

CHAPTER 1.1.2.

COLLECTION, SUBMISSION AND STORAGE OF DIAGNOSTIC SPECIMENS

INTRODUCTION

Laboratory investigation of animal disease is critically dependent on the quality and appropriateness of the specimens collected for analysis. This chapter sets out the general standards involved in specimen collection, submission, and storage. The individual disease chapters in this Terrestrial Manual provide specific information on appropriate specimens needed to test for particular pathogens or toxins. Sampling may be from individual animals, from animal populations, or from the environment for a variety of purposes, such as disease diagnosis, disease surveillance, health certification, and monitoring of treatment or vaccination responses. To provide scientifically and statistically valid results the specimens collected must be appropriate for the intended purpose, and adequate in quality, volume, and number for the proposed testing. Additionally, the animals and tissues sampled must be appropriately representative of the condition being investigated.

Specimens must be collected using appropriate biosafety and containment measures in order to prevent contamination of the environment, animal handlers, and individuals doing the sampling as well as to prevent cross-contamination of the specimens themselves. Care should additionally be taken to avoid undue stress or injury to the animal and physical danger to those handling the animal. Biological materials should be packaged to rigorously control for leakage, and then labelled with strict adherence to the applicable regulations guiding their transport as outlined in Chapter 1.1.3 Transport of biological materials.

A. COLLECTION OF SAMPLES

1. General considerations

Careful consideration must be given to the collection, containment, and storage of the specimens, including biosafety measures that must be in place to prevent contamination of the environment or exposure of other animals and humans to potentially infectious materials (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). For information on transport of specimens see Chapter 1.1.3 *Transport of biological materials*.

The reliability of the diagnostic testing is critically dependent on the specimen(s) being appropriate, of high quality, and representative of the disease process being investigated. Prior to sampling, consideration must be given to the type of specimen(s) needed including the purpose of the testing and the test technologies to be used. The volume or quantity of specimen must be sufficient to perform initial testing, to perform any subsequent confirmatory testing and to provide sufficient residual specimen for referral or archival purposes.

The purposes of testing will be aligned with the purposes for which tests are validated, as listed in Chapter 1.1.6 *Validation of diagnostic assays for infectious diseases of terrestrial animals*, namely:

- i) Demonstration of freedom from infection in a defined population.
- ii) Certification of freedom from infection or presence of the agent in individual animals or their products for trade/movement purposes.
- iii) Eradication of disease or elimination of infection from defined populations.

- iv) Confirmatory diagnosis of suspect or clinical cases.
- v) Estimation of prevalence of infection or exposure to facilitate risk analysis.
- vi) Determination of immune status of individual animals or populations (post-vaccination).

Epidemiologically appropriate sampling plans should be developed prior to collection of specimens, as described in Section B and Appendix 1.1.2.1. These will specify the number of animals or other sampling units to be sampled.

Specimens must be collected according to a sound knowledge of the epidemiology and pathogenesis of the disease under investigation, or the disease syndrome to be diagnosed. This will lead to the sampling of tissues or fluids most likely to contain the infectious agent or evidence of the infection. Considerations will include the tissue predilection(s) or target organ, the duration and site of infection in each tissue type and the duration and route of shedding, or the time frame in which evidence of past infection, such as an antibody response, can be detected reliably by the tests to be deployed. These considerations will also indicate the method(s) of collection to be used. In many herd or flock-based disease investigations it is beneficial to collect specimens from a healthy cohort for comparative epidemiological or baseline testing (e.g. case-control and cohort approaches for diagnostic testing) and for validation purposes.

Where chemical euthanasia or anaesthesia is required for animal restraint, the impact of the chemical on the test result (e.g. toxicology testing) must be considered. Some laboratory tests are not compatible with specific blood anticoagulants and tissue preservatives, such as heparin, formalin, dry ice (exposure of the test sample to elevated levels of CO₂), or even freezing. While it is critical to collect specimens as aseptically as possible, equal care must be taken to avoid contamination with detergents and antiseptic treatments used to clean the collection site on the animal, as these agents may interfere with the laboratory test procedures. Procedures requiring tissue culture of pathogens, as well as many molecular-based tests, can be negatively affected by chemicals or detergents commonly used in the manufacture or preparation of collection tools (e.g. chemicals used in manufacture of some types of swabs and detergents used in cleaning glassware).

Specific information on diagnostic test methodologies and the recommended specimens, preservatives, and specimen handling procedures can be found in the individual *Terrestrial Manual* disease chapters or through direct consultation with the laboratory that will be performing the required testing. Procedures for collection and submission of specimens are available from most diagnostic laboratories, including national and international authorities, where the information is frequently accessible via the specific laboratory's web page. The WOAHP web page provides contact information for all WOAHP reference laboratories¹.

It is critical not only to collect the most diagnostically-appropriate specimens, but to also inform the laboratory of the associated disease epidemiology in order for the laboratory scientists to assign the most appropriate tests or panels of tests. Epidemiological information to be submitted with specimens is outlined in Section C of this chapter.

Where investigating diseases of unknown cause multiple different specimens that represent the different stages of the disease progression in an animal or the population of animals (e.g. the pre-clinical, early clinical, active clinical, chronically affected and convalescent phases) should be collected. Epidemiological considerations for sampling are particularly critical for diagnosing population-related diseases, as would occur with beehives, flocks, and herds. Epidemiological principles used for sampling are further introduced in Section B of this chapter.

Specimens can be collected ante-mortem or post-mortem. Specific considerations regarding different specimen types are as outlined below.

2. Blood

Whole blood samples may be collected for haematology, clinical chemistry, toxicology, direct examination for bacteria or parasites, PCR testing, immunological testing, or for culture for bacteria or viruses. Dependent on testing needs, whole blood, blood cells, and/or plasma samples can be obtained from whole blood collected into appropriate anticoagulants. In selecting the anticoagulant to be used the collector must be aware of the laboratory tests, including PCR-based diagnostics, clinical chemistry, and toxicology, which may be negatively affected by the presence of specific anticoagulants or preservatives. Specific disease chapters in this *Terrestrial Manual* provide guidance for individual tests and sample requirements. To be effective anticoagulants require that the collected

1 <https://www.woah.org/en/what-we-offer/expertise-network/reference-laboratories/#ui-id-3>

blood be thoroughly mixed with the chosen anticoagulant during or immediately following its sampling from the animal.

To obtain serum, whole blood is collected without anticoagulants and the clot is allowed to contract at ambient temperature protected from extremes of heat and cold for periods that may range from a few hours to overnight. Clear serum can be decanted or collected by pipette following physical removal of the clot, ideally following gentle centrifugation to separate cell components from the serum. In the absence of a centrifuge, separation of the clot can be facilitated by tilting the freshly collected blood tube at an approximate 45 degree angle until the clot has retracted, “ringing” the clot with a sterile rod or pipette to separate the clot from the tube surface, and then removing the clot with forceps. The results of serological testing can be compromised by the quality of the sample. Bacterial contamination and red blood cell debris in serum samples can produce false positive reactions in agglutination-type assays. Serological assays can be negatively impacted by haemolysis in the serum sample. Microbial contamination and haemolysis are significant concerns especially when obtaining blood and serum samples from post-mortem animals. Frequent causes of haemolysed serum and plasma include exposure to excessive temperatures or time delays prior to separating sera from the red blood cells, blood collection using a needle of too small gauge, or failure to remove the needle when transferring the blood sample from the collection syringe.

Whole blood should be collected aseptically, typically by venipuncture of the live animal. Depending on the animal and sampling situation jugular, caudal, brachial, cephalic, mammary veins or the vena cava may be used. Specific techniques for sampling small laboratory animals have been reviewed (Anon, 1993; Hem *et al.*, 1998). Care should be taken to collect and dispense blood samples as gently as possible to prevent damage to red blood cells, which causes haemolysis. Blood and sera are typically shipped and stored cool (or frozen in the case of sera) in non-breakable vials, tubes, or bottles; however for some laboratory tests that require viable peripheral blood mononuclear cells, the blood must be packaged, transported and stored so as to prevent exposure to temperature extremes. For some tests, aliquots of specimens can be dried onto a piece of untreated, or specifically-treated commercial filter paper designed for stabilised sample transport and storage.

3. Faeces

Faeces can be collected freshly voided or preferably directly from the rectum/cloaca for tests such as culture for microorganisms, parasite examination, or faecal occult blood determination; or can be collected for culture and molecular-based diagnostics from the rectum/cloaca using cotton, dacron, or gauze-tipped swabs, dependent on the volume of sample required by the specific test methodology. Samples collected on swabs should be kept moist by placing them in the transport media recommended for use with the specific test to be performed, which may range from sterile saline to culture media containing antimicrobials or stabilisers. Faecal specimens should be kept chilled (e.g. refrigerated at 4°C or on ice) and tested as soon after collection as possible to minimise the negative impacts on test results caused by death of the targeted microorganism, bacterial overgrowth or hatching of parasite eggs. Double-packaging of faecal samples in screw cap or sealable containers that are subsequently contained within sealed plastic bags will help prevent cross-contamination of samples and associated packaging materials. Faeces contained only in rectal exam gloves, plastic bags, or rubber-stoppered tubes are unsuitable as they are very frequently comprised by bacterial growth with gas production that can rupture plastic bags, displace stoppers, and allow leakage of the specimen.

4. Epithelium

Epithelial tissue in the form of biopsies or skin-scrapings; swabs of oral, nasal, pharyngeal, and gastrointestinal surfaces, as well as plucked hair or wool can be used variously for direct examinations or laboratory tests to identify surface parasites such as mites and lice, fungal, bacterial or viral infections, allergic reactions, and neoplasia. The specimens should be collected aseptically and preserved as specified for the intended test(s). Deep skin-scrapings obtained using the edge of a scalpel blade are useful for burrowing mites. Feather tips have been validated for use in the detection of viral antigen for Marek’s disease, and used as a sample for molecular detection of additional avian diseases. Epithelial tissues, particularly those associated with vesicular lesions and collected into viral transport media, can be critical in the laboratory diagnosis of specific viral infections such as foot and mouth disease.

5. Ocular sampling

The surface of the eye can be sampled by swabbing or ocular scraping, ensuring that cells rather than mucopurulent discharge or lacrimal fluids are collected for testing. Specimens from the conjunctiva are typically collected by holding the palpebra apart and gently swabbing the surface of the eye with a cotton, dacron, or gauze swab that has been pre-moistened with sterile saline or equivalent media. Such swabs should be kept moist in saline or transport

media specifically recommended for use with the testing to be performed. Biopsies from the third eyelid of sheep have been used as a lymphoid-rich tissue for prion detection.

6. Sampling the reproductive tract

Preputial and vaginal wash fluids and swabs of the cervix and urethra can be used as specimens for investigation of reproductive disease. The swabs should be kept moist following collection by placing in the recommended volume of transport media required by the laboratory test, typically sterile saline or specified culture media. Semen specimens are typically obtained using an artificial vagina or by extrusion of the penis and artificial stimulation. Avoid contamination of the specimen with antiseptic or detergent solutions used to prepare the animal/site for sampling.

7. Nasal discharge, saliva, and vesicular fluids

Secretions can be collected directly into a vial or tube, or can be collected using swabs. Vesicular fluids provide a highly concentrated source of pathogen for diagnostic testing, and can be collected from unruptured vesicles using a sterile needle and syringe, and immediately transferred to a securely sealed vial or tube. Specifically developed sampling tools, such as probang cups, can be used for collecting cellular material and mucus from the pharynx of livestock. Cotton ropes that animals are allowed to mouth and chew have been validated for use in collecting saliva specimens from domestic swine.

8. Milk

Milk can be collected from individual animals or from bulk milk in tanks pooled from multiple animals in a herd. The teat(s) used for sample collection should be cleaned, and any detergent thoroughly rinsed off before collection of the specimen. In collecting milk from individual teats, the initial stream must be discarded and only the subsequent streams sampled. The method of preservation prior to testing varies with the requirements of the test; in some cases it will be critical to avoid freezing or addition of chemical preservatives. The individual disease chapters of this *Terrestrial Manual* or the advice of the testing laboratory should be consulted for appropriate specimen handling and preservation recommendations.

9. Tissues collected at necropsy

Necropsies should be conducted only by qualified veterinarians and pathologists. Paraveterinary staff may be trained by veterinarians to conduct post mortem examinations for specific purposes. Importantly, the purpose of the necropsy is not only to collect specimens but to make informed observations regarding the pathology of the condition. Such observations are an important adjunct to epidemiological and clinical observations in the comprehensive veterinary investigation of the case or outbreak. It is useful for veterinary authorities to retain specialist veterinary pathologists to lead post mortem investigations in important cases. Where this expertise is managed from a veterinary laboratory the methods employed should be formally described in the laboratory's Quality Assurance Manual and the capability should be recognised in the laboratory's scope of accreditation. Detailed procedures for conducting post-mortem examinations and tissue collection are available in most pathology text books, and are additionally provided in many of the web-page accessible national laboratory testing guidelines. Specimens that are critical for the laboratory investigation of listed diseases are included in the chapters of the *Terrestrial Manual* relating to each disease.

Whether the necropsy is performed in a designated laboratory facility or in the field, appropriate biosafety and containment procedures should be followed to ensure operator safety and to provide non-contaminated and useful tissues for testing as well as to protect the environment and other animals from potential exposure to pathogens. As a minimum requirement the collector(s) must wear personal protective equipment that protects the skin and mucous membranes and that can be discarded or decontaminated. All remaining tissues or carcass parts and fluids should be contained and treated with an appropriate disinfectant or destruction method, and the immediate environment should be thoroughly disinfected.

Dependent on the suspected disease, condition of the carcass and facilities available for necropsies post-mortem specimens can be collected from one or multiple organs and submitted to the laboratory as either fresh (no preservative) or preserved specimens for further laboratory testing. The process of carcass autolysis can destroy diagnostically relevant tissues and infectious agents and so should be considered prior to collecting and submitting post-mortem specimens.

For fresh specimens particular attention must be paid to their handling and storage to avoid autolysis and overgrowth by bacterial and fungal contaminants. Ideally, freshly collected specimens are kept at a constant cool temperature from collection until processing for testing. Where such a cold chain cannot be provided fresh specimens for some test procedures can be collected into fluids such as ethylene glycol that inhibit the growth of secondary organisms. Where such strategies are compatible with the subsequent test methods the option is mentioned in the relevant chapters of the *Terrestrial Manual* for each disease.

Preservation of post mortem specimens is most frequently achieved by collection into formalin solution. Where such chemical fixative is supplied to pathology staff by laboratories or competent authorities they must ensure adequate training in health and safety aspects of the use of such chemicals and training in compliance with regulations relating to the transport of such chemicals.

10. Environment and feed

Environmental sampling may be of litter, bedding, water from troughs and drinkers, or feed which has been exposed to urine, faeces, and/or saliva of affected animals, or swabbed surfaces of facilities, ventilation ducts, drains or feed containers. If specialised equipment is available circulating air may be sampled.

11. Honey bees

Adult moribund or recently dead bees can be collected in the vicinity of colonies. Live bees can be killed by freezing. Brood specimens are typically collected by removing a piece of brood comb showing abnormalities and including dead or discoloured brood followed by wrapping in a paper towel or newspaper rather than in foil or wax paper in order to help prevent microbial overgrowth. Alternately, diseased cells in a comb may be sampled using a toothpick or equivalent. A sticky board can be used to collect hive debris, including trapping of mobile parasites. More information on the specimens that need to be collected can be found in the disease-specific chapters of this *Terrestrial Manual* related to bees.

B. EPIDEMIOLOGICAL APPROACHES TO SAMPLING

To provide scientifically and statistically valid results specimens must be appropriate for the intended purpose for the proposed investigation, and adequate in quality, volume, and number. The range of purposes for which investigations supported by laboratory testing may be conducted have been outlined in Section A above.

For the purpose of laboratory testing to establish a diagnosis it is important to sample animals that are either clinically affected, or suspected on good evidence to be infected or, for serology, to have been infected. Specimens that are most likely to give highest sensitivity and specificity to the investigation should be collected. In general, the stage of infection, as well as the route and duration of shedding will determine the appropriate animal(s), stage of clinical disease, timeline for sampling, and tissue or anatomical site for sampling. These criteria will be addressed through an understanding of the pathogenesis of the disease for known conditions and on an hypothesis of the pathogenesis of the diseases for conditions of unknown aetiology.

To detect evidence of infection in line with the other five purposes of testing as listed in Section A above the sampling should be done within the context of a surveillance programme. The criteria for the design and implementation of effective surveillance are described in Chapter 1.4 *Animal health surveillance* of the *Terrestrial Animal Health Code (Terrestrial Code)*. Identification of animals for sampling in surveillance programmes may be targeted (risk-based) or random. Risk-based sampling based on epidemiological knowledge of the infection under study or on epidemiological observations of the population under study is intended to result in the most likely detection of infected individuals.

Inferences on the status of a population such as estimation of prevalence, immune status or disease freedom should be based on random sampling. Random sampling ensures that the animals sampled are representative of the population, within the practical constraints imposed by different environments and production systems. Additionally, random sampling allows extrapolation of the findings of the study to the overall population (with an appropriate confidence interval). The epidemiological principles for sample size estimation are addressed in Appendix 1.1.2.1.

The specific requirements for surveillance to demonstrate freedom from disease/infection, and the associated sampling requirements, are addressed in detail in Article 1.4.6 of Chapter 1.4 of the *Terrestrial Code*.

Sampling and laboratory testing may also be used in support of epidemiologically based diagnostics and studies such as case control, structured longitudinal, and cohort studies (Fosgate & Cohen, 2008; Mann, 2003; Pfeiffer, 2010). The number and selection of the animals to be sampled and the nature of the specimens will be part of the study design.

C. INFORMATION TO BE SENT WITH SPECIMENS

Individual specimens must be clearly identified using appropriate methods. Marking instruments should be able to withstand the condition of use, i.e. being wet or frozen. Use of an indelible marking pen is required. Pencil may rub off containers. Labels attached to plastic may fall off when stored at -70°C .

Information regarding the location and contact information of the submitter and the premises sampled, the case information and associated epidemiological information, as detailed below, should always accompany the specimens to the laboratory. Such documentation should be placed in a plastic envelope on the outside of the shipping container so as to be available for reference during transport and should also be duplicated inside the shipping container between the secondary and the outer packaging (see also Chapter 1.1.3 *Transport of biological materials*). It would be advisable to contact the receiving laboratory to obtain an appropriate submission form and other relevant shipping and handling information.

Necessary information includes:

1. Location and contact information

- i) Name and address of owner/occupier of the animal owner and/or the sampled premises and the geolocation (latitude and longitude, if available) where disease occurred, with appropriate contact information (telephone number, e-mail address).
- ii) Name, postal and e-mail address, telephone and fax numbers of the sender.

2. Case information

- i) Disease agents suspected and tests requested.
- ii) Species, breed, sex, age and identity of the animals sampled, and trackability number when available.
- iii) Date samples were collected and submitted.
- iv) List and type of samples submitted with transport media used.
- v) Case history:
 - a) The clinical signs and their duration including the temperature of sick animals, condition of mouth, eyes and feet, and milk or egg production data as relevant.
 - b) A list and description of the animals examined and the findings of the ante- and post-mortem examinations.
 - c) The length of time sick animals have been on the premise; if they are recent arrivals, from where did they originate.
 - d) The date of the first cases and of subsequent cases or losses, with, for tracking, any appropriate previous submission reference numbers.

3. Epidemiological information

- i) A description of the spread of infection in the herd or flock.
- ii) The number of animals on the premise by species, the number of animals dead, the number showing clinical signs, and their age, sex and breed.

- iii) The type and standard of husbandry, including biosecurity measures and other relevant factors potentially associated with the occurrence of cases.
- iv) History of foreign travel by owner or of introduction of animals from other countries or regions.
- v) Any medication given to the animals, and when given.
- vi) Vaccination history describing the type of vaccines used and dates of application.
- vii) Other observations about the disease, husbandry practices and other disease conditions present.

D. RECEIPT, STORAGE AND ARCHIVES OF LABORATORY SUBMISSIONS

1. Reception of samples

Receiving, unpacking and aliquoting specimens must be done in a way to avoid cross-contamination in order to guarantee reliable testing of samples and prevent exposure of personnel.

A risk assessment (RA) as outlined in chapter 1.1.4 should be performed before systems for handling biological agents and toxins are established in order to define the appropriate biosafety and laboratory biosecurity measures. The RA should lead to the development of stated policy and procedures for the operation of the whole process of receiving submissions to the laboratory.

Submissions delivered to the laboratory should be received in accordance with specified standard operating procedures by staff who are appropriately trained, and when possible are made aware of potential arrivals so that parcels are treated correctly upon reception. To enable appropriate specimen tracking the following information should be logged: a) the time of arrival, b) the sender, c) the person receiving the samples, and d) the shipper with the tracking number. Where a specified chain of custody is required for the purposes of legal action or investigation the consignment should remain unopened and secured in a cool, dry place away from direct sunlight until authorised laboratory personnel are notified and available to receive the package and continue chain of custody. Laboratories should have a written operating procedure detailing the requirements to meet national legal requirements for such submissions.

1.1. Specimen reception area

Specimen reception areas should be equipped to facilitate the safe handling and processing of diagnostic submissions to avoid contamination of the work area, the personnel, cross-contamination among specimens and to allow easy disinfection in situations where specimen containers may have leaked. The specimen reception room should be clean with adequate bench space for organising submissions and paperwork. Depending on the number of submissions expected and depending on the RA the specimen reception area may be either a dedicated part of the diagnostic laboratory or a separate space outside the diagnostic laboratory.

The receiving room should contain an area dedicated to the unpacking of the specimens, with easily cleanable surfaces and trays and/or a biosafety cabinet, depending on the RA. There should be adequate and appropriate space to store specimens, either refrigerators or freezers, taking into consideration the time the specimens are to be stored. Specimen registration equipment such as computers, printers or logbooks should be available. A bar code system can be used to identify and track the specimens.

1.2. Submission unpacking, registration and preparation for further processing

Consignments should remain unopened until transferred to the specimen reception area for further processing. The submission should be unpacked and opened according to defined standard operation procedures. Surface decontamination should be considered to avoid cross-contamination and be part of the designated procedures arising from the RA.

Information to be recorded at specimen log-in includes the delivery source, the date the submission was consigned, the condition of the outer package, and the condition of inner packages (noting the presence of leaks or breakage), the condition of the specimen material, the inner package temperature, and any specific requests from the sender.

Further activities may include labelling of specimen containers, transfer of specimens to the laboratory and storage of specimens.

Packaging material should be disposed of appropriately according to national regulations which may include autoclave destruction of all packaging materials, depending on the RA.

Personal protective equipment (PPE) should be provided to protect the personnel. Minimal PPE is a laboratory coat and gloves. Depending on the relevant RA respiratory protection or effective splash protection (e.g. protective glasses) may be required as well.

After it has been determined that the submission contains the appropriate paperwork matching the specimens and that the specimens are in good condition appropriately trained laboratory personnel become responsible for transfer to the appropriate laboratory area, including maintenance of the chain of custody of the specimens as required. Only properly contained registered (identified) specimens should be transferred into the diagnostic laboratory. It is good practice, and at times a requirement for biosafety and laboratory biosecurity dependent on the RA, to enclose the submitted specimen containers in a secondary container to transfer the specimens safely within the laboratory.

1.3. Emergencies

A comprehensive RA will identify credible and foreseeable emergency scenarios, and be the basis for preparing a response plan. In particular, leaky samples represent a biohazard for the laboratory personnel and could contaminate the environment and other samples. Written instructions on how to deal with broken or leaky tubes should be available in the sample reception area. Personnel should be trained and regular emergency exercises and simulations should take place.

Samples that are degraded or in a condition unacceptable for testing should be decontaminated and appropriately disposed of according to the laboratory's response plan as noted in the prior paragraph. Contaminated laboratory surfaces should be decontaminated with the appropriate disinfectant. Sample rejection and discrepancies between the sample and accompanying paperwork should be resolved by contacting the sender immediately to resend a duplicate sample or to clarify paperwork.

2. Storage and archives

Collections of well characterised specimens including infectious agents, infected tissues and fluids, as well as negative control tissues and fluids, are critical for future research and development efforts, for retrospective studies, epidemiological investigations, and for providing critical reference materials used in assay standardisation, validation, and proficiency testing programmes. In addition, specimens being investigated for legal purposes should be banked.

Materials routinely needed as reference standards and for assay validation are described in Chapter 1.1.5 *Quality management in veterinary testing laboratories*. The materials maintained in laboratory archives should be representative of the agents handled and the types of samples used in the different testing methods performed, which would variously include fresh and fixed tissues and fluids, paraffin-embedded tissues, and stabilised or otherwise preserved cultures. The World Federation for Culture Collections (WFCC: www.wfcc.info/collections) is a useful source of information and reference documentation for developing, maintaining, and sharing culture collections, and has published a comprehensive guide for establishing and operating microbial culture collections (WFCC, 2010). It is part of the remit of WOH Reference Laboratories to supply reference materials.

The principal components of any laboratory archive include the appropriate means of stabilisation and storage, a complete system of documentation and inventory of the material stored, and implementation of biosafety and laboratory biosecurity measures needed to manage the collection.

2.1. Stabilisation and storage

The method of preserving tissues, fluids, and cultures will depend on their anticipated use(s). Samples stored for periodic access such as assay reference materials should be aliquoted to avoid potential problems associated with repeated retrieval and return to storage. They may be stored separately from specimens or samples stored for historical, long-term preservation. Storage conditions should be managed to maintain viability, biochemical, and immunological properties of the samples to the

maximum extent possible. Considerations for preserving the integrity of the samples must include protection from desiccation (e.g. as can happen in certain freezers), frequent or extreme temperature fluctuations, UV degradation, humidity, contamination, and the potential for loss of identification and associated archive documentation. Unique or valuable isolates and materials should be stabilised and stored using at least two different procedures and storage locations.

Storage at ultra-low temperatures (e.g. liquid nitrogen, cryopreservation in freezers at -140°C or lower) is considered the optimum method for long-term storage of biological materials. Storage at low-freezer temperatures of -80°C and -20°C is common for periods that may range from months to 5–10 years. Ultra-low freezing may not be a practical choice as it is expensive to maintain, but cost must be balanced with the fact that biological degradation of the sample over time is an increasing risk at warmer freezer temperatures. Reference materials that are to be accessed with any regularity should be stored in appropriately-sized aliquots to allow access while minimising the number of times the “master stock” is handled. Repeated freezing and thawing of samples should be avoided as it can denature antigens, result in loss of viability of fastidious agents, and can precipitate the over-growth of contaminants or unwanted microorganisms in the sample.

Methods for stabilisation and storage of samples at room temperature range from commercially available technologies that largely target nucleic acid stabilisation, lyophilisation processes, to the relatively low-tech versions of drying fluids on filter paper disks or storage of biological samples in the presence of desiccating agents such as silica gel or grains of rice to absorb moisture.

Considerations such as speed at which a sample is frozen or chemically preserved, size and density of the material to be preserved, storage container and media, and also protocols for reconstitution, thawing, and reviving agents will vary with different tissues and agents. Whether the plan is to store samples frozen or at ambient temperature, for most tissues and groups of infectious agents there are specific preservatives, stabilisation protocols, and storage conditions that are considered optimal; the current published literature should be consulted.

2.2. Documentation and Inventory

Agents and tissues maintained in an archive must be correctly identified and sufficient supporting data that characterises the sample or agent must be recorded. For reference materials, further documentation that authenticates the agent or tissue is required. The unique identity of the tissue, fluid, or agent and the storage location are best maintained in an electronic or paper inventory record which also documents the date the material was obtained, date and method of preservation, volume of material stored, source of the material including associated species, geographical location, and the clinical history of the donor animal and the disease situation of the flock or herd. Additional information is extremely useful and generally includes the original method of isolation/recovery, characterisation (e.g. available data on biochemical properties, antibody or antigen titre, and genetic sequence) as well as additional history on handling of the material (e.g. number of passages for infectious agents and cell lines, dates the archived material was frozen-thawed or rehydrated and the dates it was transferred to different storage conditions or containers). Inventories are most often organised by assigning a unique identifying number or alphanumeric code to each sample (sample container) that is cross-referenced to a database or inventory log. Inventory records can be manual data logs, computerised spreadsheets, or specialised computer programs. However the records are managed, they must be kept current and the information entered must be traceable to its source. The identification of the individual making an entry or modification to the sample inventory should be recorded.

2.3. Biosafety and laboratory biosecurity

As a first step in establishing an archive, a laboratory biorisk assessment addressing biosafety and laboratory biosecurity issues, including any control or mitigation measures to be implemented, must be completed. As further defined in chapter 1.1.4, an appropriate risk assessment for archived samples should address the technical competency needed of staff handling the tissues, fluids, and agents, with particular emphasis on those materials that are potentially infectious or toxic to workers, animals, and the environment in and around the laboratory. The laboratory should consider all biorisk management measures needed to protect the integrity of the sample, as well as the health of the workers and environment, from the time the original sample is received through the long-term storage and ultimate use or destruction of the material(s).

The appropriate level of laboratory biosecurity, including controlled access to the archived samples and inventory records, is an important consideration for laboratories maintaining biological inventories and archives. The laboratory should also have a back-up plan for the transfer or destruction of potentially dangerous archived materials in the event of power failures or other compromises to the storage environment. National and international regulations and legislation, including requirements for permits and licenses to receive, maintain, work with, and distribute specific agents and tissues must be followed for all laboratory archives. Current regulatory information in regards to receiving and storage of biological materials can be found on the European Biological Resource Centre Network website (www.ebrcn.net/), in the WFCC guidelines (2010) and from relevant national government agencies.

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NB: FIRST ADOPTED IN 2008 AS COLLECTION AND SHIPMENT OF DIAGNOSTIC SPECIMENS.
MOST RECENT UPDATES ADOPTED IN 2013.

APPENDIX 1.1.2.1.

EPIDEMIOLOGICAL APPROACHES TO SAMPLING: SAMPLE SIZE CALCULATIONS

The type and number of samples needed depends on the desired purpose. Sample size calculation for each of the main purposes of testing, where random sampling has been used, may be approached as follows:

1. Demonstrate freedom from infection in a defined population (country/zone/compartment/herd where the prevalence is apparently zero)

Frequently, the objective of sampling is to determine if a disease is present or absent in a population at a specific threshold (design prevalence). These sampling methods are needed to perform the scientifically based surveys specified in the WOAH *Terrestrial Animal Health Code* in order to determine freedom with and without vaccination as well as to re-establish freedom after outbreaks.

It is possible to calculate how many animals should be sampled from a herd/flock of a certain size, to achieve a 95% probability of detecting infection or previous exposure assumed to be present in a certain percentage of the animals. The following formula can be applied:

$$n \cong \frac{(1 - (1 - CL)^{1/D})(N - 1/2 (SeD - 1))}{Se}$$

Where

- n*: is the required sample size
- CL*: is the confidence level (generally 0.95)
- N*: is the population size
- D*: is the number of diseased animals expected in the population
- Se*: is the diagnostic sensitivity of the test used

For example, to determine the sample size required to detect with 95% confidence at least one infected animal in a herd of 500 animals at a design prevalence of 10%, the formula above would be used as follows (assuming perfect diagnostic sensitivity):

$$n \cong (1 - (1 - 0.95)^{1/50})(500 - 1/2 (50 - 1)) \cong 28$$

If the laboratory results are negative for all samples the epidemiologist can conclude, with 95% confidence, that the prevalence is lower than 10%. If the disease in question is highly infectious and it is unlikely that only 10% of the animals would be infected, the herd could be considered free. If, however, one or more samples are positive the epidemiologist may conclude, with 95% confidence, that the disease prevalence is at least 10%.

2. Certify freedom from infection or presence of the agent in individual animals or products for trade/movement purposes

The *Terrestrial Code* provides specific recommendations for trade purposes. Some are based on demonstration of disease freedom at a herd or flock level and others on testing of individual animals for export. When certification of a disease free herd or flock is recommended, the approach described in point 1 above can be followed to calculate the number of samples required.

In the case of testing individual animals, it is generally expected that all animals will be tested. The critical question in this case is related to the negative predictive value (NPV) of the test and the probability of having at least one false negative individual in a group. The negative predictive value of a test is defined as the probability that an animal

is not infected given that it tested negative. The NPV is a function of the diagnostic sensitivity and specificity of the test(s) used and the prevalence of the infection in the population where the animals come from. In general, the probability of having at least one false negative in a group is calculated as:

$$P(x \geq 1) = 1 - (1 - NPV)^n$$

The negative predictive value is calculated as follows:

$$NPV = \frac{TN}{TN + FN} = \frac{(1 - p)Sp}{(1 - p)Sp + p(1 - Se)}$$

Where

- TN: is the true negative
- FN: is the false negative
- Se: is the diagnostic sensitivity
- Sp: is the diagnostic specificity
- p: is the prevalence
- n: is the number of animals in the group

Additional information on quantifying these types of probabilities can be found in the WOAHA *Handbook on Import Risk Analysis for Animals and Animal Products, Quantitative Risk Analysis*.

3. Eradication of disease or elimination of infection from defined populations

The objective of surveillance in the event of an outbreak is to try to find any remaining pockets of infection. Sampling should be directed at populations having higher risk of exposure and where the agent is most likely to be found, such as animals exhibiting clinical signs or suspected to have been in contact with infected animals. If animals within a selected herd or flock are not exhibiting clinical signs, a representative sample, based on the formula presented under point 1 above for presence or absence of disease, should be collected.

4. Confirmatory diagnosis of suspect or clinical cases (includes confirmation of positive screening test)

Suspect or clinical cases with a positive screening test result should be retested with a confirmatory test. Usually, a test with high diagnostic sensitivity is used for screening purposes and one with high diagnostic specificity for confirmation. If the status of the herd or flock is of interest, animals exhibiting clinical signs compatible with the disease of interest should be sampled. This will increase the probability of confirming the infection. If required, the formula for presence or absence in point 1 above can be used to calculate the number of samples required. Given that sample collection is directed to animals exhibiting clinical signs, the design prevalence used can be relatively high, yielding a lower sample size.

5. Estimate prevalence of infection

Disease control programmes may need to periodically assess the impact of control measures. One of the key indicators of success is a reduction in the prevalence of the disease. To determine the prevalence of disease within a group of animals, the following formula can be used to determine the number of samples required.

$$n = \frac{Z^2 pq}{L^2}$$

Where

- n: is the required simple size
- Z: is the value of the Z distribution for the desired confidence level (usually 95%)
- p: is the expected prevalence in the population
- q: is 1-p
- L: is the level of precision (or acceptable error)

There are no fixed rules to determine the level of precision (sometimes called also margin of error), the choice of it is left to the epidemiologist conducting the survey. However, finer levels of precision require larger sample sizes.

The corresponding value of the Z distribution for 95% confidence is 1.96. To determine the prevalence of a disease with 95% confidence in a herd of 500 animals with an expected disease prevalence of 20%, at a level of precision of $\pm 3\%$, the required sample size can be calculated:

$$n = \frac{1.96^2 \times 0.2 \times 0.8}{0.03^2} = \frac{0.6146}{0.0009} \cong 683$$

Note that in this case the required sample size is larger than the population, so the sample size will need to be adjusted to take account the population size (N):

$$n_{adj} = \frac{1}{1/n + 1/N} = \frac{1}{1/683 + 1/500} \cong 289$$

Therefore, 289 animals would have to be randomly sampled.

6. Determine immune status of individual animals or populations (post-vaccination).

Disease control programmes often rely on vaccination as a tool, in such cases it is important to assess immunity coverage and not merely count the number of animals or herds that have been vaccinated. The proportion of animals that need to be immunised to stop the spread of disease in a population is a function of the number of secondary infections arising from a single infected case (the reproductive number, R_0). For many infectious diseases the proportion needing immunity in order to control, disease spread is around 80%. Two approaches can be applied. If the objective is finding the proportion of immune animals, the formula for determining prevalence in point 5 above, can be applied. If, however, programme managers want to assess if herds have an immunity level at or above a certain threshold, the formula for presence or absence, in point 1 above, should be used. The difference in sample size varies greatly depending on the objective.

If the immune status of a herd of 500 animals that have all been vaccinated wants to be estimated, the following approaches can be followed.

6.1. Estimating the proportion of immune animals in a group

- Expected prevalence (of immune animals) 80%
- Precision, for this example assume 3%
- Confidence level 95%

$$n = \frac{1.96^2 \times 0.8 \times 0.2}{0.03^2} = \frac{0.6146}{0.0009} \cong 683$$

$$n_{adj} = \frac{1}{1/n + 1/N} = \frac{1}{1/683 + 1/500} \cong 289$$

6.2. Estimating immunity at a defined threshold

- Expected prevalence (of immune animals) 80%, i.e. 400 out of 500 animals
- Confidence level 95%
- Perfect diagnostic sensitivity

$$n \cong (1 - (1 - 0.95)^{1/400})(500 - 1/2(400 - 1)) \cong 3$$

If at least one of the three samples is positive to the test, the interpretation is, with 95% confidence, that the proportion of immune animals in the herd is at least 80%. If none of the samples test positive, the herd cannot be considered adequately immunised. Such an approach can be used to determine geographical locations or types of production systems where immune coverage is low and might need to be re-vaccinated.

On-line resources

Open Epi: <http://www.openepi.com/OE2.3/Menu/OpenEpiMenu.htm>

Free Calc: <http://epitools.ausvet.com.au/content.php?page=FreeCalc2>

Win Episcopy: <http://www.winepi.net/>

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