PROTOCOLS FOR COLLECTION, STORAGE AND TRANSPORTATION OF BIOLOGICAL SAMPLES OF FRESHWATER MACRO-FAUNA

नमामि गंगे

भारतीय वन्यजीव संस्थान
Wildlife Institute of India

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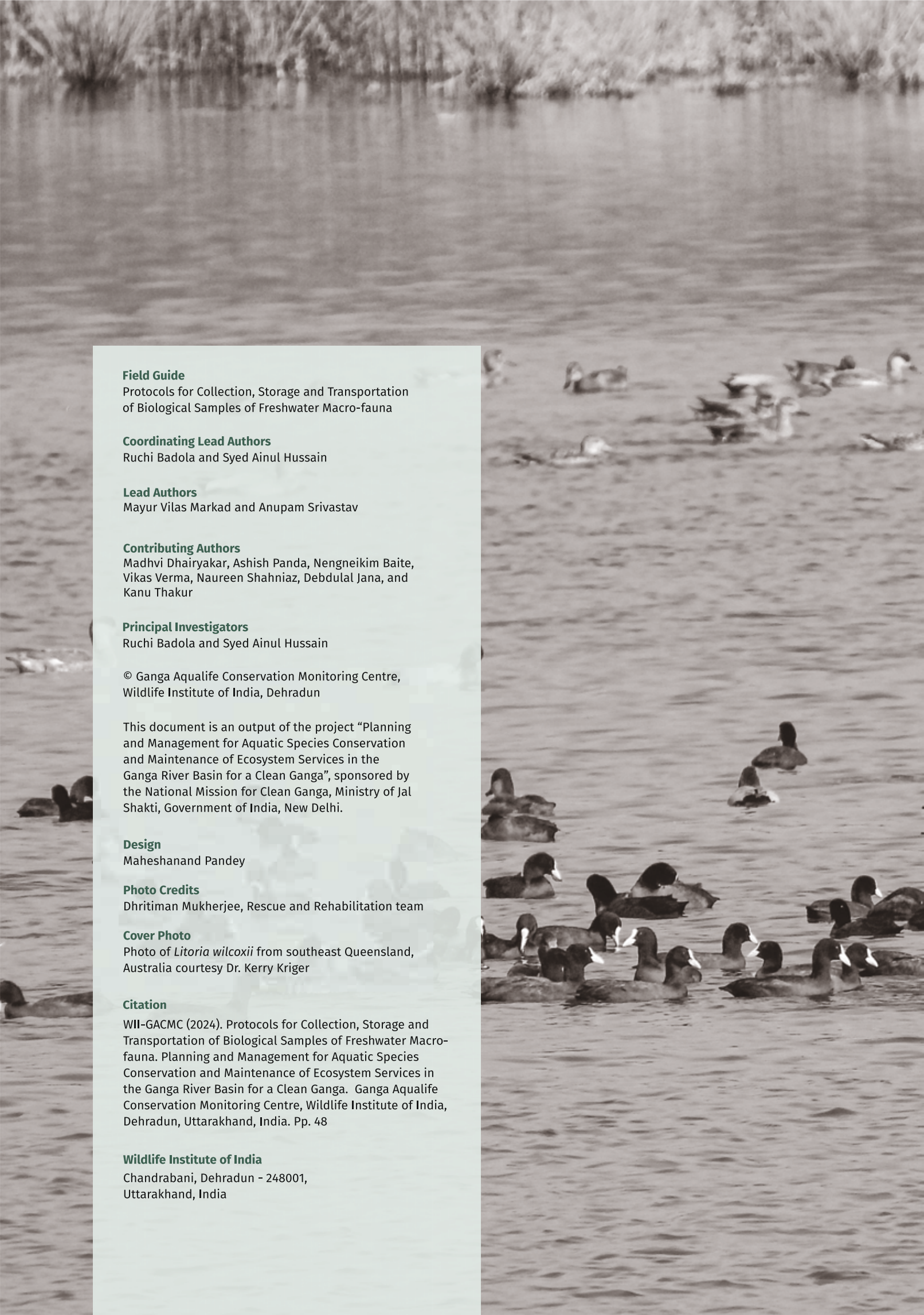

Field Guide
Protocols for Collection, Storage and Transportation of Biological Samples of Freshwater Macro-fauna

Coordinating Lead Authors
Ruchi Badola and Syed Ainul Hussain

Lead Authors
Mayur Vilas Markad and Anupam Srivastav

Contributing Authors
Madhvi Dhairyakar, Ashish Panda, Nengneikim Baite, Vikas Verma, Naureen Shahniaz, Debdulal Jana, and Kanu Thakur

Principal Investigators
Ruchi Badola and Syed Ainul Hussain

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Maheshanand Pandey

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Wildlife Institute of India
Chandrabani, Dehradun - 248001,
Uttarakhand, India

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Bhaskar Dasgupta, *Executive Director (Finance)*
Brijendra Swaroop, *Executive Director (Projects)*
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INTRODUCTION

Wildlife diseases greatly impact the conservation and preservation of biodiversity and affect domestic animals. They also significantly impact the economy and public health. 60 per cent of emerging infectious diseases are zoonotic in nature, and many of the new world diseases in the recent past have originated from wildlife.

"Even the most reliable test, performed in the most reliable facility and interpreted by the most skilled diagnostician, cannot overcome the error introduced by an inappropriate technique used in sample collection or handling."

-Thrall et al., 2004

Disease diagnosis in wild animals is a challenging task, more so in freshwater fauna. An accurate diagnosis of disease is the basis for its treatment and for ensuring the well-being of the animal. Symptomatic assessment of sick animals is a challenging task; however, recent advancements in pathological techniques are effective aids in disease diagnosis in wild animals. Freshwater fauna presents unique challenges for diagnostic sampling due to their diverse physiology, environmental adaptations, and sensitivity to handling. Additionally, pathogens such as viruses, bacteria, fungi, and other parasites require specific protocols and sample types, including blood samples, tissue body fluids, swabs, etc. The collection, storage, and transportation of these biological samples play a crucial role in disease diagnosis.

With increasing threats from pollution, habitat degradation, climate change, and emerging pathogens, it is imperative to adopt standardized protocols that ensure the integrity and accuracy of diagnostic results. Various studies have documented the effects of pre-analytical factors, such as sample collection site, handling, storage, and preservatives used for understanding the health of animals (Stewart et al., 2012; Eshar et al., 2018; Muro et al., 1998). All methods require some level of technical skill to achieve accuracy, and methods may not be directly comparable with one another within or between laboratories. It is ideal to use one laboratory and one or two technicians with similar training and skills, as significant variation may occur between laboratories because of the interpretations by the technicians handling the samples.

Given the diversity of species and pathogens encountered in freshwater environments, this document aims to provide guidelines for personnel engaged in collecting and handling samples from freshwater macro-fauna.

Understanding disease

Disease can be defined as "Any change in an organism's normal structural and functional state, generally associated with chronic signs and symptoms differing from physical injury" (Grossman, 2013). Anthropogenic activities have given rise to many emerging and remerging diseases. The impact of disease can range from mild discomfort to severe collapse or even death. Understanding the disease mechanism, including its causes, progression, and effects on the body, is critical for its effective diagnosis, treatment, and prevention.

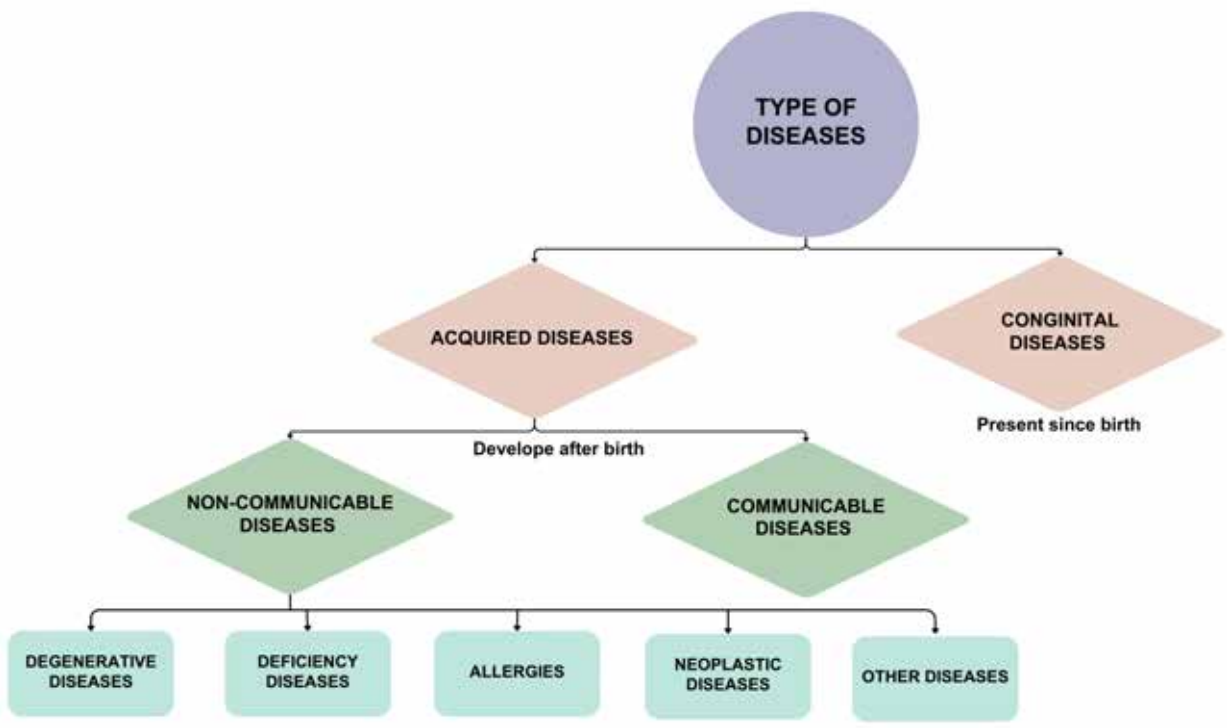


Figure 1. Types of diseases

DEFINITIONS

Health:	It is a state of complete physical, mental and social well-being and not merely the absence of disease or illness
Disease:	A departure from a state of health. Any impairment in health results in physiological dysfunction. ("dis-ease" literally means a departure from the state of ease)
Zoonosis:	An infectious disease caused by a pathogenic agent (an infectious agent, such as a bacterium, virus, parasite or prion) that spreads between animals and humans (e.g., influenza virus)
Emerging disease:	Infectious diseases that have newly appeared in a population or have existed but are rapidly increasing in incidence or geographic range (e.g., SARS-CoV-2 or COVID-19)
Re-emerging disease:	Infectious diseases that were previously under control but are now showing an upward trend in incidence or have reappeared in new geographic locations.

Diseases based on their origin

Infectious or communicable diseases: Disease due to the presence of an infectious agent that is capable of being transmitted to another host. (e.g., Avian influenza and brucellosis, including zoonotic disease)

Non-infectious or non-communicable diseases: Diseases that are not caused by pathogens, such as bacteria, viruses, or fungi, and cannot be spread from one person to another.

Toxic: Disease which can be caused by the presence of toxins or poison (e.g., heavy metal poisoning, pesticides)

Nutritional: Disease caused by nutritional deficiencies or imbalance (e.g., starvation and metabolic bone disease)

Traumatic: Disease caused by traumatic or physical injury (e.g., collision and electrocution)

Developmental: Disease that disturbs normal development in growing animals. It may affect a particular part of the body or affect different systems.

Congenital: Disease inherited genetically during embryonic development.

Neoplastic: Disease caused by abnormal cell growth (e.g., tumor).

The manifestation of disease is a complex process often associated with environmental stressors such as reduced nutrition, toxin exposure, weather change, infectious agents, and congenital anomalies, either alone or in combination with other factors.

Three epidemiological concepts of understanding disease are:

1. Disease never occurs at random
2. All diseases have a multifactorial origin
3. Diseases occurrence has an association among pathogenic agents, hosts, and environment.

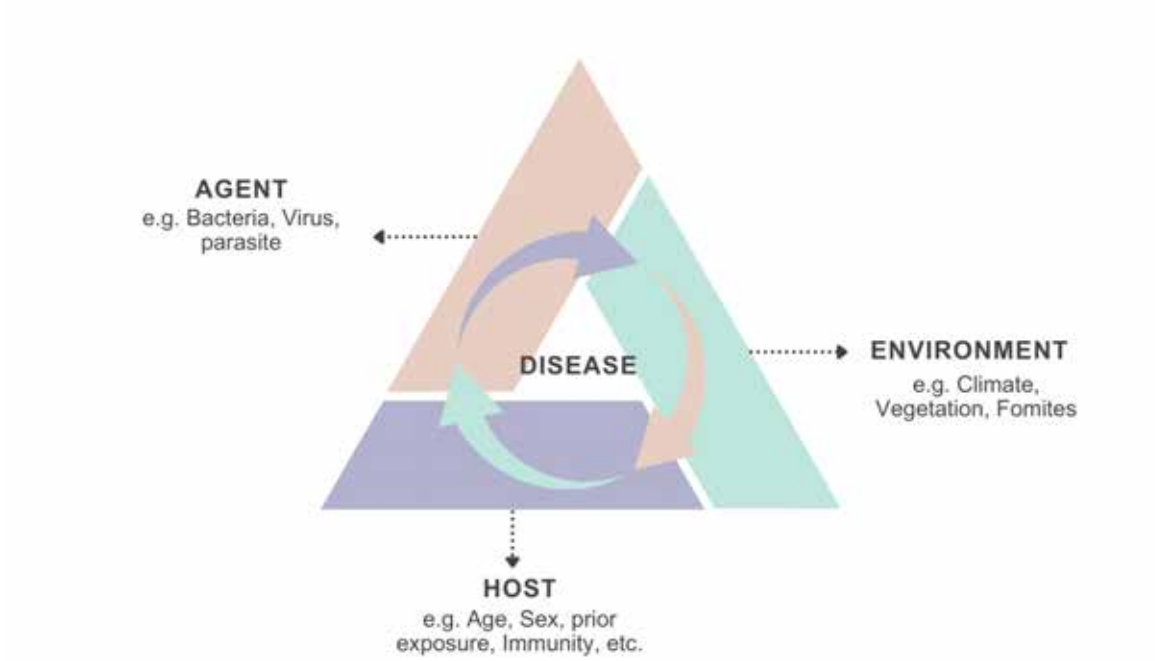


Figure 2. Disease triangle showing interaction among agents (Bacteria, viruses, etc.), host, and environment

Disease emergence

The growing human footprint has increased interspecies interaction due to the much larger human-wildlife interface, i.e., the interface between humans and wildlife and domestic animals. This has opened possibilities for transmission of novel pathogens, leading to the emergence of new diseases.

Globalization and climate change are key environmental changes that might be associated with the emergence of wildlife diseases. Specific environmental and anthropogenic changes, such as habitat fragmentation and degradation, have amplified the role of disease in wildlife conservation. In some cases, nutritional deficiencies related to unbalanced nutrition may lead to nutritional diseases in animals. Increased drought and excessive heat can lead to a congregation of wildlife at a limited water source, and increase the risk of disease transmission.

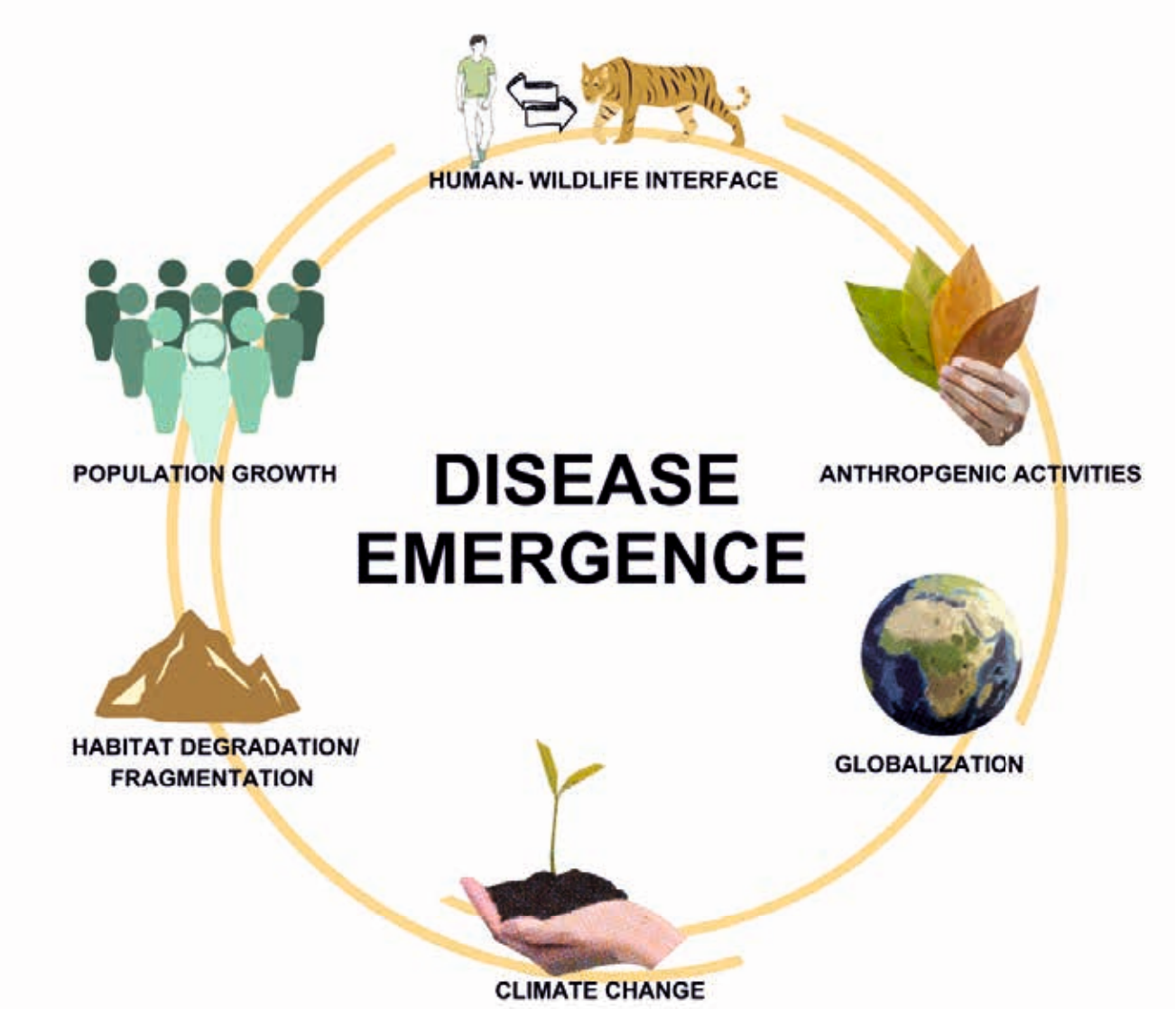


Figure 3: Factors influencing disease emergence

"The growing demand for agricultural and livestock expansion at the expense of natural habitats increases the human-wildlife interface, facilitating the transmission of novel pathogens from animals to humans."

Daszak et al. (2001)

2

HANDLING OF TARGET SPECIES FOR SAMPLE COLLECTION

Even though non-invasive techniques have been developed in recent years, animal capture remains a fundamental component of wildlife disease study. Modern scientific techniques allow us to understand disease epidemiology using DNA from naturally shed tissue samples to gather hormone data information from feces, but actual physical capture of animals for sample collection offers a wealth of data on animal health and understanding disease dynamics. Handling animals for sample collection is an important aspect; the handler should take utmost care to avoid any form of injury to both animals and humans. Handling leads to an increase in corticosteroids and adrenal catecholamines (Lance and Lauren, 1984). Therefore, the handler should have a good understanding of animal physiology, anatomy, and behavior to ensure safety. Proper preparation in advance minimizes stress and facilitates sampling.

Amphibians

Most amphibians are quiet, but few of them may inflict injury through bite. Handling amphibians should be carried out only if blood samples, skin scrapes, and swabs are needed or to administer therapeutics. Handling is always inversely related to amphibian size, i.e., the smaller the animal, the less it will tolerate handling. Aggressive, improper handling may cause stress or even death of the animal. The use of vinyl (non-powdered) gloves over latex (powdered) gloves is advisable for handling to avoid adverse reactions. Amphibians with slimy and slippery skin are handled with a wet towel or tissue. Frogs and toads can be handled by grasping the animal firmly, just cranial to the hind legs. Utmost care should be taken to wear PPE because some species of amphibians have toxins that may cause irritation or intoxication.

Reptiles

Aquatic chelonians are aggressive and can inflict serious injury to the handlers by scratching and biting. In some cases, urination, defecation, and musking can be unpleasant. Handle chelonians by their shell, and never grab their appendages or tails. Large chelonians may require more than one person to handle. Handlers should never turn chelonians upside down rapidly while handling as this may result in rapid changes in hemodynamics and cause respiratory distress by compressing lungs under the weight of coelomic viscera or may result in torsion.

Other aquatic reptiles like Gharial, Mugger, and Saltwater crocodiles are also very risky to handle and are among the largest reptiles in the world. Considering their large body size and powerful musculature, restraining can be performed by trained personnel only. Approaching crocodilians is always performed from behind rather than front or side. A minimum of one person is required for every one meter of the body for animals up to 2 meters in length (tip of the snout to tip of the tail), and beyond 2 meters, two people for each additional meter. Manual restraining can be performed up to 3.5 meters by experienced staff.

Animals between 1-3 meters can be safely captured with the help of a catchpole or snare pole. Animals, more than 3.5 meters can be captured simply by forming a single line from behind and quickly landing on top of the animal. Make sure to use special aids such as ropes and nets before attempting to tape their mouth.

Figure 4: Procedure for restraining amphibians

Figure 5: Restraint of juvenile Three-striped roofed turtle (*Batagur dhongoka*).

Figure 6: Restraining an adult Indian soft shell turtle (*Nilssonia gangetica*) for sample collection.

Figure 7. Restraint of an Indian Mugger (*Crocodylus palustris*) of ~1 meter. The neck and tail are held in order to support the animal. The animal is restrained close to the handler's body

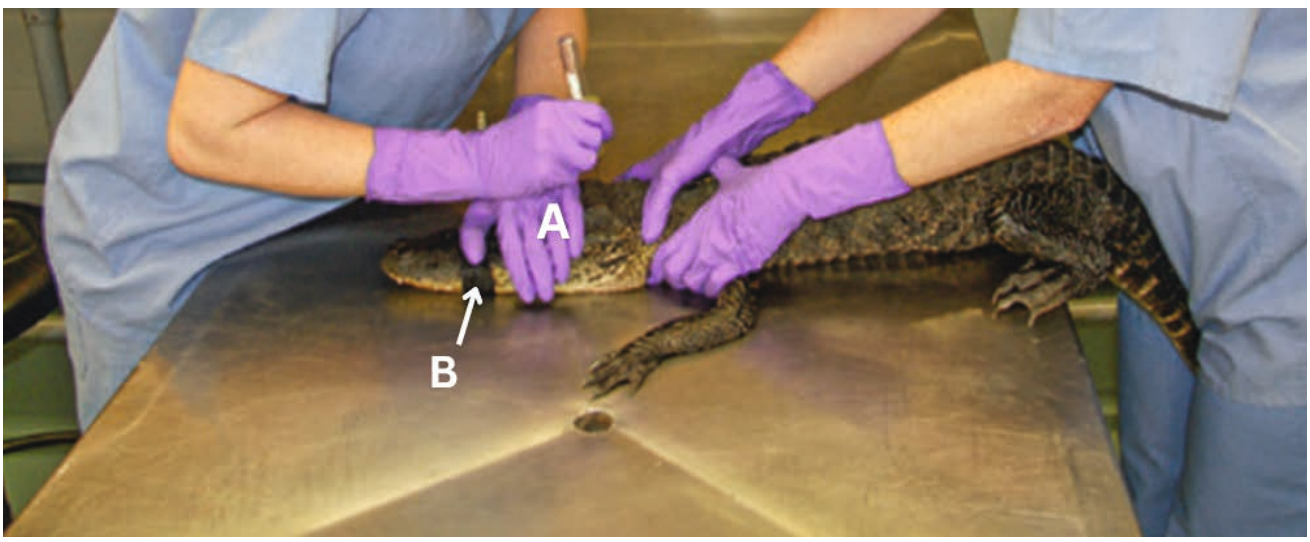
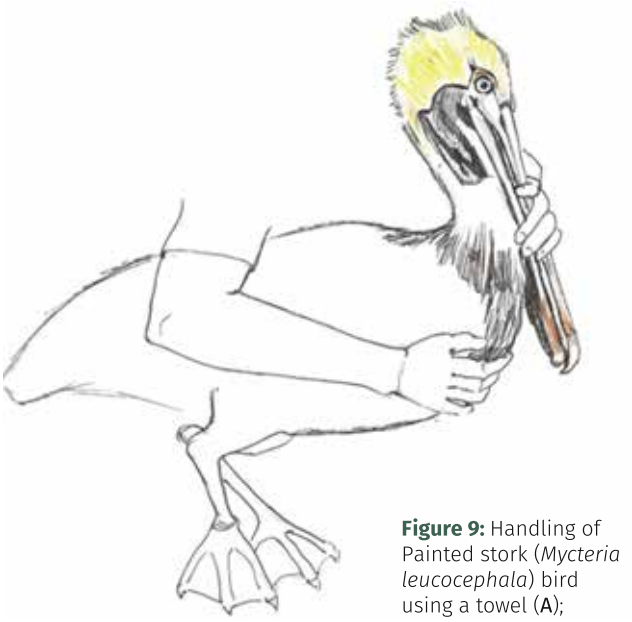


Figure 8: Restraint and handling of crocodile for paravertebral venipuncture. Eyes being covered by handlers' hand to decrease visual stimulation (A), tape around the mouth (B) (adapted from Divers and Stahl, 2018).

Aves

In contrast to amphibians and reptiles, birds are active and fast-moving, and handling them always requires gloves, traps, and nets. Other than raptors and waterfowl, most other birds can be best handled by wrapping their bodies in towels and securing their heads. Birds of prey can cause serious injury with their talons and beaks. While handling, talons must be secured all the time to avoid injury. Covering eyes may have a calming effect on raptors and lower stress.



B

Figure 9: Handling of Painted stork (*Mycteria leucocephala*) bird using a towel (**A**); handling larger birds like pelican (**B**).

Figure 10: Handling of smaller birds and pigeons

Note-*Most birds can either bite, peck or scratch during handling. Birds of prey may inflict serious injuries with their talons as their grip is 100 times stronger than the human fist. Aquatic birds like cormorants, ducks, coots, and herons, etc., are not the most cooperative birds to handle. It is always advisable to keep them at a distance from the face and protect the hands and eyes.*

Aquatic mammals

Dolphins and otters are protected under Schedule I of the Indian Wildlife Protection Act (1972) and listed as endangered and vulnerable, respectively in the IUCN Red List of Threatened Species. Therefore, handling may be undertaken only with prior permission in rescue situations. The Ganges River Dolphin (GRD) is India's national aquatic animal and is provided the highest level of protection. Sampling may only be carried out after permission from a competent authority. Opportunistic samples can be taken while the animal is being handled during rescue and release operations. Handling should be carried out by trained personnel such as veterinarians and wildlife biologists. Use proper equipment while handling and minimize the time out of water. Support the body evenly, especially around the abdomen, to avoid internal injuries due to gravitational pull, support jaws, protect the blow hole, etc. These are some of the key factors to keep in mind while handling GRD.

Otters

Handling otters requires care and expertise to ensure the animal's and the handlers' safety. Otters are agile, strong, and aggressive creatures when threatened. Always assess the situation and handle only when necessary. Wear protective gear to protect hands from sharp teeth and claws. It is always advisable that two people handle one animal. Use techniques which minimize stress such as covering the eyes. If direct handling is necessary (e.g., in the case of an injured otter), use a towel or soft net to restrain the animal gently. While lifting and carrying animals always support the head and body. Avoid holding animals with their tail.

Figure 11: Restraining a raptor by supporting with one hand and securing talons in the other hand using a welder's gloves

Note- *If the animals are extremely stressed, in pain, or not used to handling, tranquilization/sedation before examination, sampling, and diagnostic testing may be considered.*

3

DISEASES OF AMPHIBIANS

The class Amphibia has more than 7,000 identified species, included in three orders: Anura, Caudata, and Gymnophiona. The order Anura alone has more than 6,000 species and includes frogs and toads. Their population is declining due to interrelated issues, including habitat loss, climate change, infectious disease outbreaks, pollution, and exposure to toxins. There are many infectious and non-infectious diseases of amphibians. Poor hygiene and temperature outside their preferred optimal temperature zone (POTZ) are significant contributors to diseases in amphibians. Parasitic diseases of amphibians include many protozoans, metazoans, nematodes, cestodes, and trematodes, which do not necessarily cause any disease unless the host is stressed or immunocompromised. They may act as final or intermediate hosts for these parasites. Additionally, amphibian nutritional diseases are common because their food source is devoid of macro and micronutrients. All amphibians show an altered calcium: phosphorus ratio. Apart from this, many neoplasias are also reported in amphibians, such as ranid herpesvirus.

Certain fungal diseases of amphibians are difficult to differentiate because they produce similar clinical signs, including lethargy and ulcerative lesions on the skin or integuments. Some fungi require a wet mount prepared from skin scrapings, whereas others require culture, histopathological examinations, and staining.

3.1 Diseases of viral origin

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic methods
Ranavirus	Anurans and caudates	Iridoviridae	Skin swabs, liver, spleen, and kidney tissues	PCR assay, primary cell culture, microscopy
Renal adenocarcinomas	Anurans	Ranid herpesvirus-1	Kidney, tissue, and blood samples	Histology (Intranuclear inclusion bodies)

3.2 Diseases caused by bacteria

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic methods
Brucellosis	Amphibians	Brucellaceae	Abscesses, bone, and tissue	culture, and Isolation Histology
Chlamydiosis	Amphibians	*Chlamydia pneumoniae, C. psittaci, C. abortus, or C. suis*	Liver and spleen	Histology (Intracytoplasmic basophilic inclusion bodies are found), PCR
Mycobacteriosis	Captive amphibians	*Mycobacterium fortuitum, M. marinum, M. chelonea, M. abscessus, M. avium, M. szulgai, M. xenopi, and M. liflandii*	Feces or oropharyngeal mucus	Bacterial culture isolation from fresh tissue or PCR assay
Red-leg Syndrome	All amphibians	*Aeromonas spp., Pseudomonas spp., Proteus spp., Elizabethkingiam eningoseptica, Klebsiella spp., Streptococcus spp., and Citrobacter spp.*	Liver, spleen. coelomic organs, blood, or coelomic fluid	Tissue for histology, blood or coelomic fluid for isolation
Flavobacteriosis	All amphibians	*Flavobacterium odeverans, F. indologenes, F. meningoseticum*	Infected tissue for culture	PCR

3.3 Other diseases of amphibians

Most fungi that cause infections in amphibians are difficult to distinguish on the basis of gross examination, as they produce similar clinical effects. Treatment requires the use of topical and systemic antifungal agents, and proper hygiene.

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic methods
Fungal Diseases				
Chromoblastomycosis	Anurans	*Cladosporium spp., Fonsecaea spp., Phialophora spp., Ochroconis spp., Rhinocladosporium spp., and Wangiella spp.*	Skin scrapes, post mortem organs	The presence of disseminated granulomas with fungal cells and hyphae in culture, pigmented hyphae visible on post-mortem gross lesions
Chytridiomycosis	Anurans, caudates, and caecilians	*Batrachochytrium dendrobatidis and Batrachochytrium salamandrivorans*	Skin scrapes, swabs of integument, digits, or drink patch	Real-time PCR assay of integument or skin swabs
Saprolegniasi	Aquatic amphibians	*Saprolegnia, Aphanomyces, and Achyla*	Skin scrapes	Thin-walled zoospores in skin scrapes confirmed by microscopy, histology, the culture of water mould & molecular confirmation
Zygomycosis	Captive and wild Anurans	*Mucors pp, Basidiobolus spp, Fusarium spp, and Rhizopus spp*	Skin scrapes	Thin-walled zoospores in skin scrapes confirmed by Microscopy
Parasitic Diseases				
Rhabdiasis	All amphibians	*Rhabdias spp.*	Fecal samples, oral or nasal swabs	microscopic examinations, fecal examination using flotation or sedimentation techniques
Strongyloidiasis	All amphibians	*Strongyloides spp.*	Fecal samples	Eggs or adult worms can be found on fecal examination
Cutaneous/ epidermal capillariasis	All amphibians	*Pseudocapillaroides xenopi*	Skin scraping or biopsy	PCR to detect *P. xenopi*
Amoebiasis	All amphibians	*Entamoeba ranarum*	Fecal samples or colon washes	Trophozoites on wet mounts
Cryptosporidiosis	All amphibians	*Cryptosporidium fragile*	Fresh fecal samples	Acid-fast stain of fresh fecal samples or via endoscopic sampling of the gastric mucosa
Rhabdiasis				Detection of parasitic eggs through —
Filariasis	Wild-caught adult amphibians or adult amphibian colonies exposed to vector	Onchocercidae family, *Trypanosima spp.*, *Hepatozoon spp.*, and *Haemogregarine spp.*	Blood samples	Identification with blood smear
Microsporidiosis	Wild and captive amphibians	*Microsporidium spp.*, *Fleistophora spp.*, and *Allogluegae spp.*	Blood, infected tissue	Blood smear, PCR, histology/ cytological examination of infected tissue

4

SAMPLE COLLECTION FROM AMPHIBIANS

Amphibians are highly sensitive to stress, and minimal handling is preferred. Anesthesia is required in certain procedures like blood sampling or biopsy. Various types of samples are collected for diagnostic purposes. Below is the overview of samples collected in amphibians for disease diagnosis.

Blood samples

The blood collection process is challenging due to their small size and delicate nature. A 27-30 gauge needle collects blood from the ventral abdominal vein, the femoral vein, or cardiac puncture (usually in larger amphibians or during post-mortem examination).

Figure 12: Blood collection by cardiac puncture in Indian bull frog

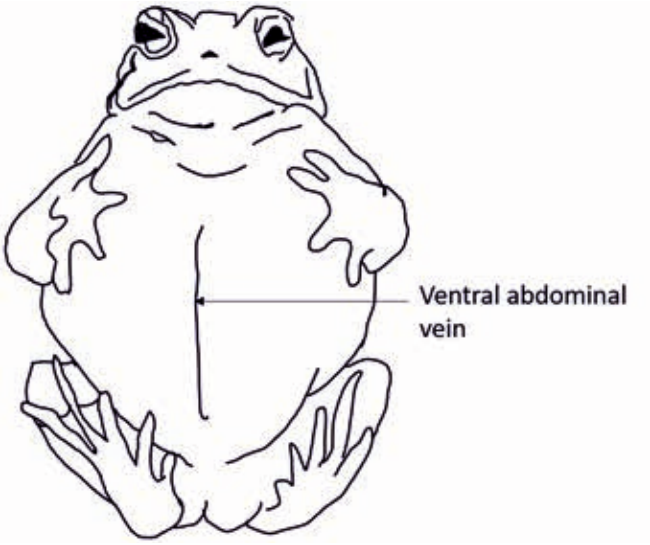


Figure 13: Location of ventral abdominal vein for blood collection in frogs and toads

Skin swabs

Skin swabs can be directly collected from amphibian skin, particularly from the belly, thighs, digits, etc.

Figure 14: Indian bull frog being swabbed for chytrid fungus.

Other Swabs

Sterile swabs are inserted into the nasal cavity or mouth to collect respiratory tract cells. The moistened swab is inserted for cloacal swabbing, and it is gently rotated to collect cells and fluid. Samples are placed in sterile containers until further use.

Fecal samples

Fresh fecal samples can be collected from the environment or by gentle pressure on the abdomen to stimulate defecation.

Tissue samples

Tissue samples are collected from abnormal growth and skin lesions using biopsy techniques, and internal organs (post-mortem).

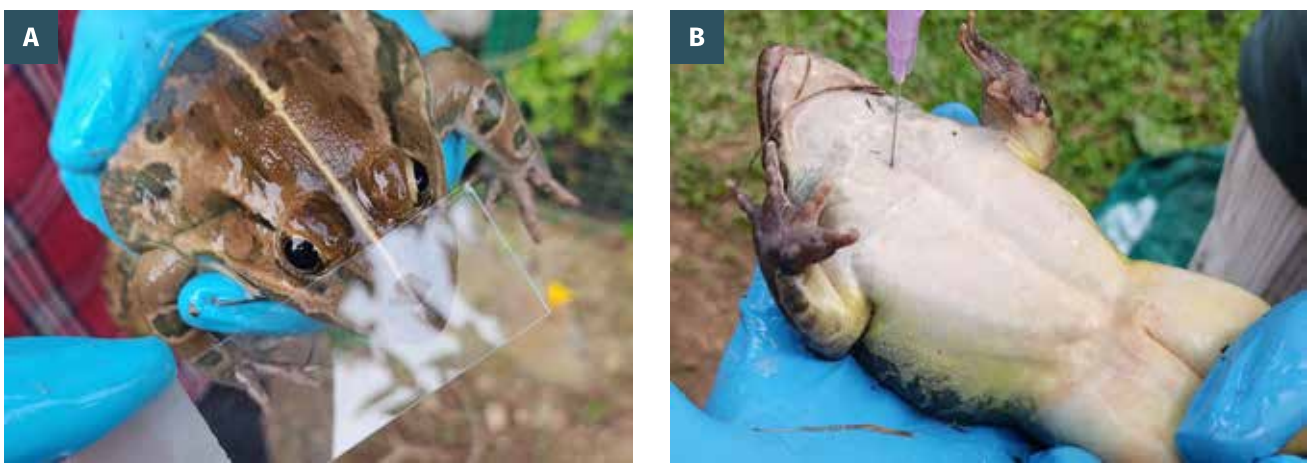


Figure 15: Collecting impression smear on rostrum (A), cardiocentesis through sternum (B)

Samples and their preservation and storage protocol:

Sample Types	Uses	Preservative	Storage
Blood Sample	DNA/RNA Isolation	DNA/RNA later	Refrigeration (4°C)
	Complete blood count	EDTA/heparin coated vials	Refrigeration (4°C)
	Serum Biochemistry	Clot activator vials	Refrigeration (4°C)
	Cytology	EDTA/heparin coated vial	Refrigeration (4°C)
	Microbiological culture	Appropriate media	Refrigeration (4°C)
Tissue	DNA/RNA isolation	DNA/RNA later or liquid nitrogen	Frozen (-80°C)
	Histopathology	10% formaldehyde	Room temperature
	Cytology	No preservative	Refrigeration (4°C)
Swabs	Cytology	No preservative	Refrigeration (4°C)
	Microbiology	Suitable transport media	Refrigeration (4°C)
Skin scraping	Parasitology	Direct examination using a microscope	Immediate use
	Microbiology	No preservative	Refrigeration (4°C)
Fecal sample	Microbiology DNA	70% ethanol	Room temperature
	Parasitology	10% formaldehyde	Room temperature

5

DISEASES OF REPTILES

Reptiles are diverse groups of species living in different habitats worldwide. They can even be found in hot deserts and the mountains of the Himalayas. Each reptile has specific bodily requirements.

Reptile diseases include infectious, parasitic, nutritional, metabolic, and traumatic injuries. Most infectious diseases may be prevented by screening before quarantine. Routine physical examination, fecal sedimentation and flotation, screening for infectious disease, radiography, complete blood count (CBC), blood biochemistry, etc., should be carried out during quarantine. The ill animal should be separated from the healthy ones until full recovery because some infectious diseases have the potential to spread via fomites. In captivity, excessive humidity, low or high environmental temperatures, dietary changes, inappropriate therapies, and other stressors may influence disease. Traumatic injuries, burns, dysecdysis, and prey-induced trauma may cause secondary bacterial infections.

Bacterial diseases are most common in reptiles and are mainly caused by opportunistic organisms that infect malnourished, immunosuppressed hosts. The concerned authority should make timely decisions to manage this situation, which often requires a comprehensive approach. The success of the therapeutic plan depends not only upon identifying causative agents but also on correcting predisposing factors. Cytology (histology), culture and sensitivity, gram stain, etc., are useful tools for determining appropriate treatment plans. Very few viruses are confirmed to be etiological agents of diseases in reptiles, but many have been isolated from sick reptiles. Nowadays, many viral PCR and serology tests are available.

Commonly occurring diseases of reptiles based on their origin and sampling protocols adopted are presented in the following sections:

5.1 Disease of viral origin

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic methods
Inclusion Body Disease (IBD),	Snakes - Boas	Retrovirus or reptarenavirus	Blood samples, tissue, biopsies (internal tissue)	Blood profile increased WBC count, PCR and Biopsy of Internal Tissue
Retrovirus	Snakes - Vipers, testudines, crocodilians,	Retrovirus squamata	Tissue biopsy	PCR, histology
Adenovirus	Snakes- vipers, pythons, and boas, lizards, crocodilians	Adenovirus	Fresh fecal samples	PCR from fresh faeces
Herpesvirus	Freshwater turtles tortoises and sea turtles	Herpesvirus	Skin, blood, or tissue samples	Histology
Ferlavirus and Nidovirus	Snakes- vipers lizards, testudines, crocodilians, squamata	Paramyxoviridae	Lung samples, endoscopic biopsies	Histology and electron microscopy
West Nile Virus	Crocodilians	West Nile virus	Blood, tissue samples, cerebrospinal fluid	RT-PCR, (Ig) antibody testing, NAAT
Papillomas	Lizards, testudines, pythons, boa	Papilloma-type virus	Skin scrapings, tissue biopsies	Electron microscopy
Iridoviruses	Chelonians, snake, and lizards	Iridoviruses	Skin, liver, or spleen samples, tissue biopsies kidney, lungs, intestine, oral and fecal swabs	Viral Isolation, PCR microscopic examination of Giemsa stained sections, negative staining electron microscopy
Neoplastic diseases	All Reptiles	Oncogenic viruses	Tumor tissue, blood, or organ biopsies	Cytology, histopathology (Biopsy) and viral isolation
Pox virus infection	Testudines, crocodilians, squamates	Pox virus	Skin lesions	TEM of skin lesions, PCR
Japanese encephalitis	Testudines, crocodilia, squamata	Flavivirus	Tissue and blood samples	Serology, PCR

5.2 Diseases caused by Bacteria

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic
Septicemia	Chelonians, snakes crocodilians	*Aeromonas spp., Pseudomonas spp.*	Swab samples from infected regions	Bacterial isolation
Septicemic Cutaneous Ulcerative Disease (SCUD)	Aquatic turtles	*Citrobacter freundii, Serratia spp.*	Skin swabs	Bacterial isolation
Ulcerative or Necrotic Dermatitis	Snake, lizards	*Aeromonas spp., Pseudomonas spp., Vibrio chitinovora*	Swab samples from infected regions	Bacterial isolation

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic
Abscesses	Freshwater turtles and tortoises	*Pepto streptococuccus, Pseudomonas spp. Aeromonas spp., Serratia spp., Salmonella spp., Micrococcus spp., Erysipelothrix spp., Citrobactor freundii, Morganella morganii, Proteus, Staphylococcus, Escherichia coli, Klebsiella spp., and Dermatophilus spp.*	Swab samples from infected regions	Bacterial isolation
Pneumonia	Snakes, lizard, and turtles	*Aeromonas spp. and Pseudomonas spp.*	Swab samples or respiratory fluid from infected region	Bacterial isolation
Mycoplasmosis	Chelonians, crocodilians, and squamates	*Proteus spp., Psudomonas spp., Citrobactor spp., Morganella morganii, Enterobactor spp.*	Serological samples	PCR and serological samples, ELISA, culture
Spinal Osteopathy/ Osteomyelitis	Snake	*Salmonella spp.*	Biopsy samples and tissue	Histopathology samples or Blood Culture
Mycobacteriosis	Chelonians, snake lizards, and crocodilians	*Mycobacterium spp.*	Nasal swabs, tissue biopsies	PCR, histology, bacterial culture

5.3 Other Diseases of Reptiles

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic
Mycotic				
Mycotic	Chelonians, snakes, crocodilians	*Microsporidia spp., Zoopagomycota spp., Mucormycota spp., Basidiomycota spp., and Ascomycota (including Ophidiomyces, Nannizziopsis)*	Tissue biopsy,	Fungal culture, histopathology, PCR, microscopic examination and culture
Metabolic, Endocrine and Nutritional Diseases				
Gout	All reptiles	Urate microcrystals, or excessive protein diet	Blood samples, joint fluid	Identification of urate crystals, hyperuricemia on blood tests, radiographs

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic
Metabolic Bone Diseases (MBD)	All reptiles	Low calcium: phosphorus ratio, vitamin D3 deficiency, Low Ultraviolet (UVB) light	Blood samples	Radiographs, blood calcium levels, bone density analysis
Parasitic Diseases				
Ectoparasite	All reptiles	Mites, ticks, leeches etc.	Skin scraping or physical examination	Microscopic examination
Endoparasites (Cestodes/ Nematodes)	All reptiles	*Strongyloides spp., Ascaris spp.*, capillarid, trichurid, oxyurid etc	Fecal samples	Fecal flotation, identification of ova or larvae under microscopy
Protozoal	All reptiles	*Entamoeba spp., Hexamita spp., Eimeria spp., Cryptosporidium spp.*	Fecal samples, tissue biopsies	Identification of trophozoites or cysts in a wet preparation of fresh faeces or tissue impressions or in histologic sections

A large number of pathogens have been isolated from reptiles; however, limited information on the diseases they cause and their diagnosis are available. It thus becomes imperative to conduct a detailed necropsy of all unexplained mortalities with collection and through laboratory examination of relevant biological samples.

6

SAMPLE COLLECTION IN REPTILES

Sample collection for viruses, bacteria, and protozoa is easier in mammals than in reptiles. To ensure the safety of both animals and humans, anesthetise the animal only in the presence of a veterinarian. Otherwise, use proper handling techniques and equipment.

The types of samples collected are

Fresh blood samples

Venipuncture is a blind technique in the case of reptiles. The sample should not exceed 0.5 ml of blood for every 100 gm of body weight of debilitated reptiles. Sodium or lithium heparin is the preferred anticoagulant for reptiles over EDTA because it is less likely to lyze erythrocytes in some reptiles.

Blood collection sites- The ventral tail vein is the most common site in snakes and lizards, but cardiocentesis can also be performed on snakes. A subcarapacial sinus, jugular vein, or tail vein is preferred for turtles. For Gharials, samples can be collected from the ventral aspect of the tail vein, and also from the supra-occipital sinus. (Care must be taken while collecting blood samples from the sinuses as there are chances of sample contamination from the lymphatic fluid)

Pre-requisite- Syringes, appropriate gauge needles (depending upon size and species), EDTA vials, Heparin vials, serum vials, cotton, alcohol or rectified spirit, ice box marker, pen, etc.

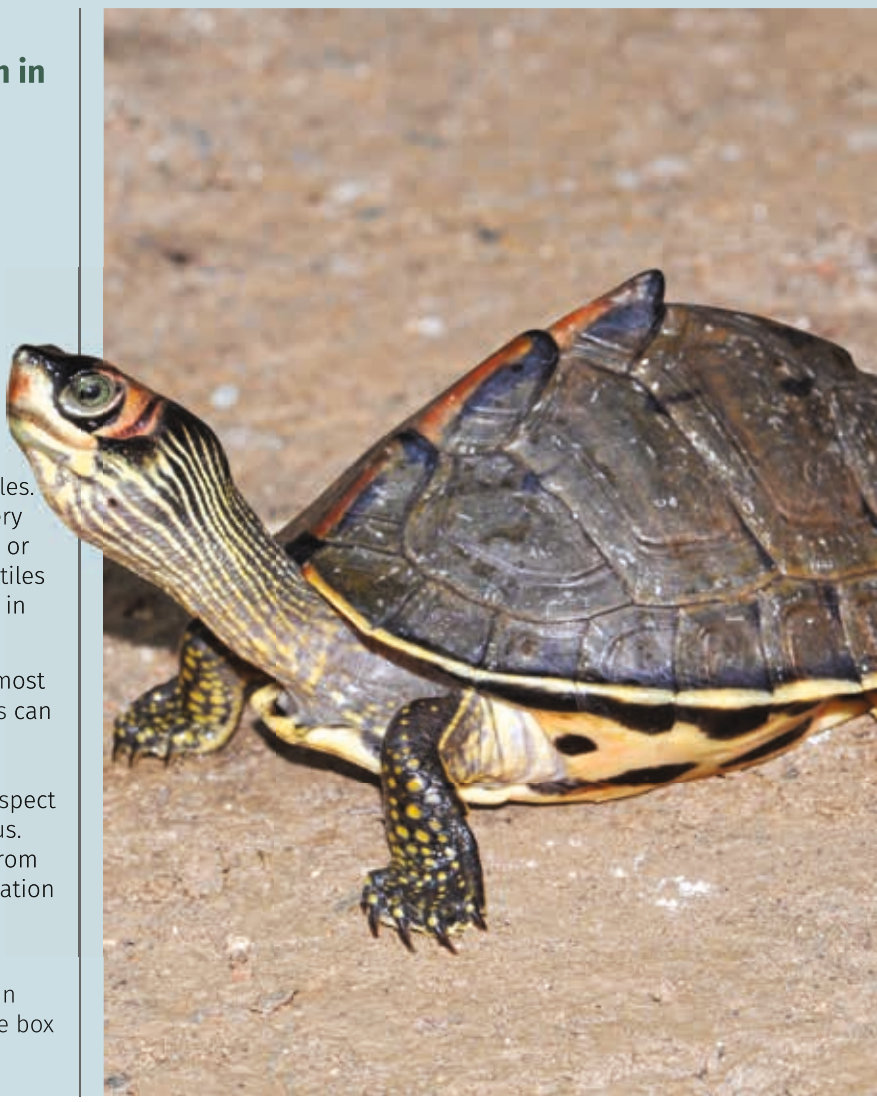


Figure 16: Blood sample collection of Indian flap-shell turtle from the subcarapacial sinus.

Figure 17: Blood collection from the jugular vein from tortoise; note the handlers' hand restricting forelimbs (adapted from Divers and Stahl, 2018)

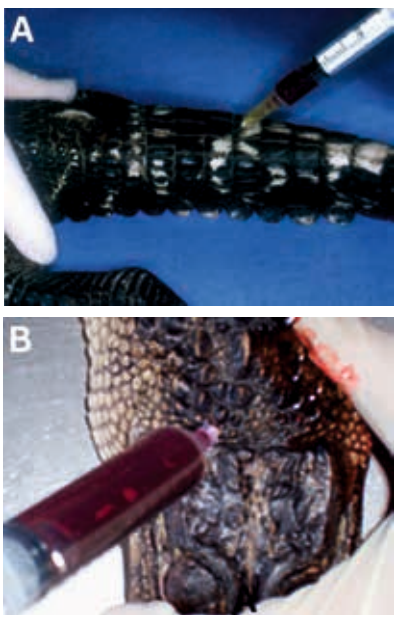


Figure 18: Blood collection from caudal or ventral tail vein in crocodiles (A). Blood collection site for crocodilians dorsally from supra-occipital sinus (B). (Adapted from Divers and Stahl, 2018)

Tissue samples

Use a sterile biopsy punch or scalpel blade to collect a piece of skin. Usually, a small portion of the lateral body or tail is taken. If necessary, anesthetize the animal.

Pre-requisite- Anesthetics, restraining tools, aseptic solutions, sterile biopsy punches, scalpels, forceps, gloves, sutures, containers, etc.

Figure 19: Collection of cloacal swab from Red-crowned roofed turtle.

Oral, nasal, or cloacal swabs

Sterile cotton swabs from oral, cloacal, or other mucosal surfaces can be taken into an appropriate medium. If you collect samples from multiple species, ensure proper handling of samples post-collection to avoid cross-contamination.

Pre-requisite sterile cotton tip swabs, transport media, marker, pen, etc.

Figure 20: Procedure for collection of pharyngeal swabs.

Skin Scraping

If there are multiple lesions, the sample is usually taken from the most recent one. In the case of fungal lesions, the sample is taken from the leading edge of the rash. Pinch the skin so that parasites move closer to the skin surface, scrape the skin against the blunt edge of the scalpel with enough pressure to bleed the skin slightly and transfer the debris to the slide for further examination.

Pre-requisite aseptic solution, gloves, mask, mineral oil, scalpel, slides, microscope, etc.

Fecal samples

Defecating in water is common for reptiles such as turtles, but this can result in a contaminated sample, making it less ideal for analysis. Collect a fresh sample within one to two hours of defecation. If possible, collect directly from the cloaca.

Tail clipping or scale snipe

A small portion of the tail or scale can be collected safely with minimal harm to the reptile.

Samples and their preservation and storage protocol:

Sample Types	Uses	Preservative	Storage
Blood sample	DNA/RNA isolation	DNA/RNA later	Refrigeration (4°C)
	Complete blood count	EDTA/heparin coated vials	Refrigeration (4°C)
	Serum biochemistry	Clot activator vials	Refrigeration (4°C)
	Cytology	EDTA/heparin coated vial	Refrigeration (4°C)
	Microbiological culture	Appropriate media	Refrigeration (4°C)
Tissue	DNA/RNA isolation	DNA/RNA later or liquid nitrogen	Frozen (-80°C)
	Histopathology	10% formaldehyde	Room temperature
	Cytology	No preservative	Refrigeration (4°C)
Swabs	Cytology	No preservative	Refrigeration (4°C)
	Microbiology	Suitable transport media	Refrigeration (4°C)
Skin scraping	Parasitology	Direct examination using a microscope	Immediate use
	Microbiology	No preservative	Refrigeration (4°C)
Fecal sample	Microbiology DNA	70% ethanol	Room temperature
	Parasitology	10% formaldehyde	Room temperature

7

DISEASES OF AVES

Pediatric and geriatric diseases are well-studied in avian medicine. In pediatric cases, the cause is mainly associated with parent health, nutrition, environmental conditions, and exposure to infectious diseases.

Advancements in avian medicine have changed the approach to infectious diseases, wellness care, and emergency medicine. Behaviour and nutritional requirements are vital components of health that play an important role in bird wellness. Management of birds is problematic as they can mask their clinical signs until the disease has progressed to an advanced state. Moreover, they have higher metabolic rates than mammals and are at a high risk of oxygen deprivation during handling, treatment, or diagnostic sampling.

Fungal diseases are also quite common in Aves, because it acts as a secondary infection in an already ill or immunocompromised bird. *Aspergillus spp*. causes respiratory tract infections, and *Candida spp.* causes GI tract infections. They are the most common types of fungal diseases.

7.1 Diseases of viral origin

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic methods
Avian Influenza	Charadriiformes and Anseriformes	Alphainfluenza virus or Influenzavirus A	Tracheal or cloacal swabs, blood samples	Detection of viral RNA, antibodies and viral isolation
Fledgling disease/ Polyoma disease	Psittaciformes	Polyomavirus	Cloacal swab, blood samples	Cloacal swab/blood samples for antibody test

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic methods
Psittacine Beak and Feather Disease (PBFD)	Psittaciformes	Psittacine circovirus	Feces, feather dander, blood samples	PCR assay of feces, feather dander or blood
Pacheco's disease	Psittaciformes	Psittacine herpesvirus	Combined oral and cloacal swabs, blood samples	DNA probe of combined oral and cloacal swabs and blood sample
Proventricular Dilatation Disease (PDD)	Psittacifromes	Avian bornavirus	Cloacal swab, fecal samples	PCR assay of cloacal swab or feces
West Nile Virus (WNV)	Passeriformes, Charadriiformes, and Falconiformes	Flaviviridae	Blood samples, brain and kidney tissues	PCR assay, histology examination
Avian pox virus disease	Sphenisciformes, Procellariiformes, Pelecaniformes, phoenicopteriformes, Procellariiformes, phaethantiformes	*Avipoxvirus*	Affected skin or mucosa	Histopathology (presence of Bollinger bodies), PCR
Duck plague	Anseriformes	Herpes virus	Swabs from lesions, biopsies, or tissue samples	Button-like lesions in the gut on PM examination and hepatomegaly through PCR, virus isolation
Eastern equine encephalitis	Pelecaniformes, Gruiformes, Sphenisciformes	Tagovirus	Affected tissue (brain, heart, spleen)	Virus isolation through culture, RT-PCR, immuno-histochemistry along with histopathology
Wellfleet Bay Virus disease	Anseriformes	*Quaranjavirus*	Tissue samples	Virus isolation through culture, RT-PCR, immuno -histochemistry along with histopathology

7.2 Diseases caused by bacteria

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic
Chlamydiosis	Psittaciformes	*Chlamydia psittaci*	Blood samples, cloacal or conjunctival swabs	ELIZA, PCR assay
Avian mycobacteriosis	Captive, exotic wild and domestic birds	*Mycobacterium avium and M. genovense*	Blood samples, tissue biopsies (liver, intestine, spleen, fecal samples)	DNA sequencing, PCR assay, acid-fast staining.
Salmonellosis	Sphenisciformes, Anseriformes, Pelecaniformes, Charadriiformes	*Salmonella typhimurium, S. anatis*	Affected tissue (spleen, liver, pancreas)	Bacterial isolation through culture, rtPCR, histopathology along immunohistochemistry

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic
Avian cholera/ pasteurellosis	Sphenisciformes, Pelecaniformes, Gaviiformes, Suliformes, Podicipediformes, Procellariforms	*Pasteurella multocida*	Heart blood, liver, bone marrow, or affected tissue	Histopathology along with immune-histochemistry, bacterial culture, and confirmation by PCR
Pododermatitis/ Bumblefoot	Galliformes, Anseriformes	*Staphylococcus aureus, Escherichia coli, Proteus mirabilis, Enterococcus spp., Clostridium spp.,* or fungi	Aspirates from nodules, swabs from ulcers, biopsy of granulomas	Bacterial culture and PCR

7.3 Other diseases of Aves

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic
Mycotic diseases				
Candidiasis	Young or immuno-compromised birds	*Candida albicans*	Feces, crop or regurgitated content, skin scrapes, or impression smear	Gram's staining, skin scrapping or impression smear, fungal culture, cytology
Aspergillosis	Psittaciformes	*Aspergillus flavus* and *A. niger*	Blood samples	Blood for - CBC, biochemistry. serology and PCR assay
Nutritional Diseases				
Metabolic Bone Disease (MBD)	Young birds	Low calcium: phosphorus ratio, vitamin D3 deficiency	Blood samples	Radiographs, blood calcium and phosphorous levels
Lead toxicosis	Aquatic birds	Ingestion of shiny objects near water bodies	Bones, liver and kidney samples, blood samples, droppings	Checking of blood lead concentration, toxicological tests of bone for chronic lead exposure
Mercury toxicosis	All birds	Biomagnification due to feeding on fishes	Liver, kidney, and affected tissues	Toxicological tests of liver and kidney
Parasitic Diseases				
Giardiasis	Psittaciformes	*Giardia psittaci*	Fresh fecal smear	ELIZA and PCR assay
Trichomoniasis	Columbiformes, Galliformes, Falconiformes, Psittaciformes, and Passeriformes	*Trichomonas gallinae*	Fresh fecal smear	Microscopic examination of fecal smear, culture
Biotoxicity				
Biotoxicity from algal blooms	Aquatic birds (that feed on small fishes and invertebrates)	Neurotoxins produced by algal blooms (freshwater cyanobacteria)	Liver and gastrointestinal contents	Toxicological test of collected samples, ELIZA, liquid chromatography

8

SAMPLE COLLECTION IN AVES

The ideal sample collection technique is the one that has minimal impact on the bird during the process. A variety of samples can be collected from birds to identify a disease. Below are a few of the techniques and types of samples collected.

Fresh blood samples

Anatomical differences demand the selection of appropriate blood collection sites and proper techniques. The total blood volume in any given bird is approximately 10 per cent of its body weight. Given that 10 per cent of the entire blood volume can be safely collected for diagnostic use (i.e. 1 ml of blood can be collected for each 100 gm of body weight).

Blood collection can be done from the right jugular, basilic, medial tibiotarsal, or metatarsal vein.

Note that some labs prefer lithium heparin over EDTA for haematology. Consult with the local laboratory for their preference.

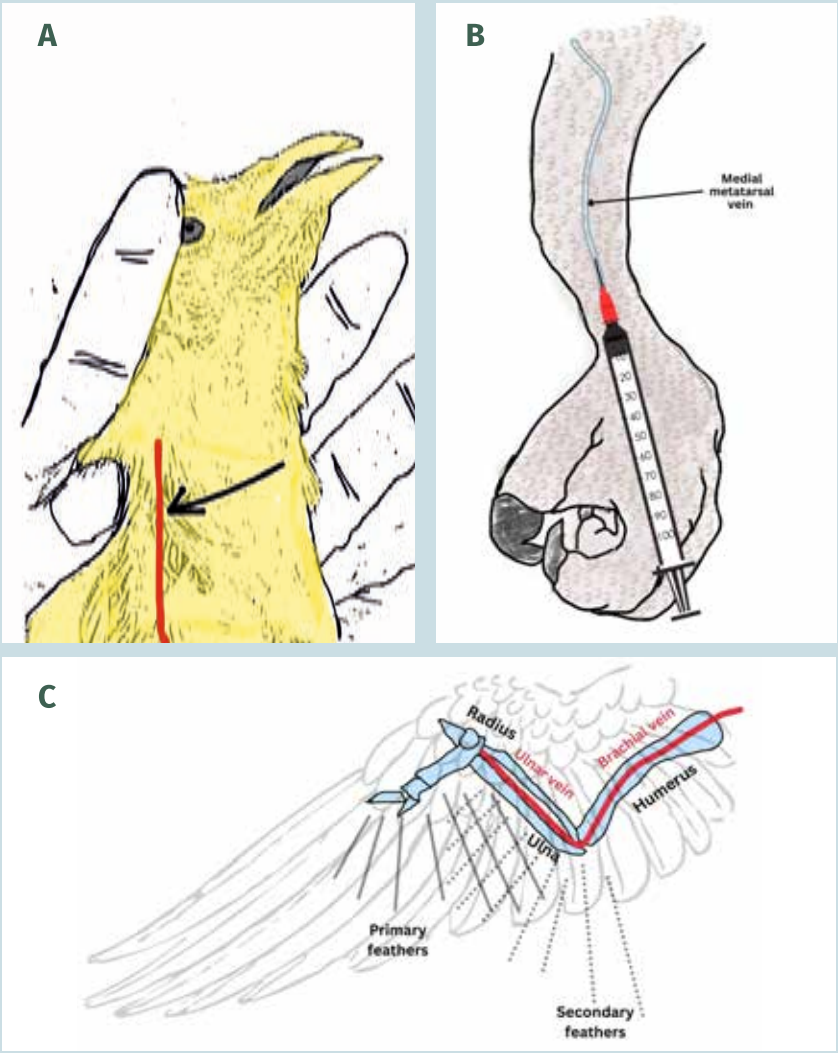


Figure 21: Location of jugular vein for blood sample collection (A); Location of metatarsal vein (B); Location of basilic or ulnar vein (C).

Swabs

Sterile swabs collected in appropriate transport media can be sent to the laboratory. They are usually from the cloaca, choana, crop, eyes, ears, and skin. Less common sites are the sinus, trachea, air sacs, and oviducts.

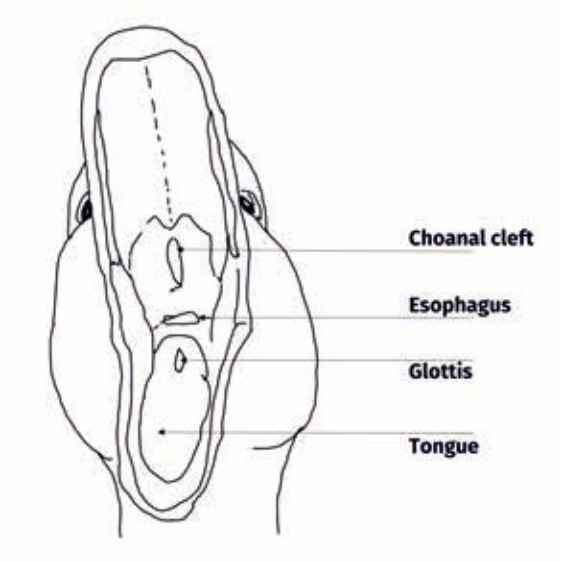


Figure 22: Location for collection of tracheal or oropharyngeal swabs

Feathers

Always wear gloves while handling birds for feather sample collection. Hold the bird securely to ensure safety, and grip the feather firmly at its base close to the skin using index and thumb fingers. Pull in a quick, smooth motion toward natural feather growth.

Tissue samples

Common tissue samples include muscle, skin, or organ samples. They are often collected during post-mortem or authorized invasive procedures. A small section of tissue is also collected using the biopsy technique.

Fecal samples

Collecting fecal samples from birds is a noninvasive technique. The sample can be collected shortly after the bird defecates, ensuring it is fresh. Always use a spatula or gloves to collect the faces to avoid contamination.

Eggshells

A small portion of the egg is collected in a sterile container using gloves shortly after hatching or when the nest is abandoned.

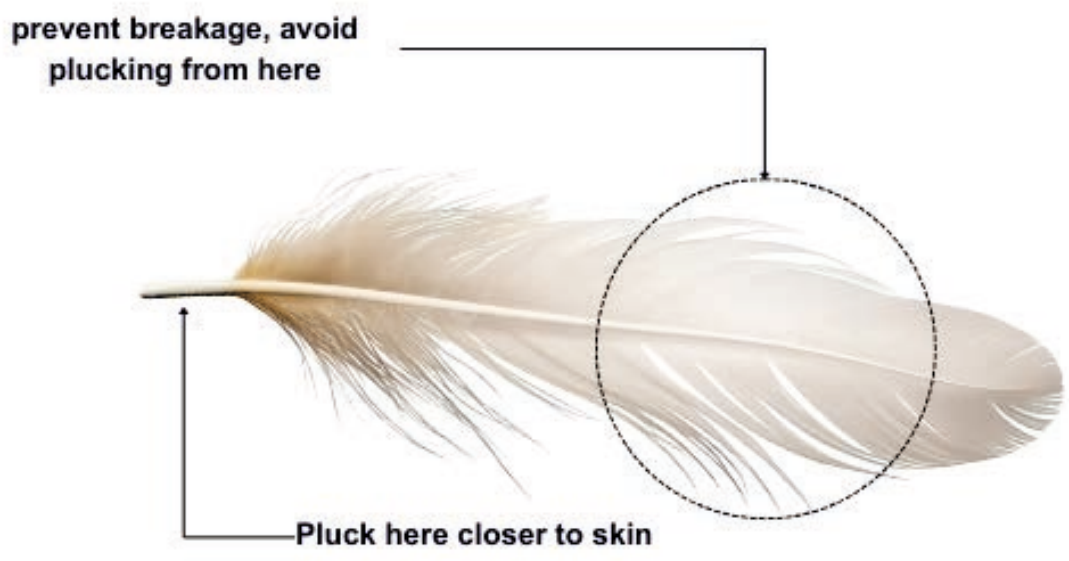


Figure 23: Feather sample collection method

Note- *Iatrogenic damage to the mucosal surface while swabbing is common. Pre-moistened swabs either with normal saline or transport medium are advisable.*

Samples and their preservation and storage protocol

Sample Types	Uses	Preservative	Storage
Blood sample	DNA/RNA isolation	DNA/RNA later	Refrigeration (4°C)
	Complete blood count	EDTA/heparin coated vials	Refrigeration (4°C)
	Serum biochemistry	Clot activator vials	Refrigeration (4°C)
	Cytology	EDTA/heparin coated vial	Refrigeration (4°C)
	Microbiological culture	Appropriate media	Refrigeration (4°C)
Tissue	DNA/RNA isolation	DNA/RNA later or liquid nitrogen	Frozen (-80°C)
	Histopathology	10% formaldehyde	Room temperature
	Cytology	No preservative	Refrigeration (4°C)
Swabs	Cytology	No preservative	Refrigeration (4°C)
	Microbiology	Suitable transport media	Refrigeration (4°C)
Feathers	DNA isolation	No preservative	Frozen (-80°C)
Fecal sample	Microbiology DNA	70% ethanol	Room temperature
	Parasitology	10% formaldehyde	Room temperature

9

DISEASES OF FRESHWATER MAMMALS

The freshwater ecosystem that includes ponds, lakes, streams, and rivers is susceptible to environmental changes and acts as a source for many of the infectious diseases. Aquatic and terrestrial mammals interact as both are dependent on freshwater. Aquatic creatures which act as vectors or intermediate hosts for many diseases, and the degradation of the aquatic ecosystem results in the transmission of many pathogens (Peter et al., 2010).

Commonly encountered diseases of aquatic mammals and their sampling methods are presented in the following section:

9.1 Diseases of viral origin

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic
Herpes virus	Cetaceans	Phocid herpes virus 1 (PhHV1) and PhHV2	Necropsy tissues or internal biopsy	PCR, viral isolation, histopathology
Canine parvovirus	Mustelidae	CPV type 1 and type 2	Fecal sample	Fecal antigen testing, PCR assay
Canine distemper	Mustelidae, cetaceans	*Morbillivirus*	Brain, tissue, lung, spleen samples	Histology of brain tissue, immunohistochemistry, RT-PCR
Canine adenovirus	Mustelidae	CAdV-1 and CAdV-2	Blood samples, nasal, and oral swabs	Serology, viral isolation or culture, PCR
Herpesvirus	Mustelidae, cetaceans	Canine herpesvirus (CHV), Phocid herpesvirus (PhHV)	Blood samples, nasal, oral, or genital swabs	Antibody testing, PCR assay, viral isolation, histopathology, and TEM
Canine parainfluenza virus	Mustelidae	Canine parainfluenza virus (CPIV)	Blood, fluid, and tissue samples	Culture, PCR assay
Canine coronavirus	Mustelidae	Canine coronavirus (CCoV)	Blood samples, nasal, oral, or genital swabs	Antibody testing, PCR assay, viral isolation
Feline parvovirus	Mustelidae	Feline parvovirus (FPV)	Fecal, blood samples	Fecal viral antigen test, blood or fecal samples for PCR
Feline herpes virus	Mustelidae	Feline herpes virus (FHV-1)	Swabs from eyes, throat, nasal discharge	Viral PCR assay
Calicivirus	Mustelidae, cetaceans	*Feline calicivirus* (FCV), *Vesivirus spp.*	Swabs or tissue samples	Culture and sensitivity test, viral PCR assay
Feline infectious peritonitis	Mustelidae	Feline infectious peritonitis virus (FIPV)	Blood, CSF	Complete blood count (CBC), A:G ratio, CSF analysis
Felineleukemia virus	Mustelidae	Feline leukaemia virus (FeLV)	Blood samples	PCR assay, ELIZA
Rabies virus	Mustelidae	*Lyssavirus spp.*	Brain tissue, CSF, saliva, blood samples	Direct fluorescent antibody test, RT-PCR
Pseudorabies virus	Mustelidae	Aujeszky's disease virus/ pseudorabies virus (PRV)	Nasal, pharyngeal secretions, necropsy tissues	PCR assay, necropsy findings, viral culture

9.2 Diseases caused by Bacteria

Diseases	Targeted species / Host	Causative agent	Samples required	Diagnostic
Nocardiosis	Mustelidae, cetaceans	*Nocardia spp.*	Secretions or abscess	Cytology and histology, PCR, aerobic culture on blood agar
Brucellosis	Cetaceans	*Brucella ceti*	Blood samples, lung tissue samples, meconium, and aborted foetus	Blood culture, serology (agglutination), and PCR
Clostridial myositis	Mustelidae, cetaceans	*Clostridium spp.*	Lesion aspirates	Gram-positive bacilli and anaerobic culture
Diamond skin disease	Cetaceans	*Erysipelothrix rhusiopathiae*	Blood, spleen, tissues	Culture, PCR, histopathology
Leptospirosis	Mustelidae, cetaceans	*Leptospira spp.*	Blood samples, urine	PCR assay and serological, microscopic agglutination test
Mycobacteriosis	Mustelidae, cetaceans	*Mycobacterium spp.*	Lesion biopsies, tracheal washes, feces	Culture, PCR, histopathology, radiography and CT
Q fever	Mustelidae	*Coxiella burnetii*	Placenta	PCR, bacterial culture, serology (immunofluorescent antibody test), histopathology

9.3 Others diseases

Diseases	Targeted species /Host	Causative agent	Samples required	Diagnostic
Mycotic diseases				
Aspergillosis, candidiasis, coccidioidomycosis, cryptococcosis	Mustelidae, cetaceans	*Aspergillus spp., Candidia spp., Trichophyton spp. or Microsporum canis*	Cytology, biopsy, fungal culture	Serological test, lactophenol cotton blue wet mount, PCR
Paracoccidiomycosis	Mustelidae, cetaceans	*Paracoccidioides brasiliensis, Paracoccidioideceti spp.*	Sputum superficial scrapings	Culture and microscopic examination
Parasitic diseases				
Acanthocephalans	Mustelidae	*Bolbosoma spp.*	Fecal samples, collocation of adult parasite during necropsy	Fecal evaluation, PCR, histopathology , identification of adult parasite
Coccidiosis	Mustelidae, cetaceans	*Coccidia spp.*	Fecal samples, intestinal smears	Floatation, microscopy, LAMP, serology
Toxoplasmosis	Mustelidae, cetaceans	*Toxoplasma gondii*	Blood, tissue biopsy	Histology, immunohistochemistry, PCR, serology, ELISA, western blots

10

SAMPLE COLLECTION IN FRESHWATER MAMMALS

Tissue samples

Biopsy- Skin, blubber, or other tissue collected using biopsy darts (primarily in dolphins), or during necropsies (in both dolphins and otters)

Hair and fur - Hair and fur samples, often from hair traps, or during handling in the wild.

Blood - Samples are typically taken from dolphins only during the rescue and release of stranded dolphins

Blood collection site- The caudal peduncle vein is the most common site for blood collection in dolphins, while the dorsal fin vein is less common. Blood can also be drawn from pectoral flippers. In the case of otters, jugular, cephalic, saphenous, and tail veins can be used for sample collection.

Figure 24: Sample collection from peri-arterial rete on the ventral aspect of the tail fluke

Excretory samples

Fecal sample- Can be collected from water when animals defecate.

Genetic sample

Swab- Oral, genital, or blowhole swabs for dolphins; skin or other swabs for otters can be collected.

Hair and fur- Used for extracting DNA in otters. Collected using hair traps or during handling.

Environmental DNA (eDNA)- Collected from water and sediment samples to detect DNA shed by otter and dolphin in their habitat.

Post-mortem samples

Collected from deceased animals while conducting post-mortem examination.

Respiratory samples

Breath or blow samples are collected from dolphins using a petri dish or similar device to capture exhaled breath air for bacteriology or virology studies.

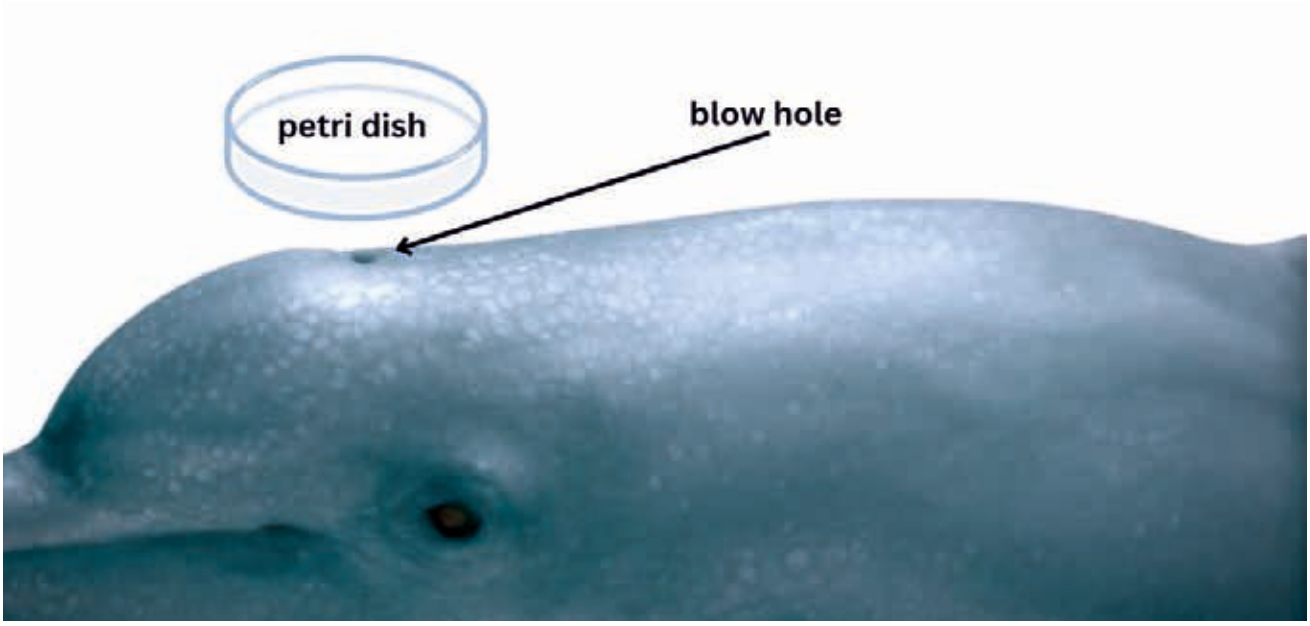


Figure 25: Process of collection of breath samples in dolphin

Samples and their preservation and storage protocol:

Sample Types	Uses	Preservative	Storage
Blood sample	DNA/RNA Isolation	DNA/RNA later	Refrigeration (4°C)
	Complete blood count	EDTA/heparin coated vials	Refrigeration (4°C)
	Serum biochemistry	Clot activator vials	Refrigeration (4°C)
	Cytology	EDTA/heparin coated vial	Refrigeration (4°C)
	Microbiological culture	Appropriate media	Refrigeration (4°C)
Tissue	DNA/RNA isolation	DNA/RNA later or liquid nitrogen	Frozen (-80°C)
	Histopathology	10% formaldehyde	Room Temperature
	Cytology	No preservative	Refrigeration (4°C)
Swabs	Cytology	No preservative	Refrigeration (4°C)
	Microbiology	Suitable transport media	Refrigeration (4°C)
Skin scraping	Parasitology	Direct examination using a microscope	Immediate use
	Microbiology	No preservative	Refrigeration (4°C)
Fecal sample	Microbiology DNA	70% ethanol	Room temperature
	Parasitology	10% formaldehyde	Room temperature

11

VETERO-LEGAL CASES

Veterinarian jurisprudence/ veterinary forensic/ veterinary legal medicine deals with the causes of injuries and deaths of animals brought to the veterinarian for taking up matters in a court of law.

Legal duties of a veterinarian in vetero-legal cases

A veterinarian must have a fair knowledge of veterinary science to adhere to duties, privileges, and responsibilities in veterinary legal cases as a witness and as an expert advisor. He/she must also be aware of animal-related acts and legal procedures in criminal courts. Generally, a veterinarian has the following responsibilities:

- Conducting postmortem examination of the vetero-legal cases
- Investigation of offences against the animal
- Investigation of malicious and accidental poisoning
- Evaluating the health of an animal
- To get culprits punished and help in providing justice and fair treatment to man and animal and also to save innocent persons from the false accusation of crime.
- To prevent cruelty to animal
- To protect the interests of society at large and prevent unethical practices in relation to animals in society.
- In fact, everything in which recourse to law is necessary in relation to the veterinary profession

Postmortem examination of vetero-legal cases

Postmortem is conducted to ascertain

- The time of death
- The cause of death

Rules for Postmortem Examination

- PM is only done after written permission from a police officer or the executive magistrate or forest officer.
- Carefully read the order and report of the legal authorities
- Examination should be carried out in broad daylight
- All details should be noted carefully in PM reports at the time of the PM examination
- The notes and the report to be submitted to the court must tally with each other
- Should have a fair knowledge of the normal and pathological appearance of viscera
- Time and date of arrival of carcass to be noted
- Unauthorized persons should not be allowed to be present at the time of PM except investigating officers.
- Keep preservatives- common salt and alcohol and other fixative agents like- 10% formaldehyde, 50% glycerin, liquid paraffin, mercuric chloride, chromate, osmic acid, etc.

Submission of specimens in suspected cases of poisoning

Suspected poison	Required samples
Alkaloids	Liver, urine, brain, stomach and gut contents
Ammonia	Whole blood or serum, urine, stomach contents (send frozen samples; may add HgCl3 to content)
Antimony	Liver, thyroid, kidney, urine, milk, stomach and gut content
Arsenic (acute)	Liver, kidneys, stomach and gut contents, urine, feed
Arsenic (chronic)	Hair, liver, urine, spleen, altered organs
Barbiturates	Blood, brain, liver, adipose tissue
Cadmium	Kidney, liver, hair
Chlorinated insecticide	Fat, liver, stomach contents, brain (Use only glass containers)
Copper	Liver, blood, kidney, faeces, urine
Cyanide (HCN)	Stomach and gut contents, liver, muscles, oxalated blood, brain, suspected feed (send frozen)
Fluorides	Altered parts of bone, teeth, urine, stomach contents, liver, kidney, feed, drinking water
Lead (acute)	Stomach contents, kidneys, liver, urine, whole blood
Lead (chronic)	Hair, liver, kidneys, bone, faeces, urine
Mercury	Liver, kidneys, intestinal contents, stomach contents, muscle, brain
Molybdenum	Feed, liver, kidneys, altered organs (bone), hair
Nitrate/nitrite	Stomach and gut contents, whole blood, urine, suspected feed (Dispatch quickly)
OPCs (organophosphorus compounds)	Oxalated blood, whole blood, liver, stomach and gut contents
Oxalates	Fresh feed, kidneys (Do not macerate; freeze, fixed in formalin)
Phenols and cresols	Liver, kidneys, oxalated blood, stomach and gut contents
Phosphide	Stomach and gut contents, suspected material (Dispatch quickly)
Phosphorous	Stomach and gut contents, liver, oxalated blood, altered organs (lungs)
Rodenticides	Stomach contents, liver, urine
Selenium	Suspected feed, altered organs, whole blood, liver, hair
Sodium chloride	Oxalated and whole blood, brain, stomach contents, liver
Sodium Fluoroacetate	Liver, gastric contents, feed
Strychnine	Stomach, gut contents, urine, liver, kidney, brain
Warfarin	Whole blood, liver, feed
Zinc	Liver, kidney, pancreas, faeces, bone (humerus)
Zinc Phosphide	Liver, gastric contents, feed (See phosphide)

Quantities of specimens to be sent

Sample type	Quantity
Fat	50-200 gms
Stomach content	100-200 gms in large species and available content in small species
Hair/feather	Stomach contents, liver, urine
Urine	All available
Liver	100-200 gms
Kidneys	One
Brain	Entire
Blood	5-10 ml
Intestinal content	100-200 gms

All the viscera collected during sampling should be preserved for the period of 6 months and then can be disposed of with written permission from the magistrate

12

CHAIN OF CUSTODY

Every interaction with the evidence must be documented, including who handled it, when, where, and why. Evidence must be kept in a proper container, sealed to ensure its security. For e.g., in the case of poisoning, the collection and handling of toxicological samples such as blood, stomach content, liver, spleen, etc., must be controlled to avoid contamination and tampering with samples.

Submission of sample for histopathological examination

Histopathological examination is often an important aspect of diagnosis to ascertain the cause of death. Tissue sample should be preserved and fixed as soon as it is collected. The ideal fixing agent penetrates the tissue and preserves it for a longer period.

Some of the fixing agents are formaldehyde, alcohol (ethanol), mercuric chloride, chromates, osmic acid, and picric acid. The frequently used fixing agent is 10 per cent formalin. The sample collected for histopathology should represent the lesion and include some part of the healthy tissue. The sample should be 0.5 to 1 cm thick. The container should be filled with fixative around 10 times the volume of the tissue.

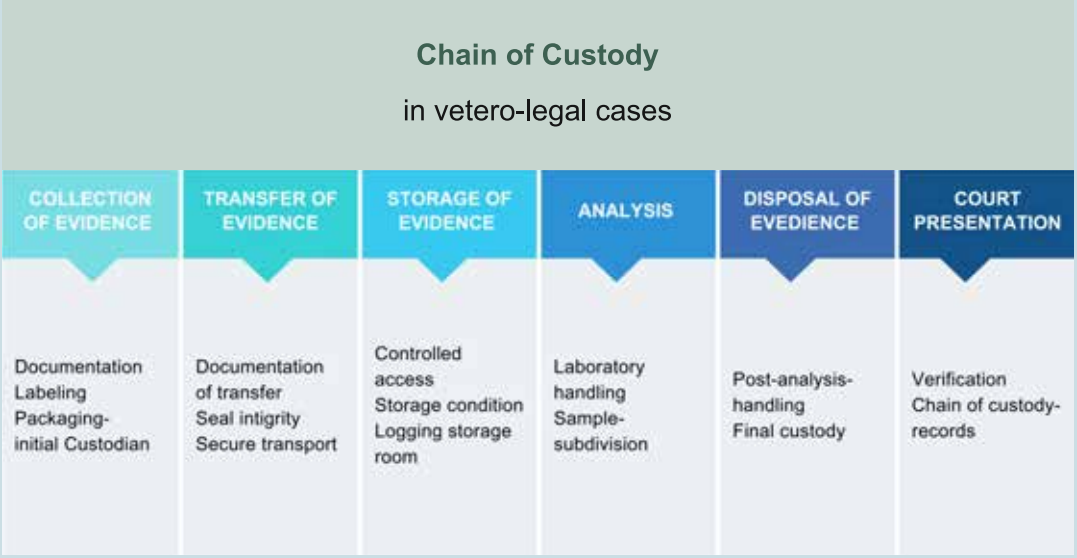


Figure 26.
Chain of custody in vetero-legal cases

13

BIOSAFETY MEASURES DURING SAMPLE COLLECTION

Wildlife biosecurity can be defined as "the set of precautions taken to minimize the risk of introducing a pest or infectious disease into the animal or human population." In other contexts, wildlife biosecurity can be defined as managing the risk associated with infectious disease transmission from wild animals to humans (and vice versa), wild animals to domestic animals (and vice versa), and wild animals to other wild animals. Lack of biosafety measures may contribute to the mechanical transmission of pathogens from infected animals to the personnel engaged in sampling and analysis.

Biological waste, such as animal carcasses, can pose a biosafety risk. Such waste products need a suitably designed disposal system to prevent disease risks to animals, humans, and the environment. The level of biosecurity risk for an animal that dies due to an infectious disease is considered the same as that for an animal suffering from an infectious disease. Animal products, samples, and carcasses should not be stored in the same refrigerator where consumables are stored.

Key considerations

- Appropriate management of animal waste material.
- Documented procedures of animal handling, sampling, storage, and disposal of carcasses and waste.
- Appropriate use of PPE while conducting necropsies and collecting biological samples.
- Cleaning and disinfecting instruments before and after use.
- Cleaning spills of body fluid and blood.
- Follow strict hygiene and keep the specimens and samples in leak-proof containers (screw-cap vials, zip lock bags) during transportation.
- Store the specimen in an appropriate temperature and controlled environment.
- Careful and minimum handling of specimens to avoid contamination.
- Keep the work environment clean and tidy.
- Protection against the diseases caused by ticks, flies, and biting insects.
- Safe handling of animals during sample collection.
- Safe use of animal handling equipment and disposal.
- Safe use of disposables such as needles, syringes, scalpels, blades, etc.
- Safely managing accidental cases of animal bites and other injuries caused by animals.
- Vaccination of workers, with respect to possible disease exposure.

Personal Protective Equipment (PPE)

PPE can reduce the risk of contracting infectious diseases and the amount of risk involved in handling wildlife during sample collection. It is advisable to use PPE based on the method of transmission of the infectious agent. PPE items include:

- Disposable gloves and aprons,
- Long-sleeved shirt or apron,
- Gumboots,
- Welders' gloves while handling,
- Face mask and shield, etc.

14

DISINFECTION AND SANITATION

Proper sanitary measures can reduce the spread and exposure of infectious agents. Sanitation involves preventing animal contact with the disease's physical, chemical, biological, and microbiological agents. Ignoring the demands of sanitation and poor sanitation causes disease. Following simple sanitary measures is proven to have a significant impact on animal and human health.

Disinfection prevents the mechanical transmission of disease agents from one person to another by eliminating many microorganisms except bacterial pores. Disinfecting inanimate objects also prevents infection. It also helps maintain good sanitary and hygiene protocols.

Key measures

- Maintain standardized hygiene and sanitation protocol.
- Avoid direct contact with samples, domestic, agricultural, or industrial waste.
- Any material that was in direct contact with infected waste material should be sanitised carefully and disposed of after use.
- Animal enclosures should be cleaned and disinfected at regular intervals.
- Some bacteria, viruses, and other disease-causing pathogens can remain persistent in the environment for an indefinite period of time.
- Disinfection is the only practical solution to control the spread of pathogens in limited areas.
- Disinfecting the local environment is advisable to prevent the reoccurrence of the disease.
- It is advisable that disinfection of the area containing animals suspected of harbouring notifiable communicable diseases should be carried out under the direct guidance of a disease control expert.
- Disinfection should be carried out wearing all necessary personal protective equipment.
- A person working in disease control should remain in quarantine for a minimum of seven days.
- The disinfectant and containers for dilution are required. Appropriate dilution and suitable dilution techniques must be followed throughout.

Health and Safety

While some disinfectants are harmless, some can be toxic and even lethal to animals, humans, and plants. Therefore, all chemicals should be used in accordance with the appropriate safety measures. Key factors of accidental exposure to the chemicals depend upon area, duration, route, and time of exposure. The use of appropriate PPE all the time is advisable for safety workers

15

SAMPLE TRANSPORT AND HANDLING

Proper transport, handling, and storage of the sample are essential to ensure that test results are not compromised. Once the sample has been collected, it should be packed and stored properly, which ensures that the sample integrity is maintained properly. Most samples that are being collected for microbiological tests can be transported at ambient temperature, while blood samples and other body fluids need controlled temperature. At all laboratories, technicians or assistants are responsible for preparing samples for transportation. Care must be taken to:

- Secure the caps of containers and aliquot tubes tightly to prevent leakage. (Advised to use screw cap vials).
- Use parafilm to secure caps.
- Pack all specimen containers in appropriate biohazard bags.
- Keep specimens in designated cooler for transport.

Blood samples

Sample type	Storage condition (During Transport)
Fresh frozen plasma	Using ice pack
Whole blood	Ice pack (2-8°C)
Serum samples	Ice pack (2-8°C)
EDTA samples	Ambient temperature (25-25°C)

Microbiological samples

Sample type	Storage condition (During Transport)
Skin scraping	20-25°C
Hairs	20-25°C
Nails	20-25°C
CSF	20-25°C
Eye swab (juveniles)	20-25°C
Cloacal swab	20-25°C
Blood culture	Incubator 35-37°C
Semen	Incubator 35-37°C
Eye swab	Ice pack/ fridge 2-8°C
Ear swab	Ice pack/ fridge 2-8°C

Sample type	Storage condition (During Transport)
Oral swab	Ice pack/ fridge 2-8°C
Wound/pus/abscess/ulcer	Ice pack/ fridge 2-8°C
Pleural fluid	Ice pack/ fridge 2-8°C
Cloacal swab/ stool	Ice pack/ fridge 2-8°C
Urine	Ice pack/ fridge 2-8°C
Saliva	Ice pack/ fridge 2-8°C
Nasal swab	Ice pack/ fridge 2-8°C
Skin swab	Ice pack/ fridge 2-8°C

Virological samples

All virological samples are transported at 2-8 degrees Celsius to maintain viral viability, while some may require freezing at -20 degrees Celsius.

Parasitological samples

Sample type	Storage condition (During Transport)
Stool (ova and parasite)	Room temperature (20-25°C)/ fridge (2-8°C) of delay

Sample type	Storage condition (During Transport)
Blood smears	Room temperature (20-25°C)
Whole blood (EDTA for parasite)	Ice pack/ fridge 2-8°C
Urine (for *Schistosoma*)	Ice pack/ fridge 2-8°C
Skin snip	Room temperature (20-25°C)
Saliva	Ice pack/ fridge 2-8°C
CSF	Ice pack/ fridge 2-8°C
Tissue biopsy	Ice pack/ fridge 2-8°C
Eye swab	Room temperature (20-25°C)
Cloacal swab	Ice pack/ fridge 2-8°C
Skin biopsy	Ice pack/ fridge 2-8°C

16

DISPOSAL OF BIOMEDICAL WASTE

Sharps

- Dedicated containers should be maintained for needles. Additional attachments should be discarded in red biohazard bags. The entire unit of butterfly scalp needle should be thrown into the sharps container, as the sharps container does not facilitate detachment of the plastic line.
- Other sharps containers should be maintained for broken glass slides, glass tubes, and pipettes.
- All broken glassware should be discarded properly to avoid accidental poking.

Infectious porous materials

- Materials that include used cotton, plasters/ paper towels, clothes, and other linen should be handled with the utmost care and following PPE.
- All of these materials should be discarded in red biohazard bags.

Other infectious material

- Material that includes histopathology samples, urine, blood, fecal, and microbiological samples.
- All of these containers and vials should be sealed with parafilm first and then discarded in the red biohazard bags.

Note- Biohazard bags should always be disposed of in the designated outdoor disposal receptacles.

Table 10: Representation of the most common colour code for biomedical;

Type of colour code	Instructions
Red 	Represent biohazard material, which is why biohazard waste is referred to as red bag waste. Any medical waste that has been contaminated with infectious material, such as blood or body fluid. • Blood or blood products (serum, buffy coat, etc.) • Body fluid • Sharps waste • Personal protective equipment (PPE) that has come into contact with infectious material.
Yellow 	Yellow containers are for the trace amounts of chemotherapy waste. It can be medical waste that has come in contact with chemotherapy waste. It should be clearly labelled as "chemo waste" • PPE that has come in contact with chemotherapy waste • IV bags or catheters used in chemo • Sharp waste used during chemo
Blue 	Blue containers are for non-hazardous pharmaceutical materials. Anything prescribed or over-the-counter medication that has gone unused. • Pills • Injectable • Antibiotics
Black 	Black bins represent hazardous waste, which is any waste that is ignitable, corrosive, reactive, or toxic in nature. • Hazardous medications • Certain chemical agents • Disinfectant or chemicals • Radioactive waste

17

COLLECTION AND CARCASS DISPOSAL

Approved methods through appropriate channels depending upon the animal species, disease, scale of mortality, and national and state wildlife laws should be followed in the collection and disposal of carcasses. Animal health authorities can be contacted to suggest the best possible practices to ensure the safety and health of the personnel involved.

Carcass collection

In most ideal scenarios, the carcass is collected and investigated *in situ* to reduce the spread of infection. In field situations, if the mortalities are higher, carcasses can be brought together to a centre location for investigation and disposal. There are chances of a leak of body fluid, which can lead to the possible spread of infectious agents in the environment. A double plastic zip-lock bag is preferred for transporting carcasses from one place to another.

Carcass disposal

Burial

Burial is a commonly used method of carcass disposal that is relatively safe, quick, inexpensive, and convenient. Certain precautions can be taken to ensure that the pit does not contaminate the ecosystem and underground water source. Care should also be taken to avoid direct or indirect contact of carcasses with animals and humans after burial. Historically, open pit was the ideal method for burial, but groundwater contamination and exposure to scavengers are certain limitations associated with this. The closed pit is a widely adopted method which addresses issues associated with the open pit. Animal health advisory group can be contacted for a proper guideline

The following aspects are to be considered:

- Accessibility of the site
- Available facilities
- Equipment needed
- Safety measures for personnel
- Distance from residences and roads
- Visibility and protection from the public and scavengers
- Slope of the surface
- Proximity to watercourses or wells
- Height of the water table
- Biosecurity factors
- Cultural or historical concerns
- Permission from the landowner

Incineration

Burning the carcass is the technique that produces solid, non-pathogenic inorganic waste. However, the following aspects are to be considered:

- Site accessibility
- Availability of fuel
- Environmental factors
- Number of carcasses and their size for disposal
- Location of the site
- Wind direction at the site
- Type of animal carcass involved

Open-air burning is a common type of burning, while the other two types are fixed-facility incineration and air curtain incineration.

Additional flammable agents, such as timber or fuel, are used in open-air burning to achieve a higher temperature and burn all carcasses. Fixed-facility incinerators of different sizes are available at administrative levels. All produce controlled high-temperature heat that burns down the carcass to ash. Air curtain incinerators function by creating a curtain of air that forces high-velocity airflow over an open field, which enhances combustion and reduces particulate emissions.

Composting

Composting is the controlled decomposition of carcasses by microorganisms. It involves a longer-duration initial phase of higher temperature followed by lower temperature for several months, which results in the production of carbon dioxide, water vapour, heat, and compost. Composting is relatively cheap and causes a low rate of pollution, and fertilizer is the end product.

Rendering

Rendering involves cooking carcasses to separate animal fats and protein. The resulting product is sometimes used as a foodstuff and for other industrial processes. Rendering is not considered an appropriate technique in infectious disease situations, as it has the highest risk of disease spread.

SUGGESTED READING

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ANNEXURE I

List of laboratories/Institutions with details for sample processing

Sr. No.	State	Name of Laboratory/institution	Contact details
1.	Bihar	Bihar Veterinary College Campus, Patna-800014, Bihar	Mobile: 9431060262 basu.edn@icar.gov.in
2.	Uttar Pradesh	Centre for Wildlife Conservation, Management and Disease Surveillance, ICAR-IVRI, Izatnagar, Bareilly, Uttar Pradesh 243122	Office: +91 0581-2303284 cwlincharge@gmail.com
		Central Disease Diagnostic Laboratory Centre for Animal Disease Research and Development (CADRAD) Indian Veterinary Research Institute (IVRI), Izatnagar-243122 (U.P.)	Office: (O) 0581-2302188/2310074 Mob. 09412288343 jdcadrad@rediffmail.com
3.	Madhya Pradesh	National Institute of High Security Animal Diseases(NIHSAD), Indian Veterinary Research Institute, Anand Nagar, Bhopal-462021 (MP)	Office: 0755-2759204 Fax: 0755-2758842 ddkulkar@rediffmail.com
		State Animal Disease Investigation Laboratory, Veterinary Hospital Campus, Jahangirabad, Bhopal-462 008	scd_11@yahoo.in Fax: 0755-2767583 statedilab07@rediffmail.com
4.	West Bengal	Regional Disease Diagnostic Laboratory Institute of Animal Health & Veterinary Biological (IAH&VB), 37, Belgachia Road, Govt. of West Bengal Kolkata-700037	Office: 033-25328033 Fax: 033-25565476 Mobile: 09433960213
		Collaborating unit of AICRP ADMAS, ICAR-Indian Veterinary Research Institute, Eastern Regional Station, No-37 Belgachia Road, Kolkata-700 037	Office: 033-25582965 Fax: 033-25565725 dahvswb@darahwb.org
8.	Uttarakhand	Animal Husbandry & Veterinary Department, Government of Uttarakhand, Kedar Puram, Dehradun, Uttarakhand-248115	Office: 0135-2450154 Fax: 0135-2450083 kvk.Dehradun@icar.gov.in/ dirahuk@gmail.com
9.	Himachal Pradesh	State Veterinary Hospital Complex Near lift, Cart road Shimla-171001 Himachal Pradesh	Mobile: 9817051951, 8988151951 dd-epid-hp@nic.in
10.	Jharkhand	Institute of Animal Health & Production Kanke, Ranchi-834006 Jharkhand	Mobile: 9199739351 iahpkankeranchi@gmail.com
11.	Haryana	Veterinary Public Health and Epidemiology, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS), Hisar, -125 004, Haryana	Mobile: 9416107869 dg.ahd@hry.nic.in
13.	Rajasthan	State Disease Diagnostic Centre Department of Animal Husbandry Government of Rajasthan, Panch Batti, Jaipur-302001	Fax: +91 141 2374617 Mobile: 9829791073 ddpathologistraj@gmail.com

Any other state Govt., Vet College Laboratories (Depending on test requirement and facility)

ANNEXURE II

Types containers for sample collection

Container types	Use
	**Plastic bottles**
	**Glass jars**
	**Screw cap vials**
	**Preservation supplies**

Types containers for sample collection

Container types	Use
	**Plastic bottles**
	**Screw cap vials**
	**Petri-dishes**

NOTE

NOTE

NOTE

NMCG

National Mission for Clean Ganga
Department of Water Resources,
River Development & Ganga Rejuvenation,
Ministry of Jal Shakti, Major Dhyan Chand
Stadium, India Gate, New Delhi - 110001

WII

Wildlife Institute of India
Chandrabani, Dehradun-248001, Uttarakhand
t.: +91135 2640114-15,+91135 2646100,
f.: +91135 2640117
wii.gov.in/nmcg/national-
mission-for-clean-ganga

GACMC/NCRR

Ganga Aqualife Conservation Monitoring Centre/
National Centre for River Research
Wildlife Institute of India, Dehradun
nmcg@wii.gov.in