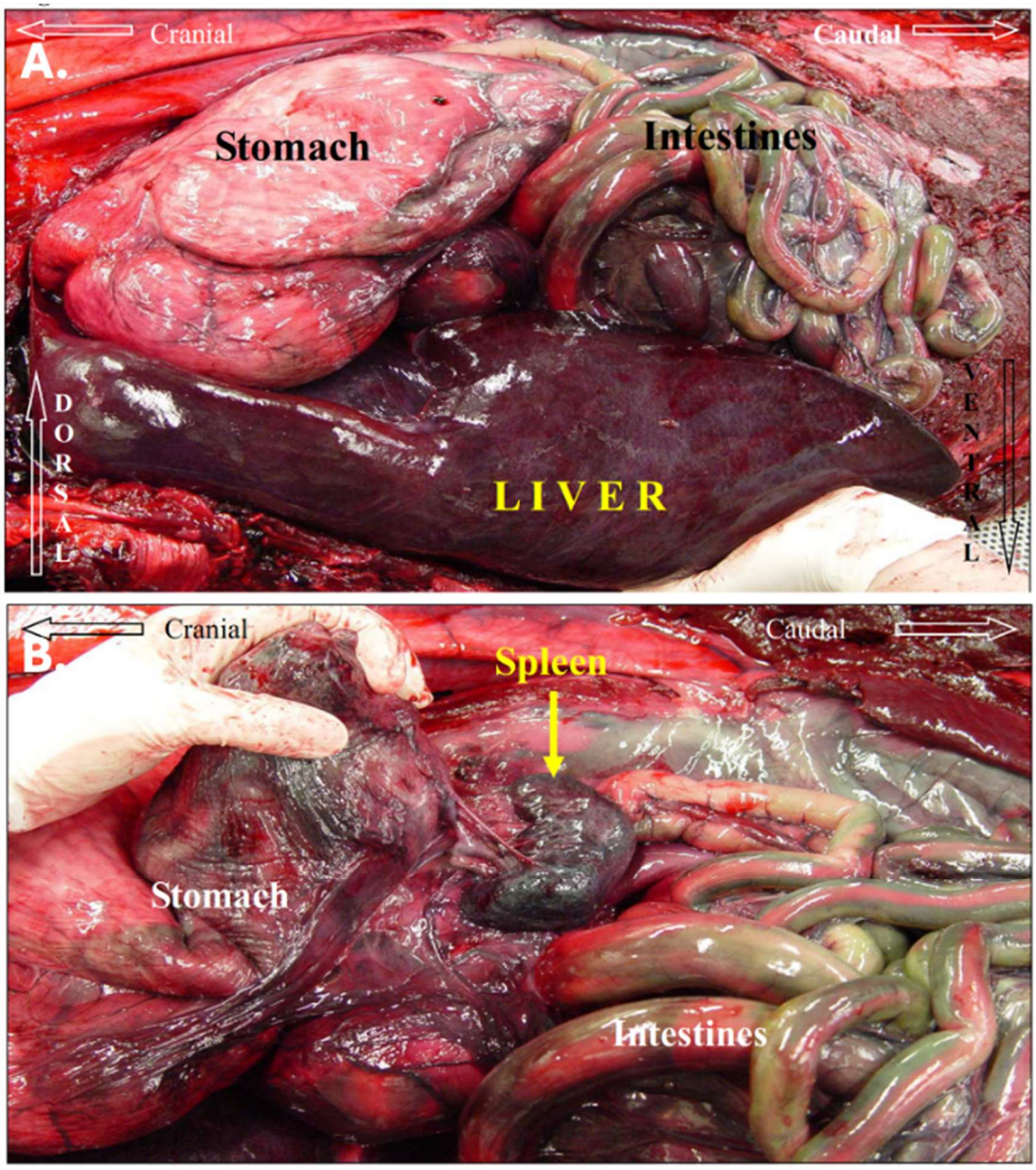


Figure 4.30 Cetacean abdominal organs in situ. Note: Cetaceans lack a gall bladder. A) Location of liver (which occupies most of the cetacean abdominal cavity), stomach and intestines; B) Location of spleen (shape/size varies among species) and intestines (with stomach lifted) (Pugliares et al., 2007).

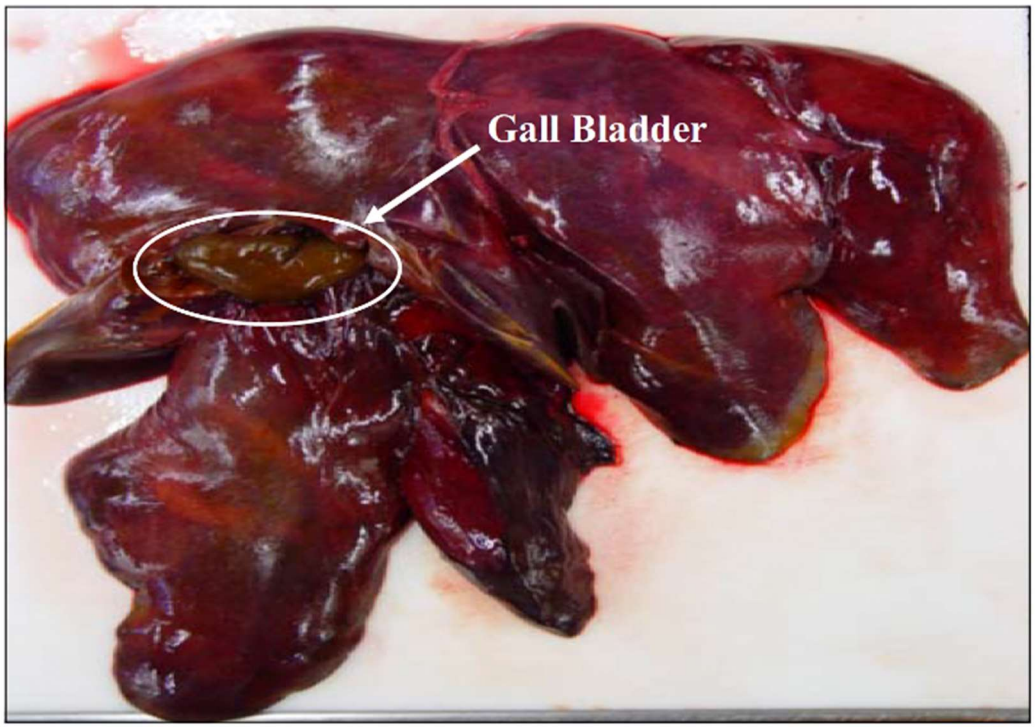


Figure 4.31 Pinniped liver with gall bladder (Pugliares et al., 2007).

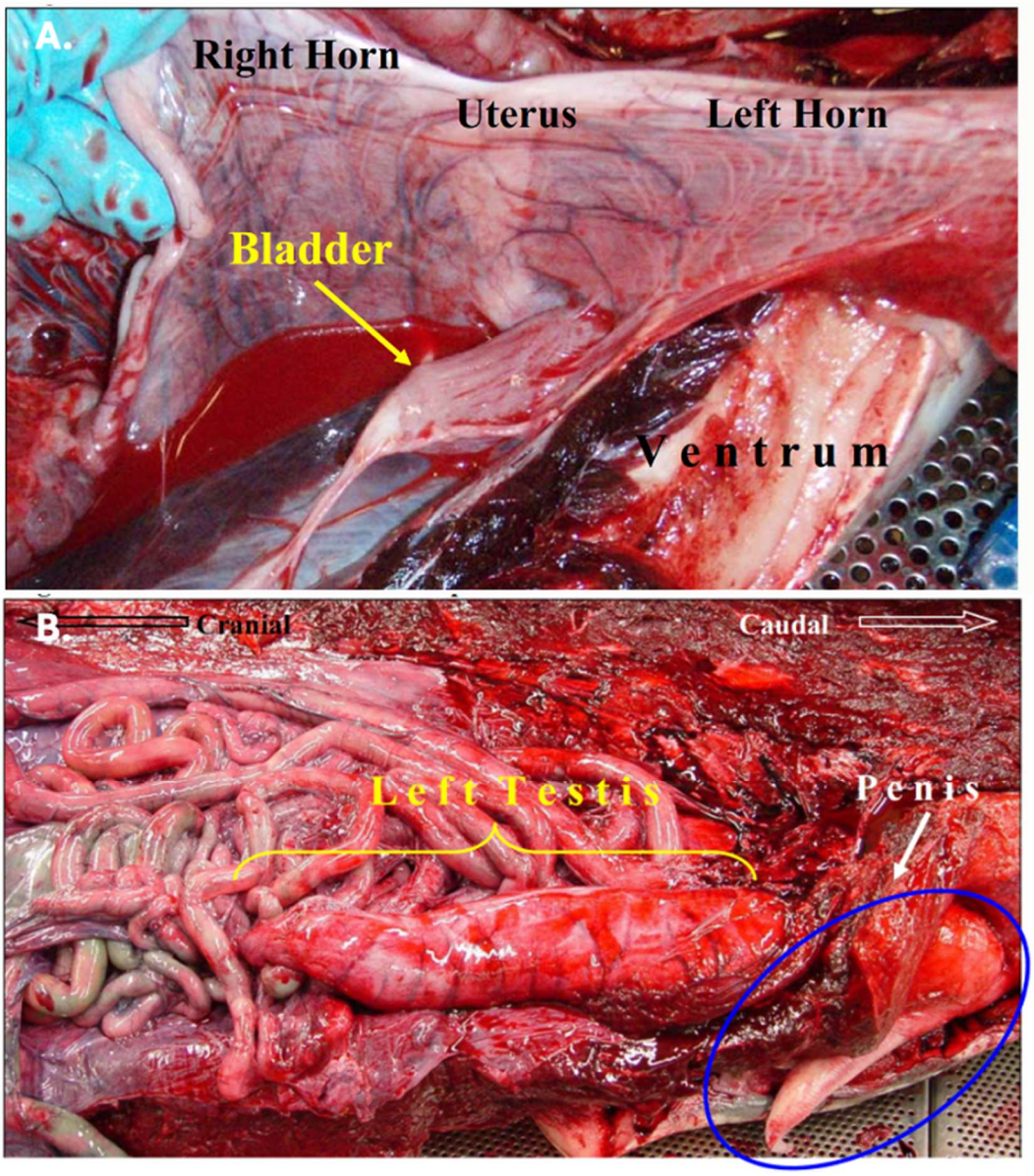


Figure 4.32 A) Location of cetacean female reproductive tract and urinary bladder in situ; B) Location of cetacean male reproductive tract in situ (Pugliares et al., 2007).

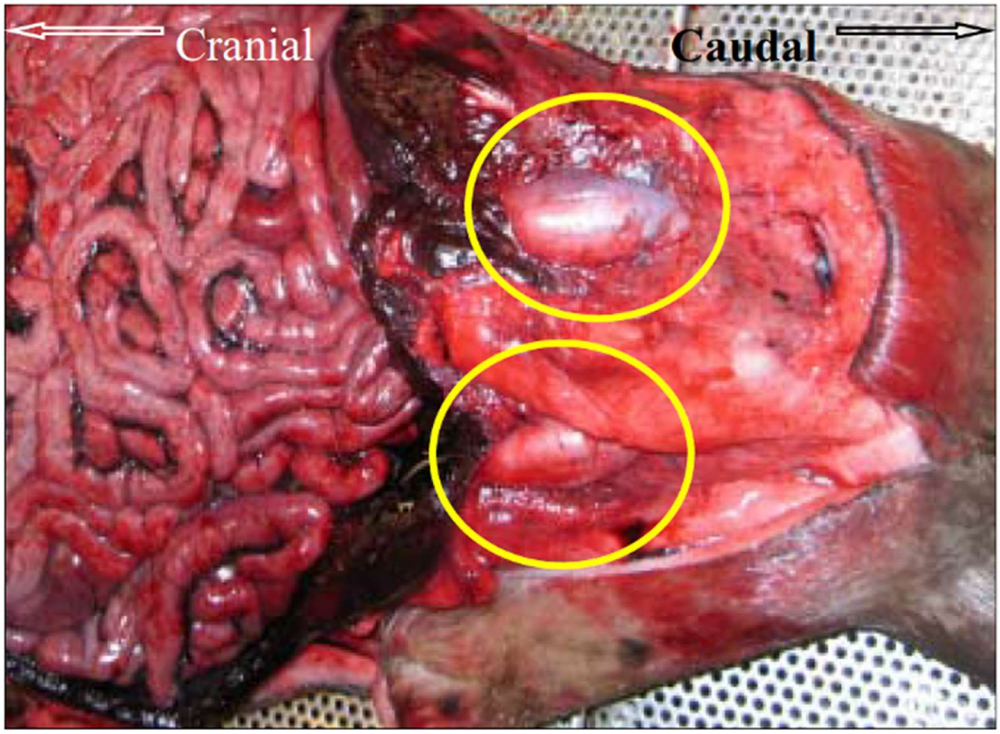


Figure 4.33 Paired pinniped testes are located outside of the abdominal cavity along the ventral body, proximal to the hip bones (Pugliares et al., 2007).

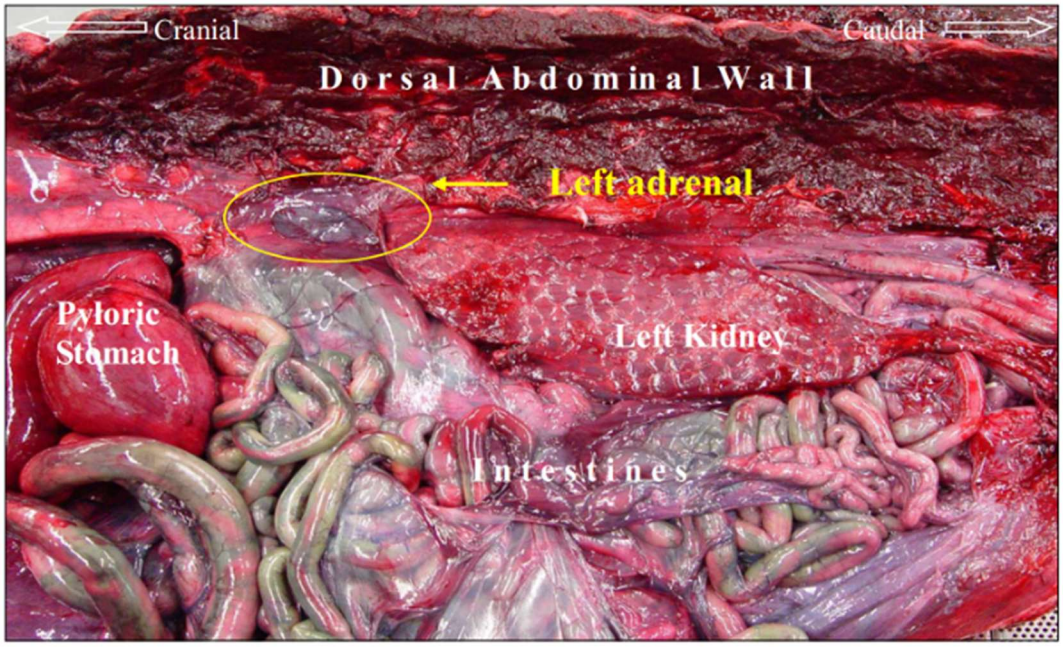


Figure 4.34 Location of cetacean adrenal gland and kidney in situ (Pugliares et al., 2007).

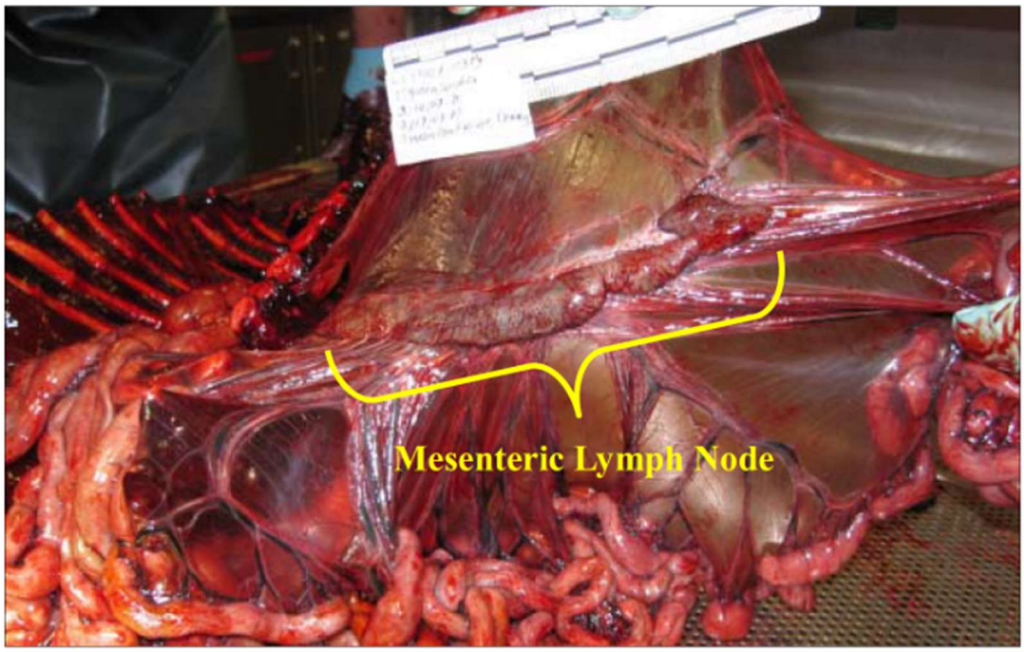


Figure 4.35 Mesenteric lymph node in a pinniped (Pugliares et al., 2007).

Tie the small intestine twice where it leaves the stomach and cut between the ties. Dissect out spleen (under the main stomach near the left abdominal wall) (Figure 4.30B) and pancreas (soft, irregular, pyramidal, tissue that is attached to the duodenal mesentery) and remove for sub-sectioning. Next, remove the stomach. If the stomach is too big to remove, collect representative samples of different regions (Pugliares et al., 2007). Collection of evaluation of stomach contents (which may include otoliths, macerated flesh of prey, skeletal remains, parasites, foreign bodies, and vegetation) is useful, both for assessment of prey and for diagnostic purposes. Fish otoliths, which are species specific and proportional to fish size, are frequently used to identify prey; however, keep in mind that neutral buffered formalin can dissolve otoliths, so it shouldn't be used to fix stomach contents in fish-eating animals; contents should instead be frozen or fixed in alcohol. For plant-eating species like manatees, stomach contents should not be frozen because fragmentation of sea grass and algae cells will occur, complicating identification. Toxicology samples should be collected from the fresh stomach contents when the carcass is first opened (then frozen for later evaluation); parasites can also be collected at this time. Foreign bodies, such as fishing gear, should be documented, photographed and saved (CRC ch13). Sampling of the intestines should be left until the end of the necropsy to avoid contaminating the other organs. Cut through attachments of the intestine to the dorsal abdominal wall and remove the intestines, dissecting out and sampling some mesenteric lymph nodes. If the GI tract is too big to remove fully then collect representative samples of each section (Raverty et al., 2018).

Sampling at necropsy (Pugliares et al., 2007):

- For culture, use a butane torch, heat a sterile stainless steel scalpel blade rinsed in ethanol, sear the intended incision site for 1 – 2 seconds, incise the tissue, swab inside the incision with a sterile culture swab, and place in appropriate culture medium. Do not freeze these samples.
- Recommended tissue samples vary by target pathogen, but for most virology assays, basic target samples include serum, lung, liver, spleen, lymph nodes and brain. Skin, mucocutaneous junctions and/or the oral cavity, rectum, and urogenital tract may also be indicated. If there is a fetus, include similar fetal tissue samples, as well as adrenal glands and placenta. See above for processing/storage recommendations. See for storage methods for various pathogens.
- Blubber, muscle, liver, and kidney are tissues typically used for detecting contaminants. Collect at least 100g and wrap each section of tissue in acetone washed aluminum foil (shiny side away from tissue). Place wrapped sample in a ziplock bag and store in a -20 ° C freezer.
- Biotoxins (often associated with algal blooms) are typically detected in the following tissues/fluids: Liver, kidney, serum, aqueous humor, stomach/intestinal contents, feces, and urine. Store tissue samples in ziplock bags and collect stomach/intestinal contents, feces, urine and aqueous humor into appropriately sized vials. Store at -80^{o}C unless testing will be performed immediately, then ship on dry ice.
- Sample transport considerations: See below for best practices.

Marine Mammal Carcass Disposal

Methods of marine mammal carcass disposal are divided into two broad categories (19):

- Allowing the carcass to remain in the environment typically involves leaving it to decompose naturally. This can take months to years; however, it is cost-effective, uncomplicated, and allows nutrients to be recycled into the natural environment. On the other hand, any toxins, medications (e.g., euthanasia drugs, particularly pentobarbital) and zoonotic pathogens can be released into the environment and/or could poison scavengers. Methods include:
    - Leaving the carcass in place- above ground, or in tidal zone/shallow water
    - Burial
    - Returning the carcass to the sea by towing it offshore
    - Sinking- the carcass is towed offshore and sunk

- Removing the carcass from the environment (typically to a disposal facility) is more logistically complicated, expensive, and often involves heavy machinery, which can damage the surrounding habitat. But, it minimizes the risk of infectious disease transmission to humans/animals and toxin release into the immediate environment. Methods include:
    - Burial in a licensed landfill
    - Rendering (at a plant)- tissues are repurposed for other uses
    - Incineration (at a licensed plant)
    - Composting at a designated compost facility

Birds

In birds, necropsy often starts with the carcass in ventral recumbency. About 70% of the feathers are cut or plucked to expose the skin and improve visualization for incisions. Careful observation of the feather tracts helps locate skin lesions and manage blood loss. Bird examination focuses on the transparency of the air sacs and the presence of fluids or plaques. There are two main approaches: 1) Complete removal of all organs from tongue to cloaca in one piece. 2) Selective removal of organs, leaving the lungs, kidneys, adrenal glands, and gonads (which lie near the vertebral column) in place for in situ examination. For gastrointestinal studies, the pancreas is often examined together with adjacent sections of the duodenum.

Key Points:

- Gross lesions are not a reliable indicator of avian influenza in wild birds.
- Died common terns in South Africa lacked visible lesions but sometimes showed microscopic brain inflammation (meningoencephalitis).
- Experimental infection of mallards with a virulent virus produced distinct signs (discrete purplish areas of lung consolidation and cloudy pleural surfaces).
- Because virulent viruses are uncommon in the wild, these severe lesions may not be typical of natural infections.

Cleanup and Carcass Disposal

Following necropsy, cleanup and carcass disposal are critical to prevent environmental contamination and disease transmission. Personal protective equipment (PPE) such as gloves,

masks, and protective clothing must always be used. Instruments and work surfaces should be thoroughly washed with soap and water before applying disinfectants. Effective disinfectants include broad-spectrum agents such as 0.5% sodium hypochlorite (bleach), 70% alcohol, or commercial products like Virkon®, which are selected based on the specific pathogens of concern and are recommended for avian influenza virus. Disposal of carcasses, sharps, and contaminated waste, whether in field or laboratory settings, should follow best-practice protocols developed in consultation with public health or wildlife authorities to ensure safe handling of suspected or confirmed infectious cases.

Disposal methods must consider personal safety, environmental impact, carcass size, proximity to water sources, the ability to transport carcasses safely, logistical or political challenges, and available facilities such as landfills or incinerators. Common disposal options include on-site incineration or burning, burial, rendering, landfill, fermentation, alkaline hydrolysis, composting, or natural decay at the necropsy site. On-site incineration or burial is often the most practical for mass mortality events. Burial should ideally be at least two meters deep to prevent scavenging and may require covering with lime to reduce pathogen persistence. If environmental factors make incineration or burial unfeasible, carcasses may need to be protected from scavengers until disposal is possible.

Sample Preservation and Storage

- Specimen preservation
  - For virus isolation
    - Swabs
      - Use sterile nylon flocked swabs (preferred)
      - Place immediately into 1–3 ml Viral Transport Medium (VTM)
      - Insert swab tip fully, break/cut shaft, and close cap tightly
      - Avoid to pool different swab types (e.g., oropharyngeal + cloacal) – cloacal swabs are more prone to contamination
      - If pooling, no more than 3 swabs of the same type from different birds should be combined, unless otherwise specified by the surveillance protocol.
    - Tissues
      - Cut 0.5–1 cm^3 pieces from target organs
      - Place samples immediately into VTM and ensure that tissue is fully submerged.

- Storage and transport
    - If the samples will be tested within 48 hours, store them upright at 2–8°C and maintain the cold chain with ice packs during transport.
    - If the samples will be tested after 48 hours, keep them upright on dry ice and store at −80°C as soon as possible.
    - Never leave the sample at room temperature for >1–2 h after collection

- For molecular detection
    - Swabs
        - Use sterile nylon flocked swabs (preferred)
        - Place into 0.5–1 ml VTM or nucleic acid preservation solution (e.g., DNA/RNA Shield) immediately after collection
        - Insert swab tip fully, break/cut shaft, and close cap tightly
        - No more than 3 swabs should be combined, unless otherwise specified by the surveillance protocol.
    - Tissues
        - Collect at least 0.2–0.5 cm^3 from target organ(s)
        - Place into VTM or nucleic acid preservation solution
        - Ensure sample volume ≤10% of preservative volume (v/v)
    - Storage and transport
        - If in VTM:
            - If the samples will be tested within 48 hours, store them upright at 2–8 °C and maintain the cold chain with ice packs during transport.
            - If the samples will be tested after 48 hours, keep them upright on dry ice and store at −20°C or −80 °C as soon as possible.
            - Never leave the sample at room temperature for >1–2 h after collection
        - If in DNA/RNA Shield:
            - Samples can be stored at temperatures between 4°C and 25°C for up to 30 days.
            - At 37°C, samples may remain stable for up to 3 days (however this is not recommended).
            - When stored at −20 °C or below, samples can be preserved for more than one year.

        - This stabilizing buffer has been validated to inactivate a number of pathogens

- For antibody detection

    - Transfer the blood into a clean, dry tube without anticoagulant.
    - Let it sit undisturbed at room temperature for 20–30 minutes (or until a clot forms).
    - If a centrifuge is available in the field, centrifuge the tube at 1,000–2,000 × g for 10 minutes. This will separate the serum from the clot. Store the serum upright at 2–8°C and maintain the cold chain with ice packs during transport.
    - If centrifugation is not possible at the field site, store the blood upright at 2–8°C and maintain the cold chain with ice packs during transport.
    - Store serum at 4 °C if testing within 1 week.
    - For longer storage, freeze serum at −20 °C or −80 °C.
    - Avoid repeated freeze–thaw cycles by aliquoting if necessary.

- For histopathology

    - To collect tissue samples from organs suspected of infectious disease for microscopic examination using H&E staining and other special stains.

        - **Target Organs:** Trachea, Lung, Air sacs, Brain (cerebrum, cerebellum, brainstem), Heart, Liver, Spleen, Kidney, Intestine, Pancreas.

            - Cut tissue pieces approximately 0.5–1 cm, with thickness ≤ 0.5 cm.
            - Immerse in 10% Neutral Buffered Formalin at least 10 times the volume of the tissue.
            - Fix for at least 24–48 hours at room temperature.
            - Label clearly (species, sample ID, date, organ).

- For Immunohistochemistry; IHC

    - To directly detect viral antigens in tissue sections using specific antibodies.

        - Select organs with visible lesions or suspected infection.
        - Cut tissue thickness ≤ 0.5 cm, fix in 10% NBF or 4% PFA.
        - Avoid delays > 30 minutes postmortem before fixation.

- For Immunocytology, ICC

    - To prepare cell smears from swabs or impression smears for detection of viral antigens using immunological methods.

        - Prepare a thin, even smear on a glass slide.
        - Air dry for 2–5 minutes.

- Fix in acetone (-20°C) for 10 min or 4% paraformaldehyde for 10 min.
- Store at 4°C (≤ 24 h) or -20°C for longer storage (1–3 months).

Table 4.3. Summary of sample preservation and storage

Test Type	Preservation /Fixative	Storage Temperature	Recommended Duration	Notes
Virus isolation	Submerged in 1–3 ml VTM	2–8°C	≤ 48 hours	Avoid leaving the sample at room temp for >1–2 h after collection
		-80°C	up to a year	
Molecular detection	Submerged in 0.5–1 ml VTM	2–8°C	≤ 48 hours	
		-20°C	2–3 months	
		-80°C	≥ 1 year	
	Submerged in 0.5–1 ml DNA/RNA shield	4 –25°C	≤ 30 days	Note that use of DNA/RNA shield may decrease the sensitivity of molecular testing and may not be adequate for sequencing
		37°C	≤ 3 days	Not recommended
		-20 °C	≥ 1 year	
		-80 °C	long term storage	
Antibody detection	Serum	2–8°C	≤ 1 week	
		-20 or -80°C	long term storage	
Histopathology	10% Neutral Buffered Formalin (NBF)	Room temp (20–25°C)	6–12 months (in fixative)	Replace fixative if stored >6 months

	Paraffin block (after processing)	Room temp	≥10 years	Archival storage; protected from moisture/heat
IHC	10% NBF	Room temp (in fixative)	1 month before processing	Process promptly for optimal antigen preservation
	4% Paraformaldehyde (PFA)	4°C	≤1 week before processing	Avoid prolonged storage to prevent antigen masking
	Paraffin block (processed)	Room temp	2–5 years	Antigenicity may decrease over time
ICC	Acetone (-20°C)	-20°C	2–3 months	Store in airtight slide boxes to avoid moisture
	4% PFA	4°C	≤24 h	For immediate staining only
	4% PFA	-20°C	1–2 months	Longer storage may reduce staining intensity

Sample transportation

- Within-country transport
  - Packaging and labeling
    - Follow the triple packaging system according to biosafety standards, consisting of:
      - Primary container: Leak-proof tubes containing the sample (e.g., swabs in VTM, tissue samples). Ensure caps are tightly closed and securely sealed (e.g., with parafilm).
      - Secondary packaging: A durable, leak-proof container or sealed plastic bag. For liquid samples, sufficient absorbent material must be placed between the primary container and the secondary packaging to absorb all fluid in case of leakage.
      - Outer packaging: A rigid outer box that protects the inner contents from physical damage during transport. The outer packaging must be sturdy enough to withstand normal transport conditions.

Basic triple packing system (Figure adapted from WHO Guidance on Regulations for the Transport of Infectious Substances 2021–2022).

- The outer packaging must clearly display the following information:
    - Name, address, and telephone number of the sender and recipient.
    - The biohazard symbol and any hazard symbols, such as those indicating the presence of dry ice, should be clearly displayed as required.
    - Any additional handling instructions or warnings as required by regulations.

- Temperature control

- Maintain the cold chain by using ice packs for samples stored at 2–8°C or dry ice for frozen samples.

■ Documentation

- Include internal transport permits or approvals if required by local regulations.
- Ensure that the courier or vehicle used has experience in handling biological materials.

■ Notification

- Inform the receiving laboratory in advance to prepare for sample arrival and processing.
- Samples must be received and delivered only at the designated meeting point.

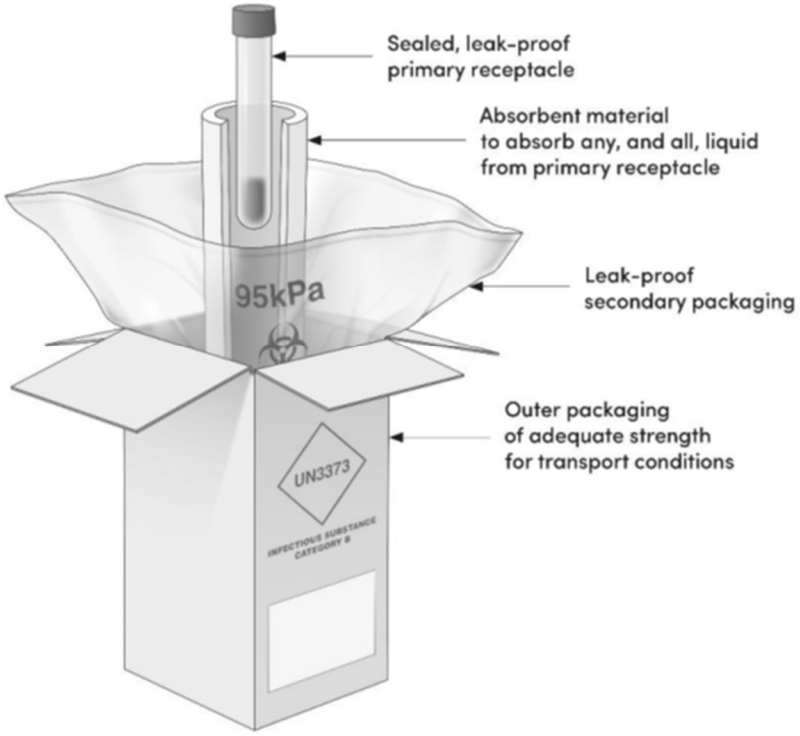
○ International transport

■ Regulatory compliance

- Comply with the International Air Transport Association (IATA) Dangerous Goods Regulations for air transport of infectious substances.
- Prepare all necessary customs declarations and secure relevant import/export permits from the competent authorities in both the exporting and importing countries.
- Ensure that all personnel involved in shipment preparation have received appropriate training to competently fulfill their responsibilities, including:
    - Procedures and methods to prevent accidents (e.g., correct operation of package-handling equipment and proper stowage of dangerous goods).
    - Accessing and applying emergency response information.
    - Awareness of general hazards associated with various classes of dangerous goods.
    - Preventing exposure to hazards, including correct use of personal protective equipment (PPE).
    - Protocols to follow in the event of accidental release or exposure to dangerous goods.

■ Packaging and labeling

- Employ the triple packaging system in accordance with Packing Instruction P650.



(Figure adapted from WHO Guidance on Regulations for the Transport of Infectious Substances 2021–2022).

- Animal samples suspected of avian influenza, when transported for diagnostic purposes, must be classified as UN 3373 – Biological Substance, Category B, with the corresponding "UN 3373 Biological Substance, Category B" label affixed to the external surface of the outer packaging.

(Figure adapted from WHO Guidance on Regulations for the Transport of Infectious Substances 2021–2022).

- When coolants are used, comply with specific packaging requirements:

- Wet ice/ice packs: Wet ice is not considered a dangerous good; however, outer packaging must be leakproof to prevent water leakage during melting.
- Dry ice (carbon dioxide, solid): Assigned UN 1845; outer packaging must be constructed from materials that permit safe venting of carbon dioxide gas (e.g., Styrofoam).
- Liquid nitrogen (nitrogen, refrigerated liquid): Assigned UN 1977; primary and secondary packaging must be able to withstand extreme low temperatures without structural compromise.
- Formalin/PFA (Fixed specimens) : clearly mark chemical hazard.

- Documentation
  - Include all shipment-related documents, such as the sample submission form (Supplementary Table 1), pathogen safety data sheets (PSDS), Chemical Safety Data Sheets (SDS) for Formalin/PFA or other chemicals used, import/export permits, and customs documentation.
  - Maintain clear and timely communication with the receiving laboratory regarding the expected arrival date and any specific handling instructions.
- Carrier Requirements
  - Engage a specialized courier service with demonstrated expertise in the international shipment of biological materials.
  - Couriers may advise senders on the required shipping documents and provide guidance for their accurate completion.
  - Monitor shipment progress through tracking systems and promptly confirm receipt upon delivery.

Quiz

Question 1: When collecting viral samples from live birds for Avian Influenza detection, which combination of factors is critical for maintaining sample integrity and maximizing diagnostic accuracy?
A) Using cotton-tipped swabs, pooling samples from multiple birds, and storing at room temperature
B) Utilizing Dacron or rayon-tipped swabs, an appropriate number of pooling samples, and maintaining a cold chain while avoiding contamination
C) Collecting only cloacal swabs, using any available swab type, and processing samples immediately
D) Prioritizing blood samples over swabs, using large-volume collection techniques

Correct Answer: B) Utilizing Dacron or rayon-tipped swabs, an appropriate number of pooling samples, and maintaining a cold chain while avoiding contamination
Rationale:

- Specifically addresses the text's recommendations about swab types (avoiding cotton or wooden-stemmed swabs)
- Reflects the guidance on an appropriate sample pooling
- Emphasizes the importance of sample preservation and preventing contamination
- Demonstrates a nuanced understanding of viral sample collection techniques

Question 2: During avian influenza surveillance necropsies, which combination of factors is most critical for maintaining sample integrity and preventing cross-contamination?

A) Using sterile instruments for each tissue, maintaining cold chain (2-8°C), and avoiding repeated freeze-thaw cycles
B) Wearing standard laboratory gloves and using general-purpose containers
C) Performing necropsy at room temperature and using any available storage method
D) Collecting samples without specific preservation guidelines

Correct Answer: A) Using sterile instruments for each tissue, maintaining cold chain (2-8°C), and avoiding repeated freeze-thaw cycles
Rationale: This answer reflects multiple critical aspects mentioned in the text:

- Preventing cross-contamination by using sterile, separate instruments for each tissue
- Preserving RNA integrity through proper temperature control
- Protecting sample quality by avoiding degradation from temperature fluctuations

Question 3: When collecting tissue samples for histopathology, what is the recommended fixative-to-tissue ratio and maximum tissue thickness?

A) 5:1 ratio, with tissues up to 1 cm thick
B) 1:5 ratio, with tissues up to 0.5 cm thick
C) 10:1 ratio, with tissues ≤0.5 cm thick
D) 2:1 ratio, with tissues up to 1.5 cm thick

Correct Answer: C) 10:1 ratio, with tissues ≤0.5 cm thick

Question 4: When collecting samples from a marine mammal carcass for comprehensive pathogen detection, which sampling strategy demonstrates the most sophisticated understanding of diagnostic protocols?
A) Collecting only fresh tissue samples and storing them at room temperature
B) Utilizing a systematic sampling approach that includes multiple tissue types, preserving samples with appropriate methods for different diagnostic targets, and maintaining a detailed metadata record
C) Focusing solely on blood and swab samples without considering tissue-specific preservation techniques
D) Randomly collecting samples without considering the potential degradation of biological materials

Correct Answer: B) Utilizing a systematic sampling approach that includes multiple tissue types, preserving samples with appropriate methods for different diagnostic targets, and maintaining a detailed metadata record

Rationale: This answer reflects the comprehensive sampling strategy outlined in the text, which emphasizes:

- Collecting samples from multiple tissue types (serum, lung, liver, spleen, lymph nodes, brain)
- Using appropriate preservation methods (e.g., flash-freezing, stabilization buffers)
- Maintaining detailed metadata (date, location, species, collection method, storage conditions)
- Considering downstream diagnostic assays during sample collection

Here's a challenging quiz question that tests advanced knowledge of sample preservation and transportation protocols:

Question 5: When preparing to transport an avian influenza virus sample internationally, which combination of factors demonstrates the most comprehensive understanding of biosafety and regulatory compliance?

A) Using a standard shipping envelope, labeling with a generic "fragile" sticker, and transporting at room temperature

B) Implementing the triple packaging system (UN 3373), using appropriate coolants, securing necessary permits, and ensuring personnel are trained in dangerous goods handling protocols
C) Packaging the sample in a plastic bag and shipping via standard postal service
D) Focusing solely on temperature control without considering packaging or regulatory requirements

Correct Answer: B) Implementing the triple packaging system (UN 3373), using appropriate coolants, securing necessary permits, and ensuring personnel are trained in dangerous goods handling protocols

Rationale:
This answer comprehensively addresses the critical aspects of international biological sample transportation outlined in the text

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Resources

- WOAH training manual on wildlife disease outbreak investigation: https://www.woah.org/fileadmin/Home/eng/Internationa_Standard_Setting/docs/pdf/WGWildlife/A_Training_Manual_Wildlife_4.pdfU.S. Centers for Disease Control and Prevention. Guidelines for laboratory biosafety: handling and processing specimens associated with novel influenza A viruses, including potential A (H5N1) virus [Internet]. 2025 [cited 2025 Oct 11]. Available from:https://www.cdc.gov/bird-flu/php/severe-potential/guidelines-for-laboratory-biosafety.html#:~:text=Manipulate%20diagnostic%20specimens%20in%20a%20certified%20Class%20II%20Biosafety%20Cabinet%20(BSC)&text=Avian%20influenza%20or%20bird%20flu%20refers%20to%20the%20disease%20caused%20by.
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Chapter V: Environmental Surveillance

Pre-course Materials

- Introduction to Environmental Surveillance
- Sample Types and Sources in Environmental Surveillance for Avian Influenza
- Sampling and Handling
- Data Management & Interpretation

Skills and Knowledge to be Acquired

- Learning outcomes:
  - To explain the principles of detecting pathogens in environmental DNA samples
  - To interpret data and identify key biosafety practices related to pathogen detection in field-derived eDNA.
- Contents:
  - Hands-on session:
    - Environmental sample collection
    - DNA preparation for metagenome analysis
    - Pathogen detection using eDNA and interpretation
    - Avian Influenza detection by real-time RT-PCR

Evaluation

- Practical: correct sampling technique
- Quiz: Basic concepts of eDNA and sample collection & processing (See at the end of this chapter)

Introduction to Environmental Surveillance

Environmental surveillance for Avian Influenza (AI) refers to the systematic monitoring of environmental samples to identify the presence and circulation of avian influenza viruses (AIV) in areas where domestic poultry, wild birds, and other animal species interact. In contrast to clinical surveillance, which primarily focuses on detecting infection in individual birds, environmental surveillance targets shared habitats and resources, where viruses may accumulate and persist. This approach acknowledges the critical role of the environment as both a reservoir and a transmission pathway for AIV, thereby providing an essential early warning system that can detect viral activity prior to the onset of clinical outbreaks in domestic or wild bird populations.

The scope of environmental surveillance extends beyond the detection of viral RNA; it also provides critical insights into the ecology, persistence, and transmission dynamics of

AIV. Aquatic environments such as ponds and wetlands frequently serve as natural convergence points for domestic and migratory bird populations, facilitating both viral transmission and genetic reassortment. Similarly, soil, sediments, and fomites including cages, litter, and feeders which serve as potential reservoirs that enable virus persistence and promote its transmission across poultry and wildlife settings. By systematically targeting these environmental matrices, surveillance activities can elucidate the spatial and temporal patterns of virus circulation, thereby strengthening risk assessment and supporting timely interventions for outbreak prevention.

Integrating environmental surveillance within the One Health framework further emphasizes its importance. Avian influenza continues to pose a significant zoonotic threat, with highly pathogenic strains such as H5N1 and H7N9 demonstrating the capacity to spill over into human populations. Through systematic monitoring of environmental sources, authorities are able to detect early indications of viral activity, elucidate cross-species transmission pathways, and enhance their capacity to implement interventions prior to the outbreaks. This integrated approach ensures that avian, human, and environmental health are addressed holistically, thereby reducing risks at the interface between animals, humans, and ecosystems.

The practical application of environmental surveillance has demonstrated its effectiveness across diverse settings. In aquaculture ponds, analysis of water samples has identified contamination by wild migratory birds, which frequently serve as natural reservoirs of AIV. Wetlands have been recognized as critical hotspots for viral diversity, reflecting their role as congregation sites for waterfowl along migratory flyways. On poultry farms, litter, drinking water, and surrounding soils are commonly found to be contaminated with AIV during outbreaks, underscoring the necessity of routine environmental monitoring. Live bird markets, where humans and poultry interact closely, also represent key environments for surveillance, with wastewater and cage surfaces often harboring viral genetic material. Collectively, these examples illustrate how environmental surveillance complements clinical and virological monitoring, thereby enhancing preparedness and response to avian influenza at both local and global levels.

Sample Types and Sources in Environmental Surveillance for Avian Influenza

a. Water sources

Water is one of the most important samples for environmental surveillance of Avian Influenza (AI). Ponds, rivers, wetlands, and drinking water troughs used by domestic poultry or wild birds are routinely sampled because infected birds can shed avian influenza viruses (AIV) into these sources. The virus can remain viable in water for extended periods, especially in cold temperatures conditions, allowing it to persist in the environment and serve as a natural reservoir. Monitoring water sources therefore provides valuable

information on viral circulation in both farmed and wild bird populations, serving as an effective early warning system for potential outbreaks.

b. Soil and sediment

Soil and sediment collected from pastures, animal pens, market floors, and natural habitats of wildlife can serve as reservoirs of AIV. Viral particles from bird feces or other environmental deposition may contaminate these substrates and persist for variable durations depending on environmental conditions such as pH, temperature, and moisture. Testing soil and sediment helps identify contaminated sites that could facilitate transmission to domestic poultry or act as points of exposure for wild birds and other animals. This sampling is especially relevant in areas where high densities of birds are present, such as live bird markets or wetlands used by migratory flocks.

c. Feed and fomites

Feed, feeders, cages, equipment surfaces, and other fomites are important points of indirect transmission in both farms and live bird markets. AIV can survive on these surfaces long enough to infect susceptible birds when biosecurity practices are weak. By monitoring these materials, surveillance programs can detect contamination and guide interventions such as cleaning, disinfection, and improved handling of feed and equipment. Such measures are essential to reduce the risk of virus spread within and between poultry facilities.

d. Waste and excreta

Waste materials, including manure, sludge, discarded litter, and bird droppings, are commonly contaminated with AIV shed from infected birds. Because birds often excrete large amounts of virus, fecal material provides a highly sensitive sample type for surveillance. Wastewater from farms or markets can also be tested as a composite sample reflecting the health status of large bird populations. Surveillance of waste and excreta not only enables detection of ongoing viral circulation but also helps estimate the scale of infection in poultry or wild bird communities.

e. Wild bird habitats

Habitats used by migratory and resident wild birds are key sampling locations because wild birds are natural reservoirs and long-distance carriers of AIV. These include wetlands, lakes, rice paddies, and other stopover points along migratory flyways. Sampling in these areas provides insights into the introduction and spread of AIV across regions and helps identify high-risk interfaces between wild and domestic birds. Continuous monitoring of wild bird habitats is crucial for global surveillance efforts, as it supports early detection of emerging subtypes and informs preventive measures to protect both poultry industries and public health.

Sampling and Handling

f. Proper sampling techniques

The reliability of environmental surveillance results depends largely on how samples are collected. Using appropriate sampling techniques ensures that the sample truly represents the target environment and minimizes bias or error. Commonly applied approaches include:

i. Grab samples: Grab sampling involves the collection of a single sample at a specific point in time and place. This technique provides a snapshot of the viral presence in that environment and is simple, quick, and cost-effective. However, because it represents only one moment, it may not reflect fluctuations in virus concentration over time.

ii. Composite samples: Composite sampling combines multiple subsamples taken at different times or from different locations into a single pooled sample. This method provides a more representative picture of overall viral circulation, reduces sampling variability, and is often used when the goal is to monitor broader trends rather than immediate presence.

iii. Passive samplers: Passive sampling methods involve leaving absorbent or specialized materials in the environment for an extended period, allowing continuous accumulation of viral particles. This approach is useful in areas where repeated active sampling is difficult, and it increases the likelihood of detecting low levels of virus that may be intermittently shed into the environment.

g. Sample preservation, transport, and storage

The quality of laboratory results is directly linked to how well samples are preserved and transported after collection. Viral RNA is highly sensitive to degradation; therefore, proper handling is essential to maintain its integrity and detectability. Key considerations to be taken into account are as follows:

i. Preservation: Once collected, samples must be preserved under controlled conditions to prevent degradation of viral RNA. Immediate cooling to 4 °C is recommended if analysis will occur within 24–48 hours, whereas long-term storage requires freezing at −80 °C. The most critical factors to consider are maintaining a cold chain, preventing cross-contamination, and using sterile, nuclease-free containers.

ii. Transport: During transport, samples should be packed in leak-proof primary containers, placed inside sealed secondary packaging, and then secured within insulated outer packaging containing ice packs or dry ice. This three-layer system complies with international standards for transporting biological specimens.

iii. Storage: In the laboratory, samples should be stored according to their intended use. Short-term storage may be at 4 °C for a few days, but for molecular testing or virus isolation, −80 °C is required to maintain RNA integrity and viral viability.

h. Biosafety and biosecurity measures

Sampling activities may expose personnel to infectious agents and risk spreading pathogens to new environments. Implementing strict biosafety and biosecurity practices helps safeguard the health of both people and animals while ensuring the integrity of the surveillance process. Essential measures include:

i. Personal protection: Personnel involved in sampling must wear appropriate personal protective equipment (PPE), such as gloves, masks, protective clothing, and eye protection, to minimize exposure to potentially infectious materials.

ii. Safe practices: All handling should be performed in a manner that avoids generating aerosols. Tools and equipment must be disinfected before and after use, and contaminated waste should be disposed of according to biosafety protocols.

iii. Containment and compliance: Field and laboratory activities must follow institutional, national, and international biosafety regulations. Samples suspected to contain avian influenza should be managed under the appropriate biosafety level (BSL) conditions to ensure both personnel safety and prevention of accidental virus release.

i. Documentation and metadata recording

Accurate and complete documentation transforms raw samples into usable scientific data. Recording detailed metadata ensures traceability, reproducibility, and meaningful interpretation of results, both locally and across international datasets. Recommended practices include:

i. Labeling: Each sample should be clearly labeled with a unique identifier, including information such as the date of collection, sample type, and collection site.

ii. Metadata: Detailed metadata should accompany each sample. This may include GPS coordinates, environmental conditions at the site, observed bird populations, and relevant farm or market management practices.

iii. Chain of custody: A record of how each sample was handled—from collection, through transport, to laboratory receipt—must be maintained. This ensures transparency, enables reproducibility, and facilitates integration into national and international surveillance databases.

Data Management & Interpretation

j. Link environmental data with epidemiological data

Environmental surveillance generates diverse datasets, including viral detection in water, soil, feces, and fomites. To maximize their utility, these data must be integrated with epidemiological information, such as poultry population density, wild bird migratory patterns, outbreak records, and vaccination coverage. Linking environmental and epidemiological data enables a more comprehensive understanding of virus circulation, identifies high-risk areas, and supports early warning systems. For example, detecting viral RNA in a pond near a poultry farm, combined with local outbreak history, can help predict potential spillover events and guide targeted interventions.

k. Apply quality control measures.

Maintaining data reliability requires implementing rigorous quality control (QC) procedures at all stages of data collection, entry, and analysis. This includes verifying sample identifiers, checking laboratory results for consistency, and standardizing data entry protocols. Regular audits and cross-checks ensure that errors are minimized and data integrity is preserved. QC measures also extend to analytical procedures, where validation of molecular assays, proper calibration of equipment, and adherence to laboratory standard operating procedures guarantee accurate detection and quantification of viral material.

l. Report findings using standardized formats.

Effective communication of surveillance results is critical for timely decision-making and action. Findings should be reported using standardized templates that include metadata, sampling methods, laboratory results, and interpretative comments. Standardized reporting facilitates comparison across sites, time periods, and countries, supporting national and international surveillance networks. Visual representation, such as maps or graphs showing viral detection trends, can further enhance comprehension and support evidence-based management of avian influenza risks.

Quiz

Question 1: Which environmental factor most significantly influences avian influenza virus persistence in wetland ecosystems?

A) Air temperature
B) Water pH
C) Soil moisture content
D) Salinity levels

Correct Answer: B) Water pH

Rationale: Demonstrates understanding of specific environmental parameters affecting viral survival.

Question 2: In environmental AI surveillance, which sample type provides the most comprehensive viral detection potential?

A) Water samples
B) Soil sediments
C) Bird droppings
D) Feed and fomites

Correct Answer: C) Bird droppings

Rationale: Highlights the sensitivity of excreta in viral detection.

Question 3: What is the primary purpose of using composite sampling in environmental AI surveillance?

A) Reduce sampling costs
B) Increase sampling area coverage
C) Provide a more representative picture of viral circulation
D) Simplify laboratory processing

Correct Answer: C) Provide a more representative picture of viral circulation

Rationale: Reflects the nuanced approach to sampling methodology.

Question 4: Which statistical approach is most critical for correcting detection bias in environmental surveillance data?

A) Mean calculation

B) Probability adjustment
C) Median filtering
D) Standard deviation analysis

Correct Answer: B) Probability adjustment

Rationale: Addresses the complexity of interpreting environmental surveillance data.

Question 5: The One Health approach to AI surveillance primarily aims to:

A) Reduce veterinary costs
B) Integrate human, animal, and environmental health perspectives
C) Increase research funding
D) Simplify disease reporting

Correct Answer: B) Integrate human, animal, and environmental health perspectives

Rationale: Captures the holistic essence of the One Health approach.

Appendices

A. Glossary of Terms

Aerosol: a suspension of tiny particles or droplets in the air, <5um, such as dusts, mists, or fumes that may be pathogenic; these particles may be inhaled or absorbed by the skin, and can sometimes cause adverse health effects for workers. (see How did we get here: what are droplets and aerosols and how far do they go? A historical perspective on the transmission of respiratory infectious diseases) Accessed 11/08/25.

Anthropozoonotic: (out od date term) diseases or infections that primarily affect animals but can be naturally transmitted to humans; also known as reverse zoonotic disease transmission (Messenger et. al, 2014 accessed 11/08/25 NOTE: It is now acceptable to simply use the term '**zoonosis**' as a disease that can be naturally transmitted between vertebrate animals and humans. This is the more general term encompassing diseases transmitted in **both** directions. See the paper Zoonosis–Why we should reconsider "What's in a name? (accessed 11/8/25).

Biohazard: is a biological substance that poses a threat to the health of living organisms (Source)

Biosafety: methods that are used to stop a biohazard from spreading out of a place or causing harm (Cambridge dictionary accessed 11/08/25).

Biosecurity: the measures taken to minimize the risk of infectious diseases caused by viruses, bacteria or other microorganisms entering, emerging, establishing or spreading in a defined area (country, city, laboratory, building, etc.), potentially harming human or animal populations, food security and economy (Australian Government, Department of Health accessed 11/8/2025).

Direct transmission: the spread of disease directly from one infected person or animal to another. (In the Centers for Disease Control and Prevention archive accessed 11/8/25)

Droplet: pathogen-containing fluid particles >5 µm in diameter (Purens, 2020 accessed 11/08/25)

Endemic: the constant presence and/or usual prevalence of a disease or infectious agent in a population within a geographic area (See the Australian Centre for Disease Control. Accessed 11/8/25)

Epidemic: an increase, often sudden, in the number of cases of a disease above what is normally expected in that population in that area; occurs when an agent and susceptible hosts are present in adequate numbers, and the agent can be effectively conveyed from a source to the susceptible hosts (See the Australian Centre for Disease Control. Accessed 11/8/25)

Indirect transmission: occurs when a disease is passed from one individual or animal to another, without direct contact between them. (In the Centers for Disease Control and Prevention archive accessed 11/8/25)

Infectious: Capable of spreading disease; also known as communicable (See the Australian Institute for Health and Welfare. accessed 11/8/25).

Pandemic: an epidemic that has spread over several countries or continents, usually affecting a large number of people (See the Australian Centre for Disease Control. Accessed 11/8/25)

Pathogen: a biological agent that causes disease or illness to its host (Science Daily accessed 11/08/25)

Risk: The likelihood of an occurrence multiplied by the consequences of that occurrence defines the risk of the occurrence (Woodruff 2005 and the WOAH Training Manual on Wildlife Health Risk Assessment in support of Decisions and Policies accessed 11/08/25)

Stakeholder: individuals or organizations that have an interest in or are affected by your initiative and/or evaluation and/or its results (NHMRC Australia Accessexd 11/08/25

Transmission: the way pathogens move from an environment, animal, person, or object to another (In the Centers for Disease Control and Prevention archive accessed 11/8/25)

Well-being: This has many definitions depending on the discipline. This definition is widely used in public health: the state of feeling healthy and happy (Cambridge dictionary, accessed 11/08/25); for additional information on well-being as a public health tool, please see this WHO site on promoting wellbeing.

B. Regulatory Bodies and Influential Organisations in the World of Biosafety

Regulatory agencies enforce safety standards to promote and maintain human health. Sanctuaries, where animal and human interaction is frequent, involve increased risk in occupational procedures such as handling biological samples from various species of non-human primates, performing medical procedures involving non-human primates, and day-to-day husbandry activities. These procedures and settings, which are necessary for sanctuary success, result in increased risk of exposure to infectious agents at the human/animal interface. For this reason, maintaining biosafety through regulatory agencies is essential in protecting and promoting employee health and well-being. The following links are for global, european, american, asian, and african agencies and networks that may be involved at the sanctuary level or influence the day-to-day biosafety operations of sanctuaries, specifically in the context of COVID-19.

Global

1. Legal Atlas Team (pdf file): Regulatory Frameworks Governing Wet Markets, Wildlife, and Zoonotic Disease
2. World Health Organization (WHO)
3. The Food and Agriculture Organization of the United Nations (FAO) and Emergency Centre for Transboundary Animal Diseases (ECTAD) - accessed 11/08/25.
4. EEAP Flyway HPAI Archive. Accessed 11/08/25.

European Union and Independent Agencies

1. European Agency for Health and Safety at Work (EU-OSHA)
2. European Centre for Disease Prevention and Control (ECDC

United States

1. Centers for Disease Control and Prevention (CDC)
2. Department of Health and Human Services (HHS)
3. Department of Labor (DOL)

Africa

1. Africa CDC
2. Kenya Wildlife Service

Asia-Pacific

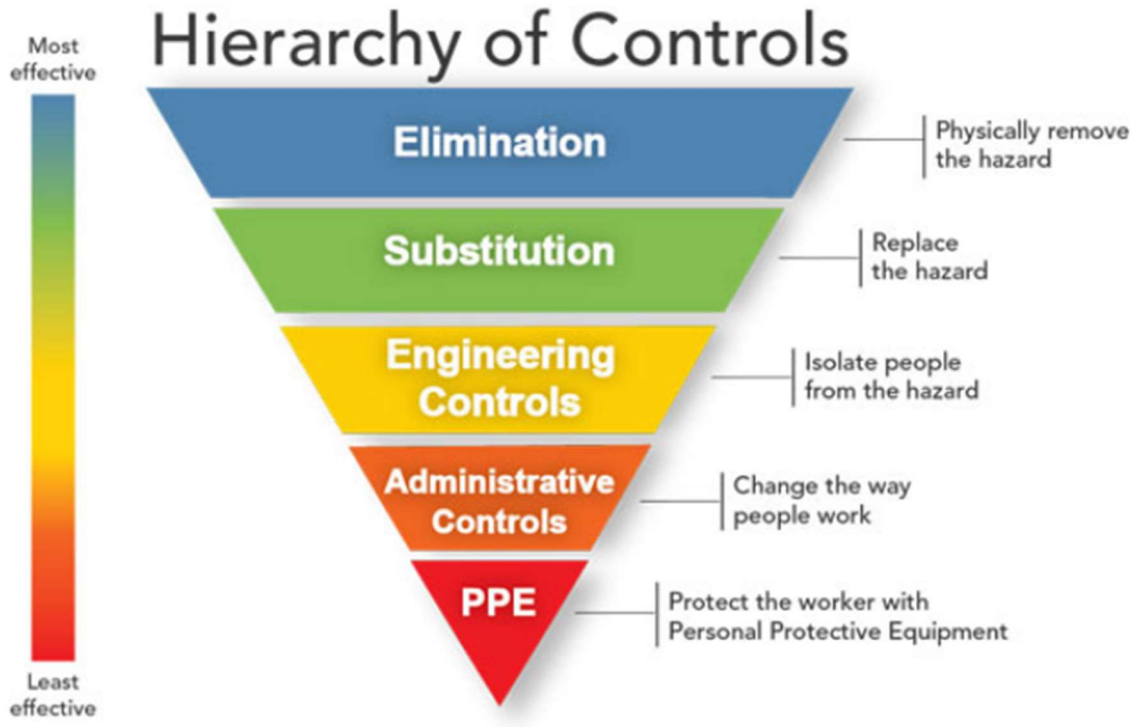
1. Indonesian Government CoVid-19 guidance
2. Government Legislation covering wildlife centre operations (Indonesia - others?)

Networks

European Biosafety Network

Asia-Pacific Biosafety Association

Modified Hierarchy of Controls for Field Settings



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Control Level	**Field-Specific Adaptation**
Elimination/ Substitution	Avoid direct contact with sick/dead wildlife
Engineering Controls	Use of containment bags, field decontamination stations
Administrative	SOPs for team roles, reporting, and health monitoring
PPE	Face coverings, gloves, boots, eye protection

Control Level	Veterinarian Adaptation	Ranger Adaptation
Elimination	Postpone non-urgent necropsies on suspect species	Avoid high-density roost areas during outbreaks
Engineering Controls	Mobile biosafety station with negative-pressure tent	Field decontamination mat and wash station
Administrative	Clinic SOPs for sample labeling, chain-of-custody logs	Patrol rotation schedules, health check-ins
PPE	N95/FFP2 respirator, goggles, disposable gowns, gloves	N95/FFP2 mask, nitrile gloves, waterproof boots

C. Avian Influenza A Virus Diagnostic Techniques

Avian influenza A viruses are highly contagious pathogens of birds, capable of causing severe economic losses in poultry and occasional zoonotic infections in humans. Rapid and accurate diagnosis is therefore essential for outbreak control, surveillance, and risk assessment. The main diagnostic approaches for avian influenza A virus are outlined below.

1. Virus isolation

Virus isolation is considered the gold standard for diagnosing avian influenza viruses (AIVs). The method involves taking a sample from wildlife and growing the virus in a controlled environment, most often by injecting a swab supernatant, or tissue suspension into the allantoic cavity of embryonated chicken eggs or into cell cultures. The process is time-consuming and labor-intensive, often taking several days to a week to complete. Virus isolation remains the definitive method for confirming the presence of an infectious and viable virus, which is crucial for fully characterizing the virus's subtype and pathogenicity.

1.1 Sample processing

Materials required

- Eagle’s Minimum Essential Medium (EMEM)
- Phosphate-buffered saline (PBS) pH 7.2-7.4
- Glass bead
- Surgical blade and scalpel
- Micropipette and tip
- Plastic plate
- Test tube
- Vortex mixer
- Centrifuge
- Class II biosafety cabinet
- Refrigerator
- Deep freezer

Procedure

1.1.1 Swab samples (Live & carcasses)

1. Add 2-3 small glass beads to the sterile tube containing the swab in the viral transport medium (VTM).
2. Thoroughly mix the tube on a vortex mixer to remove the cells from the swab.

3. Centrifuge the tube containing the swab suspension at 1500–1900 g at 4°C for 15–30 minutes.
4. Carefully transfer the supernatant into the new sterile tube. The supernatant is now ready to use without dilution, or you can dilute 1/2 in PBS or cell culture medium if needed.
5. Keep the collected supernatant at 4°C if you plan to use it within 24 hours. For longer storage, freeze the samples at –70°C or colder to preserve the virus. Avoid repeated freezing and thawing.

1.1.2 Tissue collection (carcasses only)

1. Place the tissue sample (e.g., lung) on the sterile plastic plate.

2. Mince the tissue sample into small pieces using the surgical blade and scalpel. Transfer all the minced tissue into the sterile tube.

3. Add 1X EMEM to the minced tissue in the sterile tube. Thoroughly mix the sample using a vortex mixer to homogenize suspension.

4. Centrifuge the tube containing the tissue suspension at 1500–1900 g at 4°C for 15–30 minutes.

5. Transfer the supernatant into the new sterile tube. The supernatant is now ready to use without dilution, or you can dilute 1/2 in PBS or cell culture medium if needed.

6. Keep the collected supernatant at 4°C if you plan to use it within 24 hours. For longer storage, freeze the sample at –70°C or colder to preserve the virus. Avoid repeated freezing and thawing.

1.2 Inoculation into embryonated chicken egg

Materials required

- Phosphate-buffered saline (PBS) pH 7.2-7.4
- 70% ethanol/Iodine
- Goose Red blood cells (GRBC)
- Embryonated chicken egg
- Egg incubator
- Egg candler

- Egg sealant
- Syringe and needle
- Sterile pipette
- 96-well plate V bottom
- Micropipette and tip
- Multichannel pipette
- Collection tube
- Centrifuge
- Class II biosafety cabinet
- Refrigerator

Procedure

1. Prepare 9- to 11-day-old embryonated chicken' eggs.

2. Inoculate 200 µl of the processed sample (see section 1.1.1 and 1.1.2) into an allantoic cavity (2 eggs per sample).

3. Incubate embryonic eggs at 35–37°C for up to 5 days and candle daily. Discard any eggs where the embryo dies within 24 hours of inoculation, as this is likely caused by the injection procedure rather than a virus.

4. Refrigerate embryonic eggs that died more than 24 hours after inoculation or at the end of the incubation period.

5. Harvest allantoic fluids and centrifuge at 1500–1900 g at 4°C for 10–20 minutes.

6. Transfer the supernatant to the collection tube for testing the presence of AIV with the hemagglutination (HA) test as screening assay.

HA test is performed as follow:

- Add 50 µl of allantoic fluids and PBS to the 96-well plate V bottom. The well containing PBS (well 7) will serve as GRBC control.

- Add 50 µl of 0.5% GRBC suspension to each well and agitate the plate to mix thoroughly.

- Cover the plate with sealing tape and incubate at room temperature (20-25°C) or 4°C until the distinct button has formed (30–60 minutes) in the control well.

7. Re-inoculate eggs (up to 1–2 passages) with allantoic fluid negative for HA assay. This is done to increase the chance of detecting a low concentration of virus. Egg isolation may be improved by making 2-fold to 10-fold dilutions of allantoic fluid in PBS/cell culture medium.

Interpretation of results

- If highly pathogenic avian influenza (HPAI) virus is present, it will often kill the embryo within 24-72 hours. Low pathogenicity avian influenza (LPAI) virus typically does not kill the embryo, but the embryo may appear stunted or sick.

- The harvested allantoic fluid gives positive results by HA assay.

  - HA-positive fluid: wells will have erythrocytes spread throughout the well, forming a lattice structure (agglutination).

  - HA-negative fluid: wells will have the distinct button of erythrocytes at the bottom of the well, forming a button structure (no agglutination).

- The HA-positive fluid is then tested using (M- or NP-gene) real-time RT-PCR assay, which gives positive results as confirmatory assay.

Criteria for quality control

- At least one egg should be inoculated with sterile VTM, PBS or cell culture medium instead of sample. If this control egg shows signs of hemagglutination or other viral effects, it indicates a contamination problem with the eggs or the reagents, invalidating the entire run.

- Any allantoic fluid that tests positive for HA should be re-tested using a confirmatory method like real-time RT-PCR to specifically detect AIV genes.

Troubleshooting

- Non-specific agglutination (false positive) from allantoic fluid contaminated with other hemagglutinating viruses such as Newcastle disease virus (NDV), bacteria, and fungi might cause red blood cells to clump.

- No virus growth (false negative) might be due to poor sample quality, bacterial or fungal contamination, or an incorrect inoculation location.

- Embryo death from non-viral causes the embryo dies, but the cause is not AIV infection, leading to the misleading result.

1.3 Inoculation into cell culture

Materials required

- Phosphate-buffered saline (PBS) pH 7.2-7.4
- Cell lines (e.g., Madin-Darby canine kidney (MDCK) cells)
- Cell culture medium (e.g., EMEM)
- Antibiotics (e.g., penicillin-streptomycin)
- Antifungals (e.g., amphotericin B)
- TPCK-trypsin
- Serological pipette
- 12-well plates
- Micropipette and tip
- Multichannel pipette
- Vortex mixer
- CO_2 incubator
- Inverted light microscope
- Centrifuge
- Class II biosafety cabinet

Procedure

1. Wash the confluent MDCK cell monolayers in 12-well plates three times using cell culture medium without FBS.

2. Inoculate 200 µl of processed samples (see section 1.1.1 and 1.1.2) in each well of 12-well plates (2 wells per sample).

3. Incubate of inoculated cell cultures for 2 hours at 37°C under 5% CO_2 with occasional rocking.

4. Remove the inoculum and wash the cell monolayer three times using the cell culture medium containing the final concentration of 2 µg/ml of TPCK-treated trypsin (maintenance medium).

5. Add 500 µl of maintenance medium to all well of culture plates and incubate at 37°C under 5% $CO_2$for 3–7 days with dairy observed for cytopathic effect (CPE).

- If CPE is observed, an aliquot of cell culture medium can be tested by HA assay as screening test (see section 1.2)

- If CPE is not observed at the end of the incubation period, two more blind passages will be performed.

Interpretation of results

- The appearance of CPE is indicated as positive results. This typically includes the rounding up, shrinking, and detachment of MDCK cells from the monolayer.

- The harvested culture medium gives positive results by HA assay.

- The HA-positive culture medium is then tested using (M- or NP-gene) real-time RT-PCR assay, which gives positive results as confirmatory assay.

Criteria for quality control

- MDCK cells must be free from any contaminants (mycoplasma, bacteria, fungi).

- At least one well should be inoculated with sterile VTM or cell culture medium instead of sample. If this control well shows signs of abnormal cells, hemagglutination or other viral effects, it indicates a contamination problem with the cells or the reagents, invalidating the entire run.

- The cell cultures must be regularly monitored using an inverted microscope for signs of CPE.

- When performing an HA assay on the culture medium, positive control (known AIV) and negative control (cell culture medium) should be included to ensure the reagents and procedure are working correctly.

- Any culture medium that tests positive for HA should be re-tested using a confirmatory method like real-time RT-PCR to specifically detect AIV genes.

Troubleshooting

- Non-specific agglutination (false positive) from the culture medium contaminated with other hemagglutinating viruses such as NDV, bacteria, and fungi might cause red blood cells to clump.

- No virus growth (false negative) might be due to poor sample quality, bacterial or fungal contamination, or no TPCK-treated trypsin in the culture medium.

2. Antibody detection

Antibody detection is a key method for diagnosing AIVs by identifying wildlife's immune response. When wildlife is exposed to AIVs, the immune system produces specific antibodies to fight the infection. These antibodies can be detected in a blood sample, indicating that the wildlife has either been infected in the past or has been vaccinated. This approach is widely used for surveillance to determine the prevalence of the virus in the population and to check the effectiveness of vaccination programs. Serological tests like the ELISA and HI assay are the most common methods for detecting these antibodies.

2.1 Blood processing

Materials required

- Micropipette and tip
- Test tube
- Vortex mixer
- Centrifuge
- Class II biosafety cabinet
- Refrigerator
- Deep freezer

Procedure

All steps must be performed inside of a Class II biosafety cabinet; the worker must wear the proper personal protective equipment (PPE).

1. Centrifuge the blood tube containing clotted blood/EDTA blood at 1500–1900 g for 15–30 minutes at 4°C.

2. Aliquot serum from clotted blood, or plasma from EDTA blood and transfer to the sterile tube.

Important:

- Blood should be allowed to clot after collection (for ~1 hour) at room temperature (20-25°C). It is recommended to observe the signs of blood hemolysis during serum processing.

- Serum/plasma can be stored at 4-8°C for up to 7 days for short-term periods, serum should be kept at -20 °C or lower for long-term periods.

- Avoid repeating to freeze and thaw serum to preserve the quality of antibodies.

2.2 Haemagglutination inhibition (HI) test

Materials required

- Phosphate-buffered saline (PBS) pH 7.2-7.4
- Serum/plasma samples
- Avian influenza virus (AIV)
- Goose Red blood cells (GRBC)
- Positive serum control
- Negative serum control
- 96-well plate V bottom
- Micropipette and tip
- Multichannel pipette
- Vortex mixer
- Incubator
- Water bath
- Centrifuge
- Class II biosafety cabinet

Procedure

2.2.1 Preparation of treated serum

1. Treat each serum/plasma with RDE (receptor-destroying enzyme) by adding 30 µl of serum to 90 µl of RDE.

2. Incubate the treated serum/plasma in a water bath at 37°C overnight (12–18 hours) for eliminating the nonspecific inhibitors.

3. Heat inactivation at 56°C for 30 minutes, and removal of natural serum agglutinins by absorbing with packed GRBC at 4°C for 1 hour with occasional mixing to keep the erythrocytes suspended.

4. Centrifuge the treated serum at 800 g for 10 minutes and then retain the serum. The treated serum will 2-fold serially diluted in duplicate wells of a microtiter V shaped plate at an initial dilution of 1:10.

2.2.2 Preparation of working HA antigens (8 HAU/50 µl)

5. Dilute HA antigens (H5, H7, etc.) to the concentration of 8 HA units (HAU) per 50 µl (4 HAU/25 µl) in 1X PBS, pH 7.2–7.4.

For example: To make 5 ml of an 8 HAU/50 µl virus antigen solution, you need to dilute 256 HAU/50 µl stock antigen 32-fold by mixing 156.25 µl of the stock with 4843.75 µl of PBS diluent. HA antigens should represent what is actively circulating in the region.

6. Conduct back titration (HA test) for working HA antigens to ensure that the correct HAU are present. The back titration is performed as follow:

- Dispense 50 µl of PBS in a row of 7 wells of 96-well plate V bottom.

- Add 50 µl of working HA antigen to the first well of each corresponding row.

- Make serial 2-fold dilutions of the working HA antigen with a micropipette set to deliver 50 µl. The resulting dilutions will range from 1/2 (well 1) to 1/64 (well 6). The well containing PBS (well 7) will serve as GRBC control.

- Add 50 µl of 0.5% GRBC suspension to each well and agitate the plate to mix thoroughly.

- Cover the plate with sealing tape and incubate at room temperature ((20-25°C) or 4°C until the distinct button has formed (30–60 minutes) in the negative control well.

Interpretation of results

• HA positive: wells will have erythrocytes spread throughout the well, forming a lattice structure (agglutination).

• HA negative: wells will have the distinct button of erythrocytes at the bottom of the well, forming a button structure (no agglutination).

If the concentration of working HA antigens is 8 HAU, GRBC agglutination will be seen in the first 3 wells of the back titration.

2.2.3 Procedure for HI test

7. Dispense 25 µl of PBS to all wells of 96-well plate V bottom.

8. Add 25 µl of the treated serum, positive serum control and negative serum control to first well and well 11 of 96-well plate V bottom.

9. Make serial 2-fold dilutions of the testing serum with a micropipette set to deliver 25 µl. The resulting dilutions will range from 1/10 (well 1) to 1/5,120 (well 10). The well containing the treated serum (well 11) will serve as serum control. The well containing PBS (well 12) will serve as GRBC control.

10. Add 25 µl of working HA antigens to well containing the treated serum (well 1-10) of 96-well plate V bottom except well (Walsh et al., 2018) and well (Davis and Walsh et al., 2018).

11. Cover plate(s) and incubate at room temperature (20-25°C) for 30 minutes.

12. Add 50 µl of 0.5% GRBC suspension to each well and shake/agitate the plate(s) to mix thoroughly.

13. Cover the plate(s) with sealing tape and incubate at room temperature (20-25°C) or 4°C until the distinct button has formed in the positive serum control well and GRBC control well (usually 30–60 minutes).

14. Tilt the microtiter plate to about 45-degree angle for 20–30 seconds and read the results

Interpretation of results

• The HI titer is the reciprocal of the highest serum dilution that shows complete inhibition of hemagglutination (compact button structure). The serum sample shows the HI titers ≥20 will be considered as positive results.

Criteria for quality control

• Positive serum control

• Negative serum control

• Serum control: wells will have the distinct button of erythrocytes at the bottom of the well, forming the compact button structure.

• GRBC control: wells will have the distinct button of erythrocytes at the bottom of the well, forming the compact button structure.

• Working HA antigens (back-titration): This set of wells confirms that the working concentration of the virus antigen (8 HAU/50 µl) is correct.

Troubleshooting

• The most important rule in troubleshooting is that if the controls fail, the entire test is invalid and must be repeated. You cannot reliably interpret the results of any test samples from the microplate with the failed control.

• When interpretation of incomplete inhibition is doubtful. Wells with partial inhibition will not produce tear drops. Tilt the microtiter plate to about 45-degree angle for 20–30

seconds and look for streaming, which produces tear-drop appearance and translucency around the cells in wells.

2.3 Microneutralization test

Materials required

- Phosphate-buffered saline (PBS) pH 7.2-7.4
- Cell line (e.g., MDCK cells)
- Cell culture medium (e.g., EMEM)
- Antibiotic (e.g., penicillin-streptomycin)
- Antifungal (e.g., amphotericin B)
- TPCK-trypsin
- Serum/plasma sample
- Avian influenza virus (AIV)
- Positive serum control
- Negative serum control
- 96-well plate V bottom
- 96-well plate U bottom
- 96-well plate flat bottom
- Micropipette and tip
- Multichannel pipette
- Vortex mixer
- CO_2 incubator
- Inverted light microscope
- Centrifuge
- Class II biosafety cabinet

Procedure

2.3.1 Preparation of treated serum

1. Treat each serum/plasma with RDE (receptor-destroying enzyme) by adding 30 µl of serum to 90 µl of RDE.

2. Incubate the treated serum/plasma in a water bath at 37°C overnight (12–18 hours) for eliminating the nonspecific inhibitors.

3. Heat inactivation at 56°C for 30 minutes, and removal of natural serum agglutinins by absorbing with packed GRBC at 4°C for 1 hour with occasional mixing to keep the erythrocytes suspended.

4. Centrifuge the treated serum at 800 g for 10 minutes and then retain the serum. The treated serum will 2-fold serially diluted in duplicate wells of a microtiter V shaped plate at an initial dilution of 1:10.

2.3.2 Preparation of MDCK cell

5. Wash the confluent MDCK cell monolayers in 96-well plates three times using cell culture medium without FBS.

6. Add 100 µl of maintenance medium to every well of 96-well plate U bottom.

2.3.3 Preparation of working AIVs concentration (200 $TCID_{50}$/100 µl)

7. Dilute testing AIV (H5, H7, etc.) to concentration of 200 $TCID_{50}$ per 100 µl in the cell culture medium containing the final concentration of 2 µg/ml of TPCK-treated trypsin (maintenance medium)

For example: To make 5 ml of 200$TCID_{50}$/100 µl virus solution, you need to dilute the 2 x 10^5 $TCID_{50}$/100 µl stock virus 1,000-fold by mixing 5 µl of the stock virus with 4,995 µl of maintenance medium. AIV should represent what is actively circulating in the region

8. Conduct back titration for working AIV to ensure that the correct 100 $TCID_{50}$/well are present. The back titration is performed as follow:

- Prepare virus suspension at dilutions of 100, 10, 1 and 0.1 $TCID_{50}$/200 µl in the maintenance medium.

- Discard maintenance medium from MDCK cell cultures for back titration.

- Transfer 200 µl of each virus concentration: 100, 10, 1 and 0.1 $TCID_{50}$ into the MDCK culture wells in duplicate.

2.3.4 Procedure for microNT test

9. Dispense 60 µl of 1X EMEM to every well of the two columns except the first two wells of 96-well plate U bottom.

10. Add 120 µl of the treated serum, positive serum control and negative serum control to the first well of two columns in the microtiter plate.

11. Make serial 2-fold dilutions of the testing serum with a micropipette set to deliver 60 µl. The resulting dilutions will range from 1/10 (well 1) to 1/1,280 (well 8).

12. Add 60 µl of working AIV to every well of the diluted sera; hence the working concentration of 100 $TCID_{50}$ is obtained.

13. Cover plate(s) and incubate at 37°C for 1 hour.

14. Transfer 100 µl of virus-antibody mixture to the corresponding well in the confluent MDCK cell monolayers.

15. Cover plate(s) and incubate at 37°C under 5% CO_2 for 2–3 days.

Interpretation of results

- The NT titer is the reciprocal of the highest serum dilution that shows ≤50% CPE in duplicate test wells. The serum sample shows the NT titers ≥20 will be considered as positive.

Criteria for quality control

- Positive serum control

- Negative serum control

- Working AIV (back-titration): This set of wells confirms that the working concentration of the virus antigen is correct. If the concentration of working AIV is 100 $TCID_{50}$/well, CPE will be seen in the well with 100, 10 and 1 $TCID_{50}$ of the back titration.

- Cell control: wells will have the distinct button of erythrocytes at the bottom of the well, forming the compact button structure

Troubleshooting

• The most important rule in troubleshooting is that if the controls fail, the entire test is invalid and must be repeated. You cannot reliably interpret the results of any test samples from the microplate with failed control.

• The virus stock may have an insufficient titer, low concentration, or be inactive. Perform virus titration ($TCID_{50}$) to determine the virus concentration before each assay. Use a fresh, high-titer virus stock and ensure proper storage to maintain its viability.

Reference

https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/3.03.04_AI.pdf
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0016164
https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0186962

2. Molecular detection

Molecular diagnostic methods, particularly real-time RT-PCR, have become the preferred tools for the direct detection and partial characterisation of influenza A viruses from clinical specimens. Their rapid turnaround, high sensitivity and specificity make them invaluable for outbreak management and timely decision-making by veterinary authorities. However, strict laboratory protocols are essential to minimise the risk of cross-contamination when using these highly sensitive techniques. Most laboratories adopt a stepwise real-time RT-PCR strategy for avian influenza A virus diagnosis, beginning with a highly sensitive generic screening assay targeting the conserved matrix (M) or nucleoprotein (NP) gene, and then performing subtype-specific real-time RT-PCR on the relatively conserved of the haemagglutinin genes. The following protocols are examples of molecular detection methods.

2.1. Real-time RT-PCR assays for M gene detection

Materials required

- Real-time fluorescence quantitative PCR analysis system
- Bench top centrifuge for 1.5mL Eppendorf tubes
- Bench top centrifuge for 96-well plate
- 10, 200, 1000µL pipettors and plugged tips
- Vortex
- RNA extraction kit
- Real time RT-PCR kit
- Primer set

- Other materials:
  - RNase-free 1.5mL eppendorf tubes, RNase-free 96-well plate with optical seal, powder free disposable latex glove, lab coat, tips for pipettors, carrier RNA, 70% alcohol.
- Primers and probes. The specific primers and probes for M genes are summarized in the table below.

Name	Sequence
InfA Forward	5' gAC CRA TCC TgT CAC CTC TgA C 3'
InfA Reverse	5' Agg gCA TTY Tgg ACA AAK CgT CTA 3'
InfA Probe	5' FAM TgC AgT CCT CgC TCA CTg ggC ACg 3' BHQ1

Procedure

1. Nucleic acid extraction

Inactivated samples can be handled in a BSL-2 laboratory within a Class II biological safety cabinet (BSC) in the specimen preparation area. RNA extraction should be performed following the manufacturer's instructions, and elution of the RNA in a final volume of 50 µl of nuclease-free water is recommended.

2. Quality control parameters
   - Negative control: Sterile water is extracted as a negative control at the same time as the nuclear acid extraction of the other specimens.
   - Reagent blank control: RNase-free water
   - Positive control: RNA of the Influenza A virus provided.
3. The reaction system preparation
   - Thaw the RT-PCR Master Mix, primers, and probes at room temperature in the reagent preparation area of the BSL-2 facility within PCR cabinet
   - Prepare reaction mixture.

Reagent	Volume (µl)
2x QuantiNova Probe PCR Master Mix	10
primer-forward (40µM)	0.4

primer-reverse (40µM)	0.4
Probe (20µM)	0.4
QN Probe RT-Mix	0.2
QN ROX Reference Dye	0.1
Template RNA	5
RNase Free H2O	3.5
Total volume	20

4. Aliquot the reaction mixture into a 96-well PCR plate as 15 µl per well.
5. Add 5µl of the template RNA for the negative control, test specimens, or positive control into the separate tubes with the reaction mixture in a PCR cabinet in the specimen preparation area.
6. Load the plate in the PCR cycler for Real-time RT-PCR detection and use the following programme for cycling:

Step	**Temperature (°C)**	**Time**	**No. of cycles**
Reverse transcription	45	10 min	1
PCR initial activation step	95	5 min	1
Denaturation	95	15 sec	45
Combined annealing/extension and perform fluorescence data collection	55	30 sec	
Cool down	25	2 min	1

Interpretation of results

The results are determined if the quality controls work.

1. The specimen is negative, if the value of Ct is undetectable.

2. The specimen is positive, if the Ct value is ≤38.0.
3. It is suggested that specimens with a Ct higher than 38 be repeated. The specimen can be considered positive, if the repeat results are the same as before, i.e., the Ct value is higher than 38. If the repeat Ct is undetectable, the specimen is considered negative.

Criteria for quality control

1. The result of the negative control should be negative.
2. The Ct value of the positive control should not be more than 28.0.
3. Otherwise, the test is invalid.

Troubleshooting

1. False positives may be due to environmental contamination, if there is amplification detected in the negative control and reagent blank control. The unidirectional work flow must be strictly obeyed. The following measures should be taken should there be false positives: ventilate the laboratories, wash and clean the workbench with RNA remover, autoclave centrifuge tubes and tips, and use fresh reagents.
2. RNA degradation should be taken into consideration if the Ct value of the positive control is more than 30. All materials should be RNase-free.

Cautions

1. In order to avoid nucleic acid cross-contamination, add the negative control to the reaction mixture first, then the specimen, followed by the positive control respectively.
2. Dedicated equipment for each area, including lab coats, pipettors, plugged tips and powder- free disposal latex gloves, are required.
3. Follow the instructions for maintenance of the incubator, PCR cycler, and pipettors. Calibration should be performed at least once every 12 months.

Biosafety

The lysis of the specimen (400 µl lysis buffer with 200 µl samples is recommended) should be to be carried out in a BSL-2 enhanced facility (BSL-2 facility with BSL-3 level personal protection equipment) or BLS-3 facility. Subsequent procedures can be performed in a BSL-2 laboratory which has separate rooms, including a reagent preparation area, specimen preparation area, and amplification/detection area. The DNA-free area is the clean area

and the area of amplified DNA is the dirty area. The work flow is from clean to dirty areas.

2.2. Real-time RT-PCR assays for H5, H7, H9 detection

Materials required

- Real-time fluorescence quantitative PCR analysis system
- Bench top centrifuge for 1.5mL Eppendorf tubes
- Bench top centrifuge for 96-well plate
- 10, 200, 1000µL pipettors and plugged tips
- Vortex
- RNA extraction kit
- Real time RT-PCR kit
- Primer set
- Other materials:
  - o RNase-free 1.5mL eppendorf tubes, RNase-free 96-well plate with optical seal, powder free disposable latex glove, lab coat, tips for pipettors, carrier RNA, 70% alcohol.
- Primers and probes.

Target	Name	Sequence
H5	H5 Forward	5' ACG TAT GAC TAC CCG CAG TAT TCA G 3'
	H5 Reverse	5' AGA CCA GCT ACC ATG ATT gC 3'
	H5 Probe	5' FAM-TCA ACA GTG GCG AGT TCC CTA GCA 3'-TAMRA
H7	H7 Forward	5' AYA GAA TAC AGA TWG ACC CAG T 3'
	H7 Reverse	5' TAG TGC ACY GCA TGT TTC CA
	H7 Probe	5' FAM-TGG TTT AGC TTC GGG GCA TCA TG-BHQ1 3'
H9	H9-For	5' ATG GGG TTT GCT GCC 3'

	H9-Rev	5' TTA TAT ACA AAT GTT GCA C(T)CT G 3'
	H9-Probe	5' FAM-TTC TGG GCC ATG TCC AAT GG-3' BHQ1

Procedure

1. Nucleic acid extraction

Inactivated samples can be handled in a BSL-2 laboratory within a Class II biological safety cabinet (BSC) in the specimen preparation area. RNA extraction should be performed following the manufacturer's instructions, and elution of the RNA in a final volume of 50 µl of nuclease-free water is recommended.

2. Quality control parameters
   - o Negative control: Sterile water is extracted as a negative control at the same time as the nuclear acid extraction of the other specimens.
   - o Reagent blank control: RNase-free water
   - o Positive control: RNA of the Influenza A virus provided.
3. The reaction system preparation
   - o Thaw the RT-PCR Master Mix, primers, and probes at room temperature in the reagent preparation area of the BSL-2 facility within PCR cabinet
   - o Prepare reaction mixture.

Reagent	**Volume (µl)**
2x QuantiNova Probe PCR Master Mix	10
primer-forward (40µM)	0.4
primer-reverse (40µM)	0.4
Probe (20µM)	0.4
QN Probe RT-Mix	0.2
QN ROX Reference Dye	0.1
Template RNA	5

RNase Free H2O	3.5
Total volume	20

4. Aliquot the reaction mixture into a 96-well PCR plate as 15 µl per well.
5. Add 5µl of the template RNA for the negative control, test specimens, or positive control into the separate tubes with the reaction mixture in a PCR cabinet in the specimen preparation area.
6. Load the plate in the PCR cycler for Real-time RT-PCR detection and use the following programme for cycling:

Step	**Temperature (°C)**	**Time**	**No. of cycles**
H5			
Reverse transcription	45	10 min	1
PCR initial activation step	95	5 min	1
Denaturation	95	15 sec	45
Combined annealing/extension and perform fluorescence data collection	57	20 sec	
Cool down	25	2 min	1
H7			
Reverse transcription	45	10 min	1
PCR initial activation step	95	5 min	1
Denaturation	95	15 sec	45
Combined annealing/extension and perform fluorescence data collection	55	30 sec	

Cool down	25	2 min	1
H9			
Reverse transcription	45	10 min	1
PCR initial activation step	95	5 min	1
Denaturation	95	15 sec	45
Combined annealing/extension and perform fluorescence data collection	60	45 sec	
Cool down	25	2 min	1

Interpretation of results

The results are determined if the quality controls work.

1. The specimen is negative, if the value of Ct is undetectable.
2. The specimen is positive, if the Ct value is ≤38.0.
3. It is suggested that specimens with a Ct higher than 38 be repeated. The specimen can be considered positive, if the repeat results are the same as before, i.e., the Ct value is higher than 38. If the repeat Ct is undetectable, the specimen is considered negative.

Criteria for quality control

1. The result of the negative control should be negative.
2. The Ct value of the positive control should not be more than 28.0.
3. Otherwise, the test is invalid.

Troubleshooting

1. False positives may be due to environmental contamination, if there is amplification detected in the negative control and reagent blank control. The unidirectional work flow must be strictly obeyed. The following measures should be taken should there be false positives: ventilate the laboratories, wash and clean the workbench with RNA remover, autoclave centrifuge tubes and tips, and use fresh reagents.

2. RNA degradation should be taken into consideration if the Ct value of the positive control is more than 30. All materials should be RNase-free.

Cautions

1. In order to avoid nucleic acid cross-contamination, add the negative control to the reaction mixture first, then the specimen, followed by the positive control respectively.
2. Dedicated equipment for each area, including lab coats, pipettors, plugged tips and powder- free disposal latex gloves, are required.
3. Follow the instructions for maintenance of the incubator, PCR cycler, and pipettors. Calibration should be performed at least once every 12 months.

Biosafety

The lysis of the specimen (400 µl lysis buffer with 200 µl samples is recommended) should be to be carried out in a BSL-2 enhanced facility (BSL-2 facility with BSL-3 level personal protection equipment) or BLS-3 facility. Subsequent procedures can be performed in a BSL-2 laboratory which has separate rooms, including a reagent preparation area, specimen preparation area, and amplification/detection area. The DNA-free area is the clean area and the area of amplified DNA is the dirty area. The work flow is from clean to dirty areas.

Reference

https://www.woah.org/fileadmin/Home/fr/Health_standards/tahm/3.03.04_AI.pdf

protocols_influenza_virus_detection_2024.pdf

D. Sample collection form

Supplementary Table 1: Sample Submission Form

	แบบบันทึกการเก็บสิ่งส่งตรวจในสัตว์ด้านระบาด (Record form of specimen collection for epidemiological study) คณะสัตวแพทยศาสตร์ มหาวิทยาลัยมหิดล (Faculty of Veterinary Science, Mahidol University)	FR-WI-CENT-G-001/23 แก้ไขครั้งที่ 01 หน้าที่ .....ของ.....หน้า

เลขที่สิ่งส่งตรวจ (CVD/FN) (Case number) .....

วันที่เก็บสิ่งส่งตรวจ (Date of specimen collection).....เวลา (Time).....
ผู้เก็บสิ่งส่งตรวจ (Sample collector).....หมายเลขโทรศัพท์ (Phone number).....มือถือ (Mobile phone number).....
สถานที่เก็บสิ่งส่งตรวจ (Sampling location).....ตำบล/แขวง (Sub-district).....อำเภอ/เขต (District).....จังหวัด (Province).....
สภาพแวดล้อม/ลักษณะพื้นที่บริเวณที่เก็บสิ่งส่งตรวจ (Describe an area).....สถานการณ์การระบาดของโรคในพื้นที่ที่เก็บสิ่งส่งตรวจ (Disease outbreak status).....
ชนิดสัตว์ที่พบบริเวณที่เก็บสิ่งส่งตรวจ ( )ไก่ ( )เป็ด ( ) นกเลี้ยง ( ) นกธรรมชาติ ( ) สัตว์ป่า ( ) อื่นๆ (ระบุชนิด).....
(Type of animal in this area) (Poultry) (Duck) (Captive bird) (Wild bird) (Wildlife) (Others, please specify)

หลอดที่ (Number)	ชนิดสัตว์/ชื่อสัตว์ (Animal/Name)	หมายเลขบนตัวสัตว์/ชื่อสัตว์ (Animal identification : number or name)	หมายเลขห้องปฏิบัติการ(เฉพาะเจ้าหน้าที่เท่านั้น) (Laboratory number) Lab staff only	พิกัดพื้นที่เก็บสิ่งส่งตรวจ (Coordinate)	เพศ Gender		อายุ Age		สภาพสัตว์ Health status			ชนิดของสิ่งส่งตรวจที่เก็บ Type of specimen							หมายเหตุ Remarks
												เลือด Blood		Swab					
					ผู้ Male	เมีย Female	ตัวเต็มวัย Adult	ลูกสัตว์ Juvenile	ปกติ Normal	ป่วย Sick	ตาย Death	Whole blood	Clotted blood	Tracheal/ Choanal swab	Feces/ Cloacal swab	Nasal swab	ชิ้นเนื้อ Tissue	อื่นๆ Others	

Supplementary Table 2: Environmental Sample Collection Report

Mahidol University
Faculty of Veterinary Science

Environmental Sample Collection Report

The Monitoring and Surveillance Center for Zoonotic Diseases in Wildlife, Exotic and Migratory Animals, Faculty of Veterinary Science, Mahidol University, Thailand

Date of Collection: .... Time: ....
Sampled by: .... Phone No.: ....

Collection Site: ....
Sub-district: .... District: .... Province: ....
Environment/Characteristics of Collection Area:
....
Disease Outbreak Situation in Collection Area:
....
Date of Sample Submission: ....

No	Coordination of Collection Site		Air		Sample types				Parameters				Remarks
	Lat	Long	Temp	Hum	Soil	Water	Feces	Others	pH	Temp	Hum	DO	

Supplementary Table 3: External Morphometric Measurement Data Sheet

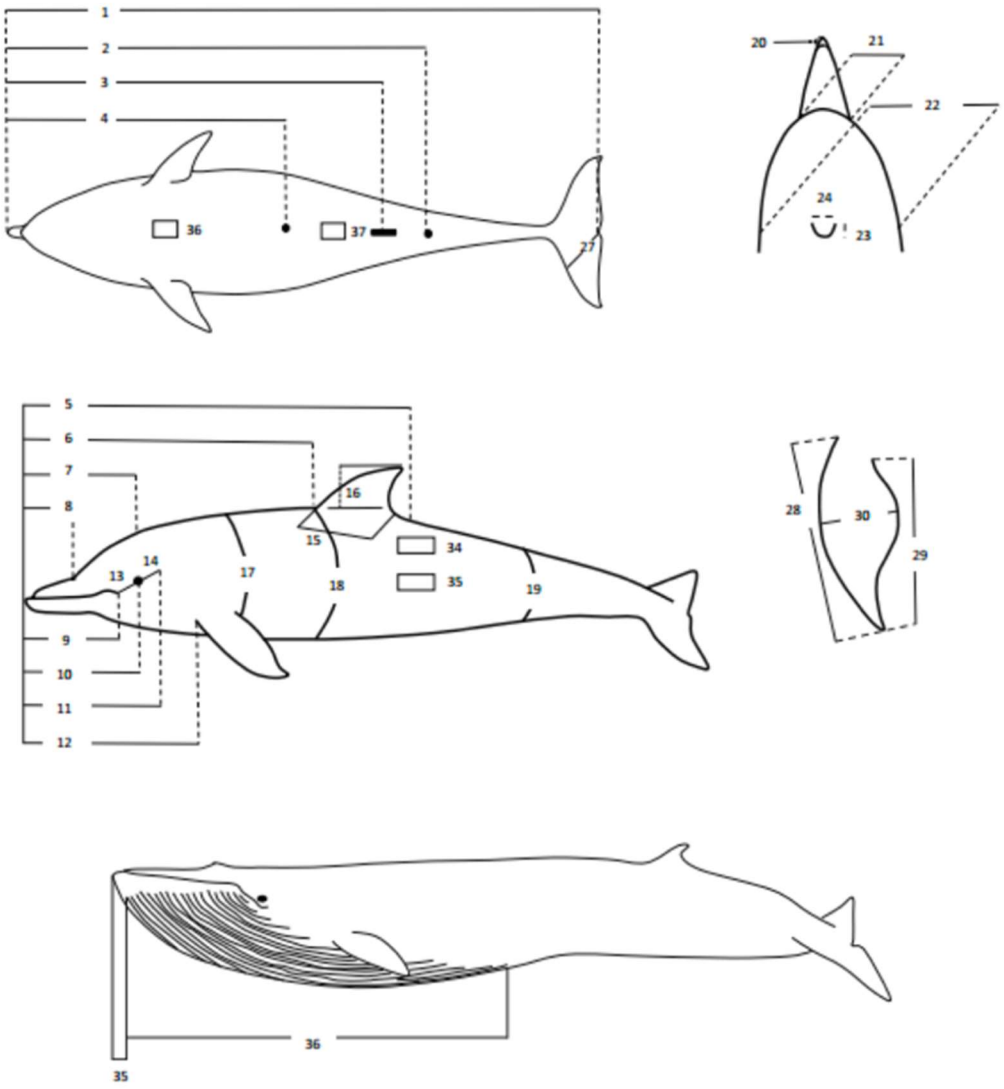
External Morphometric Measurement Data Sheet
ตารางบันทึกข้อมูลการวัดสัดส่วนภายนอก

No. (ลำดับ)		**Measurement, cm.) (การวัด, ซม.)**	**No. (ลำดับ)**		**Measurement, cm.) (การวัด, ซม.)**
1	Total Length (ความยาวทั้งตัว)		16	Dorsal Fin Height (ความสูงของครีบหลัง)	
2	Snout to Anus (ปลายปากถึงช่องก้น)		17	Girth at Posterior of Pectoral Fin (รอบตัวบริเวณหลังครีบข้าง)	
3	Snout to Center of Genital Slit (ปลายปากถึงกลางช่องเพศ)		18	Girth at Anterior of Dorsal Fin (รอบตัวบริเวณด้านหน้าครีบหลัง)	
3a	Snout to Anterior of Genital Slit (ปลายปากถึงด้านหน้าช่องเพศ)		19	Girth at Anus (รอบตัวบริเวณช่องก้น)	
3b	Snout to Posterior of Genital Slit (ปลายปากถึงด้านหลังช่องเพศ)		20	Gape Height (Distance between Upper and Lower Jaw Tip) (ระยะห่างระหว่างปลายปากบนและล่าง)	
3c	Snout to Center of Mammary Slit (ปลายปากถึงกลางร่องนม)		21	Mouth Width (ความกว้างของปาก)	
4	Snout to Umbilicus (ปลายปากถึงสะดือ)		22	Head Width at Eyes (ความกว้างของหัวบริเวณลูกตา)	
5	Snout to Anterior Insertion of Dorsal Fin (ปลายปากถึงด้านหน้าฐานครีบหลัง)		23	Blowhole Length (ความยาวของช่องหายใจ)	
6	Snout to Posterior Insertion of Dorsal Fin (ปลายปากถึงด้านหลังฐานครีบหลัง)		24	Blowhole Width (ความกว้างของช่องหายใจ)	
7	Snout to Blowhole (ปลายปากถึงช่องหายใจ)		25	Eye Length (ความยาวลูกตา)	

8	Snout to Apex of Melon (ปลายปากถึงโหนกหัว)		26	Fluke Width (ความกว้างแพนหาง)	
9	Snout to Gape (Corner of Mouth) (ปลายปากถึงปลายมุมปาก)		27	Distance from Tail Stock to Anterior Fluke Insertion (ระยะจากคอดครีบหางถึงฐานด้านหน้า)	
10	Snout to Center of Eye (ปลายปากถึงกลางตา)		28	Anterior Insertion to Tip of Pectoral Fin (ด้านหน้าฐานครีบข้างถึงปลายครีบข้าง)	
11	Snout to Ear Opening (ปลายปากถึงรูหู)		29	Posterior Insertion to Tip of Pectoral Fin (ด้านหลังฐานครีบข้างถึงปลายครีบข้าง)	
12	Snout to Anterior Insertion of Pectoral Fin (ปลายปากถึงฐานด้านหน้าของครีบข้าง)		30	Maximum Pectoral Fin Width (ความกว้างสุดของครีบข้าง)	
13	Gape to Anterior Edge of Eye (ปลายมุมปากถึงขอบตาด้านหน้า)		31	Snout to Start of Throat Grooves (ปลายปากถึงส่วนต้นของร่องใต้คาง)	
14	Posterior Edge of Eye to Ear Opening (ขอบตาด้านหลังถึงรูหู)		32	Length of Throat Grooves (ความยาวของร่องใต้คาง)	
15	Dorsal Fin Base Length (ความยาวฐานครีบหลัง)		33	Number of Throat Grooves (นับจำนวนร่องคาง)	

Blubber Thickness 34_____ 35_____ 36_____ 37_____

Diagram of External Morphometric Measurement
(แผนภาพประกอบการวัดสัดส่วนภายนอก)



Supplementary Table 3: Necropsy Form for Animal Carcasses

MU.VET.PATH.1

Veterinary Hospital for Education, Mahidol University
25/25 Phutthamonthon 4 Rd., Salaya , Nakhonpathom 73170 Tel.(02) 441-5245-6

NECROPSY REPORT

Date 20 Apr 2011
Clinic No.
Clinician Doctor xxx xxxxx
Pathologist Doctor xxx xxxxx
Address Faculty of Veterinary Science, Mahidol University
Tel. 02-441-5242-4 Ext. 1532, 1107 Fax. 02-441-0937

Accession No. HT 143/54
Animal's I.D. 53-1059 Owner Wildlife Station, Ratchaburi
Species Himalayan Griffon Breed
Sex Age Wt. Marking none
X Died ☐ Euthanasia ; method :
Date 13 Mar 2011 Time 01.00 P.M.

Clinical Diagnosis: Suspected Bacterial infection

History:

Acute death
PE : Bleeding from norstrils, collapsed and then sudden death
Stage of carcass: Fresh
Nutritional stage: Thin

Macroscopic findings:
The carcass was rather thin and presented in a good flesh. There was slight bleeding from both nostrils. The body integument was normal and there were no any wounds, fracture or foreign body on it. There were no remarkable macroscopic lesions found in the brains, stomach, intestine, air sacs, spleen and kidneys. The gastric contents consisting of undigested food were found in the stomach. The liver showing slightly pale, enlarge and necrotic areas were seen on the surface and cut surface. The ventricular hypertrophy was found in the heart. Pulmonary congestions, edema, and hemorrhages characterized by blood and fluid filled in the parenchyma and abnormal mass were found in the lungs. Blood clots were demonstrated within the tracheal lumen and thoracic cavity.

Gross Diagnosis:
monary arrest resulting from pulmonary edema and hemorrhages.

Clinical Pathology:

Bacterial culture from
Swab from air sacs: positive for *Escherichia coli*
Swab from lungs: positive for *Escherichia coli*
Haemoculture: positive for *Bacillus spp.* and *Escherichia coli*
Swab from lungs: Negative for *Mycobacterium* spp.
Note: Pending for *Mycobacterium* sp.
Viral detection from liver, lung, spleen
Negative for Avian influenza virus and Newcastle disease virus
Fungal culture
Negative for *Aspergillus* sp.

MU.VET.PATH.2

Tissue sectioned: The tissue collected from the lungs, liver, spleen, kidneys, trachea, heart, intestine were processed for the histopathological examination.

Microscopic findings:
There were no remarkable lesions seen in the kidneys, trachea, spleen and intestine. Hepatocytes showing degenerative changes and the presences of portal hepatitis characterized by leukocyte infiltration around the portal tracts were demonstrated. The liver's capsule showing edema, leukocyte infiltration, and hepatic necrosis around the liver's capsule were also seen. The presences of pneumonitis characterized by the thickening of air vesicles and inflammatory cell infiltrates were seen. The other remarkable lesions including pulmonary edema, congestion and multifocal hemorrhages within the pulmonary were also demonstrated. The special staining for the detection of acid-fast bacteria in the lung parenchyma was negative.

Final Diagnosis:
Pneumonitis with pulmonary haemorrhages, edema and congestion resulting in fatal respiratory failure

Doctor xxx xxxxx
(Signature)

MU.VET.PATH.1

Veterinary Hospital for Education, Mahidol University
25/25 Phutthamonthon 4 Rd., Salaya , Nakhonpathom 73170 Tel.(02) 441-5245-6

NECROPSY REPORT

Date 08/04/2551
Clinic No. -
Clinician -
Pathologist Doctor xxx xxxxx
Address 999 Phutthamonthon 4 Rd., Salaya , Nakhonpathom
Tel. 02-441-5236 Fax. 02-441-5236

Accession No.
Animal's I.D.
Owner -
Species Feline Breed -
Sex Male Age - Wt. -
Marking Black color
√ Died ☐ Euthanasia ; method :
Date 06/04/08 Time

Clinical Diagnosis:

History:
Domestic cat from outside the Bung Chawark zoo. The official found it died in area of the zoo.

Stage of carcass: Mild autolysis
Nutrition stage: Good

Macroscopic findings:
1. Bloody nasal discharge
2. Corneal opacity and conjunctivitis
3. Bloody saliva
4. Abdominal cavity
Serosanguineous exudate about 20 ml.
Pale liver
5. Thoracic cavity
Serosanguineous exudate
Lung congestion
Hemopericardium (Serosanguineous effusion)
6. Brain
Cerebral cortex congestion
Engorge cerebral artery

Gross Diagnosis:
Severe acute cerebral cortex and Hemopericardium.

Comments:
Suspect Avian influenza or Warfarin /Caumarin toxin

MU.VET.PATH.1

Veterinary Hospital for Education, Mahidol University
25/25 Phutthamonthon 4 Rd., Salaya , Nakhonpathom 73170 Tel.(02) 441-5245-6

NECROPSY REPORT

Date 10/04/2551
Clinic No. -
Clinician -
Pathologist Doctor xxx xxxxx
Address 25/25 Phutthamonthon 4 Rd., Salaya , Nakhonpathom
Tel. 02-441-5245-6 Fax. 02-441-0773

Accession No.
Animal's I.D. -
Owner xxx zoo
Species Lion Breed -
Sex Male Age ~ 4 yrs Wt. -
Marking -
☒ Died ☐ Euthanasia ; method :
Date 09/04/2551 Time -

Clinical Diagnosis: Per-acute death

History:

It was alert, good appetite, not depressed or sick but it died with undiagnosed cause at night (09/04/2551).

Macroscopic findings:

Skin:	Subcutaneous congestion
Oral cavity:	Pale mucous membrane, cyanosis and petechial hemorrhage on tongue
Nares:	Bloody nasal discharge
Trachea:	Hemorrhage
Lungs:	Congestion and edema (both sides)
Heart:	Hemorrhagic effusion (~50 ml.)and hemorrhagic endocadium
Liver:	Hepatomegaly, congestion and edema
Spleen:	Splenomegaly, congestion and edema
Omentum:	Pale
Intestine:	Generalized subserosal hemorrhage, mesenteric congestion and hemorrhage
Stomach:	Subserosal hemorrhage (Physiological congestion), Gastric dilatation
Kidneys:	Renal subcapsular hemorrhage (both sides), corticomedullary hemorrhage, renal pelvis enlargement
Pancreas:	Congestion/hemorrhage

Gross Diagnosis:
Cause of death is CPA form cardiac tampanad and multiple organ failure (may be from hemolytic toxicosis or severe acute infection).

Comments: