

## **DIAGNOSTIC TECHNIQUES IN AQUATIC ANIMAL MEDICINE**

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The ability to test for and gain information about potential disease agents of aquatic animals often is opportunistic depending on the tests available, a researcher's interest, or tests that have been developed in other domestic species. It therefore is important to understand the assay and its limitations when interpreting results. This guide provides a review of the basic principles of traditional and novel techniques used by clinical and anatomic pathologists, electron microscopists, microbiologists, and molecular and serologic diagnosticians in aquatic animal medicine. It also reviews the appropriate sampling and handling requirements for each technique. The goal is to increase the overall understanding of the available assays, so that results will be evaluated critically and interpreted correctly. We intend that this guide of diagnostic techniques will help maximize the information gained from each physical exam, phlebotomy, biopsy, and necropsy.

The authors encourage feedback to help enhance the usefulness and accuracy of this review of diagnostic techniques in aquatic animal medicine. We also encourage researchers and diagnosticians to submit completed forms on the different diagnostic assays available through their institutions. This information will be used to build a database of the resources available to aquatic animal clinicians and other researchers of aquatic animal disease.

Please see the IAAAM website (<http://www.iaaam.org>) for an electronic copy of this guide as well as the submission forms and instructions.

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## **GENERAL ISSUES RELATED TO DIAGNOSTIC TESTING**

### **Consistency in sampling, handling, analysis**

- One of the biggest factors affecting the results obtained from any assay is inconsistent sample collection, handling, and analysis. The period of time between collection and freezing or formalin fixation, the temperature at which samples are stored, the quality of the storage media, and the length of time of storage prior to analysis are all important considerations. Always include this information with sample submission, because it may impact how the test is run and the ability to optimize recovery.
- Additionally, it is important to note whether samples have gone through a freeze/thaw cycle and if so, how many, as this may affect analysis.
- It is important to identify labs and assays that will be used consistently to analyze all of the samples so that obtained results are comparable.

### **Comparability between labs/assays**

- Various factors regarding the labs and assays being used require consideration. These factors include: the types of results that are provided, what they mean, and how they can be compared according to titers versus antibody concentrations, establishment of cut-offs for positive and negative results, the assay's sensitivity (i.e. percentage of true positives that test positive), specificity (i.e. percentage of true negatives that test negative), and predictive values; and how those values were obtained.

### **• Issues to consider**

- **Cut-off level / point, Ranges**

When measuring test values for a continuous variable (i.e. a test that is quantitative), a cut-off level must be established, above which a test is considered positive and below which a test is considered negative. The choice of the cut-off will affect the number of false positives and false negatives for the test. A range of values also may be provided in which a sample may fall for it to be considered positive or negative.

- **Test validity, Sensitivity, Specificity**

A test has been validated when its ability to distinguish between truly infected/exposed animals and truly uninfected/not exposed animals has been determined and quantified. Therefore, assay validity has two components, sensitivity (the ability of the test to identify correctly those who have disease or been exposed) and specificity (the ability of the test to detect animals that do not have disease or been exposed). To calculate these values, test results must be compared to a "gold standard" for each species and sample type. Without validation, performing concurrent cytology and/or histology and seeking confirmatory tests is important to verify results.

- **Test reliability**

It is important for a test to be reliable or repeatable. If test results cannot be reproduced its value and usefulness are minimal. Intra-subject variation (variation within individuals) and inter-observer variation (variation between those reading the results) contribute to reliability. The latter is especially important for some immunofluorescent assays and immunohistochemistry.

- Controls

Testing of control samples should be included with the testing of any samples. Controls should include a positive and negative sample, ideally species-specific, for all assays. Molecular assays in particular also should include a DNA extraction control (to ensure no contamination between samples during the extraction process), an internal control (to ensure the ability to amplify DNA from the sample), and a PCR control (to ensure no contamination between samples).

## **CATEGORIES OF TECHNIQUES FOR DETECTING PRESENCE OF OR EXPOSURE TO DISEASE AGENTS**

1. Visualization of agent and/or its effect (ie. “footprint”) on a gross and microscopic level
2. Measurement of host antibody response using serologic methods
3. Molecular methods to detect and characterize the agent’s nucleic acid or other distinctive molecules

### **1. Visualization**

- Gross examination
  - Wet preparation (dissecting microscope): Parasites/tissue collected ante- or post-mortem
  - Cytology: Smears of tissue, fluid, or swabs collected at necropsy or biopsy
  - Histology: Tissues collected and fixed at necropsy or biopsy
- Special histological techniques
  - Special stains (not discussed in this guide)
  - Immunohistochemistry and Immunofluorescent chemistry
  - Laser capture microscopy
- Electron Microscopy
  - Scanning: Surface topography and morphology (ie. external structure) at low or high magnification of unfixed or fixed tissue or organism
  - Transmission: Tissue and cellular ultrastructure (ie. internal structure) of fixed tissue using positive or negative staining techniques, uncommonly complemented by particle enrichment
- Culture techniques: Bacteria, viruses, protozoa, fungi

### **2. Serologic Techniques**

- Antibody-based tests
  - Measurement of host antibody responses (important to note whether IgM, IgG)
  - Measure of exposure, not infection (unless paired samples are tested)
- Antigen-based tests
  - Detection of a surface molecule of an organism

### **3. Molecular Techniques**

- Aimed at detecting agents, especially potential pathogens
- Probe archived tissues samples
- Analysis of samples to be collected, ante-mortem and post-mortem
- Samples can be tested ex-situ (eg. polymerase chain reaction) or in-situ (eg. in-situ hybridization)

## FORETHOUGHTS

- Is it legal that I handle and sample this animal (ie. for endangered species and necrolegal cases)?
- Suspected disease process(es)? ⇒ Implication of predisposing condition? (ex: mycosis ⇒ cause for immunosuppression)
- What samples are needed to confirm: type (tissue, blood, CSF, urine) & handling (fixed, fresh, frozen, special media)?
- Will imaging study (radiograph, CT scan, MRI ) facilitate localization of lesion or diagnosis?
- Sterile approach required? Special container or media required?
- Should I seek additional input from toxicologist, pathologist, molecular diagnostician?

## “CYA” Samples

Lesion or Target organ

- Formalin (<1 cm thick)

-70°C (<2 cm<sup>3</sup> tissue, swab, 1 ml fluid)

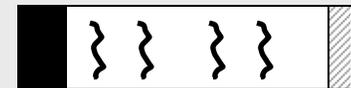
± -20°C (toxicology)

Blood -70°C: serum (5ml), whole (1ml)

## RECORD: ENVIRONMENT / POPULATION

- Weather, Water quality, Normalcy of habitat
- Nearby anthropogenic activity or facility
- Nearby body fluids / excrements
- Nearby animals (esp. con-specifics):  
presence, number, apparent condition

Impression smears with multiple tissues on one slide is good  
Lung Liver Spleen GI Blank

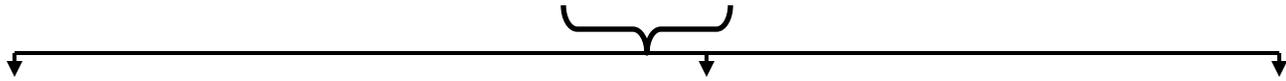


## NECROPSY

- Take notes: small details easily are forgotten
- External Exam: morphometrics, carcass condition, orifice discharge/staining, skin defects/discoloration (even minor)
- Sterile samples: tissue usually higher yield than effusion / swab
- Wet mounts for ectoparasites (protozoa, metazoa)
- Parasites: collect as many as possible and collect feces/blood
  - ❖ See attached reference guide for specific handling
- Impression smears (x3 slides) to help make a diagnosis & direct diagnostics: stain 1 slide with HE/Wrights giemsa
  - ❖ Especially useful for non-mammalian species
- GI content per region: quantity and consistency
- Complete tissue set: regardless if normal or not
  - ❖ Include fetus and placenta (chorion, allantois, amnion, stalk)
- Multiple smaller sections are better than few large sections
  - ❖ Normal: each lobe (liver, etc) or segment (GI), intact brain
  - ❖ Lesion: active & chronic region, leading edge
  - ❖ Thickness < 1cm; can be as long or wide as you like
  - ❖ Small / Special interest tissue: label / into cassette, sieve bag

## BIOPSY

- Impression smears (x3 slides) to help make a diagnosis & direct diagnostics: stain 1 slide with HE/Wrights giemsa
  - ❖ Especially useful for non-mammalian species
- Histology, cytology, and ancillary diagnostics are performed on the same sample divided or on adjacent samples
- Tru-cut, incisional, or excisional biopsies are far superior to pinch, fragment, or shave biopsies
- Multiple biopsies of affected and adjacent “normal” areas from each lesion and from different lobes (liver) or regions (stomach) within the organ
- Preferentially sample the leading edge of “active” lesions (except bone: obtain a core sample)
- Take concurrent samples from other organs that may be primarily or secondarily affected
- Mark margins with different color inks and provide key
- Small / Special interest tissue: label / into cassette, sieve bag
- Skin, Muscle: secure on flat support, like cardboard



**FIXATION**  
Formalin 10% neutral buffered  
 ❖ **10:1** formalin to tissue ratio  
 ❖ **Tissue thickness < 1 cm**  
 ❖ **Process within 1 week when possible**  
 ➢ Histology = standard  
 ➢ Special stains  
 ➢ TEM: minimal autolysis important  
 ➢ IHC  
 ➢ In situ hybridization  
 ➢ LCM  
 ➢ PCR

*Short Fixation  
Often Important*

Davidson's / Bouins Solution (not a must)  
 ➢ Ocular histology  
 ➢ Reproductive histology (Bouins)

Glutaraldehyde fixative (ex: Karnofsky's)  
 ➢ TEM: 0.1 cm<sup>3</sup>; negative / positive stain

Ethanol 70% → **Parasitologist**  
 ➢ Parasites (metazoan, insect, tick, mite)  
 ➢ Histology: try to avoid

**To Pathologist:** thorough history, gross report, photos, differential list, ± unstained slides, ancillary tests submitted

**Key:** CYA = cover your ass; IFC = immunofluorescent chemistry; IHC = immunohistochemistry; LCM = laser capture microscopy; PCR = polymerase chain reaction; VI = virus isolation  
 TEM = transmission electron microscopy

**FRESH / 4°C ± SPECIAL MEDIA**  
Unstained slides:  
 ➢ store in dark, refrigerator  
 ➢ Special stains (regular slides)  
 ➢ IHC / IFC (+ charged slides optimal)  
Fluids (whole blood unless serology)  
 ➢ Toxicology: check a reference  
 ➢ Serologic tests: antigen / antibody based  
 ➢ Culture: bacteria, fungus, VI, protozoa  
 ➢ PCR  
Tissue / Swab / Feces  
 ➢ Culture: bacteria, fungus, VI, protozoa  
 ➢ PCR  
 ➢ Antigen capture ELISA  
 ➢ TEM (negative or positive stain)

**FROZEN ± SPECIAL MEDIA**  
-70°C (tissue < 2cm<sup>3</sup>; fluids 1ml; swab)  
 ➢ IHC: place in tissue prep if possible  
 ➢ Culture: bacteria, fungus, (VI)  
 ➢ PCR  
 ➢ Serology: antigen / antibody based

-20°C (tissue, swab)  
 ➢ Toxicology: check a reference  
 ➢ Molecular diagnostics: see above  
 ➢ Not culture, IHC, or IFA

**SHIPPING**  
 ➢ Do not allow freeze-thaw cycle to occur and if it does tell the receiving lab  
 ➢ If dividing a sample, use sterile technique and do not allow to thaw  
 ➢ -70°C & -20°C samples on Dry Ice Overnight with 3:1 dry ice to tissue ratio  
 ❖ Do not mix with wet ice or with fresh, refrigerated, or fixed samples  
 ➢ Fresh / Refrigerated samples on Wet ice Overnight with 3:1 wet ice: tissue ration

# 1. VISUALIZATION

## 1.1. Gross examination, Wet preparation, Cytology, and Histology

**Caveat:** This review is not meant to be a complete outline of necropsy and biopsy protocols, but rather to highlight important considerations and sampling, handling, and shipping methods that will help you, the pathologist, molecular diagnostician, toxicologist, and parasitologist obtain as much information as possible. For more detailed information on necropsy and biopsy procedures and forms, a reference list is provided below according to major aquatic taxa.

### *Reference List*

- Handbook for Marine Mammal Medicine. 2<sup>nd</sup> Edition. Eds: LA Dierauf and FMD Gulland. CRC Press, Boca Raton, Florida, 200. Pp. 1063.
- Clinical Avian Medicine and Surgery: Including Aviculture. Eds: GJ Harrison and LR Harrison. W.B. Saunders Co., Philadelphia, Pennsylvania, 1986. Pp. 1384.
- Diseases of Poultry. 11<sup>th</sup> Edition. Eds: YM Saif, HJ Barnes, JR Glisson, AM Fadly, LR McDougald, and DE Swayne. Iowa State University, Ames, Iowa, 2003. Pp. 1231.
- Manual of Reptiles. 2<sup>nd</sup> Edition. Eds: SJ Girling and P Raiti. BSAVA, Gloucester, England, 2004. Pp. 383.
- Reptile Medicine and Surgery. Ed: DR Mader. W.B. Saunders Co., Philadelphia, Pennsylvania, 1996. Pp. 512.
- Amphibian Medicine and Captive Husbandry. Eds: KM Wright and BR Whitaker. Krieger Publishing Company, Malabar, Florida, 2001. Pp. 499.
- Fish Diseases: Diagnosis and Treatment. Ed: E Noga. Iowa State University, Ames, Iowa, 1989. Pp. 367.
- Systemic Pathology of Fish: A Test and Atlas of Comparative Tissue Responses in Diseases of Teleosts. Ed: HW Ferguson. Iowa State University, Ames, Iowa, 2000. Pp. 263.

### **General Considerations Regarding Pathology**

- Histology (anatomic pathology) and cytology (clinical pathology) of samples taken at biopsy or necropsy is a critical complement to any ancillary diagnostics, because it potentially can convey significance of the results, it is an effective means of demonstrating a direct association between a pathogen and a lesion, and it can identify confounding factors that predispose to or exacerbate disease.
- Pathologists characterize and categorize alterations in tissue and cellular morphology (ie. the nature of the host response to an insult) in order to provide a list of differential diagnoses, because tissues have only so many, and usually characteristic ways to respond to insults. Part of this assessment is looking for the presence or “footprint” of local and systemic potential pathogens.
- Refining this differential diagnosis list is facilitated by providing the pathologist with more information, including:
  - The *distribution* within the body, organ system, and tissue itself and *concurrent alterations*, which are surmised through a
    - thorough history
    - detailed gross report
    - complete tissue set submission
    - histological examination of multiple sections
  - The results from ante- and post-mortem *ancillary diagnostic(s)*
  - The potential (history) or confirmed (toxicological analysis) exposure to toxins
  - The history of trauma or exertional event
  - The history of the animal’s condition (including nutritional and reproductive status), population condition, and environmental conditions

- The history of therapy: type, duration, and time course relative to sample submission
  - The history of previous disease(s) or diagnostic results relating to the general health status or organ(s) of interest
  - Physiological or metabolic derangements cannot be determined by histopathology.
- At best, a pathologist can speculate on the presence of a derangement based on the identification of a cause or morphological alteration(s) that can result from such derangements.
- Pathologists appreciate feedback on diagnoses and interpretations – it helps us learn!

### *Thorough History*

- Died or euthanized?
- Condition of con-specific animals: Presence, Number, Number of healthy vs. sick vs. dead
- Conditions of co-housed or sympatric other species: as above according to each species type
- Environmental conditions
- Animal: Signalment, Nutritional condition, Reproductive status
  - Gross report: Carcass condition, GI content by region, Lesion description by organ system, Differential diagnoses, Samples taken by organ system
  - Biopsy report: Lesion description, Differential diagnoses, Samples taken by organ system
- Potential (history) or confirmed (toxicological analysis) exposure to toxins
- Trauma or exertional event
- Diagnostic results relating to the general health status and organ(s) of interest
- Therapy: Type, Duration, Time course relative to sample submission
- Previous disease(s)

*Complete Tissue Set:* even if the cause of morbidity / mortality seems obvious or lesions seems confined to one organ or system, because it allows for:

- Identification of factors that predispose or contribute to decline or traumatic injury
- Assimilation of information on basic biology, normal and variants of normal morphology, pathogenic and commensal / symbiotic microbes, and significant vs. background disease in that species and population
- See references for list according to taxa

*Lesion Description:* helpful information includes: (*Photos are a welcomed supplement*)

- Distribution in the body
- Location within the organ: capsule, parenchyma; mucosa, mural, serosa, transmural
- Distribution within the organ: which lobe(s) of liver, lung, etc.; which segment of the intestine; associated with a major structure (bronchus, bile duct, etc.)
- Distribution within the tissue: diffuse, multifocal, multifocal to coalescing
- Infiltrative or discrete; Encapsulated or non-encapsulated
- Ancillary changes: necrosis, hemorrhage, crusting, effusive

**“Target Samples” under CYA section of flowchart** (ie. tissues to consider to save when unsure of cause of morbidity/mortality):

- Virology: Heart, Lung, Liver, Spleen, Lymph node, Brain, ± Intestine, Serum, Whole blood (in EDTA) if possible
- Toxicology: Liver, Kidney, Fat, Stomach contents, Brain, Urine, Serum
- Histology: Complete tissue set

## 1.2. Special histological / cytological techniques

### 1.2.1. Immunohistochemistry (IHC) / Immunofluorescent chemistry (IFC)

#### *Utility:*

- Enables, confirms, or provides more specific identification of an agent, the sensitivity and specificity of which depends on the antibody used. Enables identification of agent or altered cell receptor expression in relation to localization within the body and within the tissue and in relation to alterations in histomorphology. Therefore, it facilitates the understanding of potential pathogenicity.
- Usually used with light microscopy, but with more specialized techniques can be used with transmission electron microscopy and laser capture microscopy.

#### *Samples:*

- Fresh tissue/fluids: this requires a pre-arrangement with the immunohistochemistry lab and submission within 24 hours. The tissue then will be processed by one of the methods below.
- Impression smears:
  - If not submitted within the next 48 hours, place in the refrigerator and in the dark to maintain antigenicity.
  - IFC: ideally, but not critical to place on positively charged slides.
  - IHC: requires placement on positively charged slides, otherwise tissue lifts off during processing.
- Formalin: fixed < 1 cm thick tissue in 10% neutral buffered formalin and embed in paraffin block ideally in < 1 week to reduce protein cross-linking and thus improve antigen detection.
  - Alternatively, tissues can be fixed for 48 hrs in formalin and then transferred to ethanol to preserve histomorphology and antigen detectability.

#### • Frozen:

Advantage: required for some antibodies that detect antigens that easily are cross-linked by formalin.

Disadvantage: diminished morphological detail, special storage needed, limited retrospective studies, and sectioning is more difficult than paraffin-embedded sections.

- Ideally embed tissue in tissue prep media (like OCT), place in isopentane, and freeze with liquid nitrogen
- Isopentane helps reduce freeze artifact, but is not critical
- Liquid nitrogen is greatly preferred to just placing into  $-70^{\circ}\text{C}$  freezer

#### *Method:*

- IHC / IFC is the localization of antigens in tissue sections by the use of labeled antibodies against specific reagents through antigen-antibody interactions that are visualized by a marker. Markers can be a fluorescent dye or enzyme used for light, fluorescent, or confocal microscopy, or a radioactive element or colloidal gold used for electron microscopy.
- There are numerous IHC / IFC methods that may be used to localize antigens and many techniques to attempt antigen retrieval. The selection of a suitable method is based on parameters such as the type of sample (smear, paraffin-embedded, frozen), the type of specimen under investigation, and the degree of sensitivity required; therefore, it is critical to provide the immunohistochemist with the following information:

- Fixation method
- Duration of fixation
- Differential diagnoses
- Species

*Advantage:*

- Helps visualize pathogens that can be difficult to identify on routine histology or with less specific special stains.
- Depending on specificity of the antibody, it can enable (more) precise identification of an agent.
- Potential to establish the pathogenic significance of an agent by directly determining its relationship to alterations in histomorphology and/or ultrastructure (ie. in-situ examination).

*Disadvantage:*

- The longer tissue sits in formalin (ie. not processed into paraffin embedded blocks), the greater the cross-linking of antigens, and thus the more likely to have a false negative or equivocal result.
- Requires the development and validation of specific or cross-reactive antibodies for the agent of interest. One must be certain of which antigen the antibody is labeling, the type of antibody, its potential for cross-reaction, and its validation in order to accurately interpret results.
- Some antibodies only are effective with frozen tissue.
- False positive and negative results can be dependent on the tissue quality, appropriate sample fixation (formalin vs. frozen) and duration of fixation; experience of histotechnician and pathologist with that antibody.

*Note:* **Immunocytochemistry** refers to immunohistochemistry techniques used on cytological preparations

*General Reference:*

Online information center for IHC: <http://www.ihcworld.com/introduction.htm>

### 1.2.2. Laser capture microscopy

*Utility:* Enables the precise excision of small groups (tens to hundreds) of cells from biopsy or necropsy tissue with the potential to separate infected and diseased cells from normal cells for subsequent molecular analysis, such as by DNA- or RNA-based PCR techniques and microarrays.

*Samples:*

- Targets are as outlined under PCR and microarray sections.
- Tissue fixed in formalin or by alcohol-based precipitation techniques. The shorter the fixation, the less protein cross-linking, and thus the less chance for a false negative result.
- Tissue flash frozen at -70°F in < 1cm<sup>3</sup> blocks.

*Method:* Tissue sections (5-6 μm) are prepared from paraffin-embedded or frozen tissue and are mounted on standard, non-charged glass slides. Tissue then can be stained by standard techniques (hematoxylin and eosin), IFC, or IHC to facilitate identification of tissue morphology and cell populations of interest. The section is coated with a thermoplastic film and a laser is applied for a fraction of a second to the area of interest, which transiently melts the thermoplastic

film that then binds to the target cells without detectable damage of biological macromolecules. The laser diameter can be adjusted to select either individual or clusters of cells.

*Advantage:*

- Potential to establish the pathogenic significance of an agent by directly determining its relationship to alterations in histomorphology and host cell gene expression (ie. effectively in-situ examination); therefore, it is superior to the ex-situ techniques for PCR and microarrays described subsequently.

*Disadvantage:*

- Requires expertise. Often requires an experienced histopathologist to overcome suboptimal visualization inherent in the technique.
- The isolation of minute amounts of material either limits subsequent analysis to amplification-based molecular techniques or requires the collection of numerous cells, which consequently is more time-consuming and requires the presence of numerous infected cells in the section(s).
- May require IHC or IFC to visualize the target, such that the same limitations apply as noted under the respective sections.

*General Reference:*

Brignole E. 2000. Laser-capture microdissection. *Modern drug discovery* 3(9):69-73.  
(<http://pubs.acs.org/subscribe/journals/mdd/v03/i09/html/toolbox.html>)

### **1.3. Electron microscopy**

#### **1.3.1. Scanning electron microscopy**

*Utility:* For surface examination of objects with great depth of field and at a very low or high magnification. Information is obtained on topography and morphology (such as for defining the number and features of flagella on protozoa). In other disciplines, it also is used for defining the composition and crystallographic information.

*Samples:* Plant or animal specimens (tissue, whole macroparasite, etc).

- Fresh plant or animal specimen: processed at lab
- Animal tissue: glutaraldehyde-based fixative or formalin (10% neutral buffered) – see TEM

*Method:* Microscope that uses a focused beam of highly energetic electrons (rather than light as for light microscopy) to image the specimen and gain information. Fixation, dehydration, critical point drying, and sputter coating of small samples can be accomplished by the facility.

#### **1.3.2. Transmission Electron Microscopy (“thin section EM”)**

*Method (general):*

- Biologic structures, because of low mass density, interact weakly with electrons used for imaging; therefore, they show little contrast or detail. Several methods are used to generate sufficient image contrast and resolution. The most versatile is positive and negative staining with heavy metal ions of high atomic number that serve to scatter the electrons from regions covered with the stain, e.g., lead, tungsten, and uranium ions.
- Requires photo documentation and review for accurate diagnosis.

*Samples (general):*

- Tissue with *minimal autolysis*.
- Glutaraldehyde-based fixatives are superior to formaldehyde (10% neutral buffered formalin).

*Advantages (general):*

- This is especially useful to detect agents that are difficult or impossible to detect at a light microscopic level.
- Does not require the designing of antibodies or molecular techniques for agent detection and identification.
- Will detect the outline of agents that no longer have nucleic acid or surface proteins required for detection by molecular techniques, IHC, or IFC.

*Disadvantages (general):*

- Not designed for screening of a large number of samples.
- Requires assistance of an electron microscopist or someone with a trained eye for accurate interpretation.
- Requires tissue with little autolysis in most cases.
- Requires either diffusely distributed organisms of sufficient quantity or a method to precisely identify the location of the agent within the tissue from which then to sample. Thus, a negative result  $\neq$  a negative tissue sample.

*References:*

- Hazelton PR and Gelderblom HR. 2003. Electron microscopy for rapid diagnosis of emerging infectious diseases. *Emerging Infectious Diseases Journal* 9(3) (<http://www.cdc.gov/ncidod/EID/vol9no3/02-0327.htm>)
- Personal communication with Robert Nordhausen (Thurman Laboratory, CAHFS, Davis, CA)

### **1.3.2.1. Positive staining**

*Utility:* It is the ultrastructural counterpart to histology. Used to screen for agents and to detect cellular alterations in response to agents at an ultrastructural level. Used to precisely identify agents and to characterize novel agents in some instances.

*Samples:* In order of *greatest to least resolution*:

- 0.1cm<sup>3</sup> tissue sample placed in glutaraldehyde-based fixative (preferable)
- 0.1cm<sup>3</sup> tissue sample placed in formalin (10% neutral buffered)
- Tissue embedded in a paraffin block (ie. those used for histology). This is taxing for the electron microscopist and can have significant loss of detail, so use only when really necessary

*Method:* The post-fixation process is modified according to method of tissue preservation. Samples undergo a lengthy process of fixation, incubation with heavy metal ions (osmium, uranyl), dehydration, embedment, and generation of thick sections (like histology sections). Using light microscopy, the electron microscopist or researcher chooses a small region of interest within the thick section. Then, ultrathin sections are generated and stained for examination by TEM.

*Result:* Chemical moieties in the object show differential affinities for the heavy metal stains, which results in clear ultrastructural detail, such as the outline of the viral bilayer envelope, viral envelope proteins, nucleocapsid, and the dense nucleic acid containing core.

*Advantage:* With good tissue preservation, it enables partial to complete identification and characterization of pathogens as well as cellular response at an ultrastructural level.

*Disadvantage:* Positive staining depends on chemical reactivity with the components of the object, such that too much or too little staining can obscure finer detail. It involves processing that may take 4-5 days before a sample is ready for examination. Rapid embedding protocols can reduce the time to approximately 1 day but with a loss in specimen quality.

### **1.3.2.2. Negative staining (“direct EM”)**

*Utility:* It is the ultrastructural counterpart to impression smears for cytology. A simple, rapid technique well suited for small particulate suspensions for morphological identification of agents and generation of a list of differential diagnoses; however, little to no internal structure of the agent or response of the cell is apparent.

*Samples:*

- Fluid or excrement saved at 4°C (*do not freeze*)
  - Collected by syringe or impression smears placed on multiple glass slides or TEM grids (air dry) (*not* swabs or samples placed in special media)
  - Blood ≥ 5.0ml without anticoagulant
  - Feces ≥ 1g
- Tissue: fresh if TEM available immediately or glutaraldehyde- or formalin-fixed tissue as for positive staining TEM (*not* tissue paraffin-embedded or placed in special media)

*Method:* A coated grid with the sample adsorbed to the surface is floated on a drop of negative stain for 0.5 - 2 min. Excess stain is wicked away with a piece of filter paper, and the grid is air dried for 1 - 3 min for subsequent examination by TEM. Structures on the grid are surrounded and stabilized by the negative drying stain.

*Result:* The negative stain moulds around the agent, outlining its structure, and also is able to penetrate between small surface projections and to delineate them. If there are cavities within the structure that are accessible to the stain, these will be revealed and some of the internal structure of the agent may be apparent. Thus, only electrons that pass through the specimen are involved in the formation of the final image, making the agent appear as a transparent, detailed image within a dark halo of stain – ie. the reverse of positive stained agents.

*Advantage:* Rapid, simple screening method for pathogens.

*Disadvantage:* Little internal structure of the agent – careful for false interpretation. No information on response of the host cell.

### 1.3.2.3. Particle Enrichment

*Utility:* The use of concentration or immunologic procedures to markedly increase sensitivity of TEM diagnostics for samples that have lower particle concentrations (immunologic or concentration procedure) or to identify a specific agent (immunologic procedure).

*Methods:*

Non-immunologic:

- Ultracentrifuge concentration
- Agar diffuse
- Direct centrifugation to the electron microscopic grid

Immunologic: with genus or species-specific antibodies (may require frozen specimens – see IHC section).

- Serum in agar (immunoaggregation)
- Solid phase immunoelectron microscopy

*Disadvantage:* Not readily available in veterinary medicine. Procedures take from 0.5 to 16 hours and are labor and training intensive. May require development of specific reagents.

## 1.4. Culture techniques

*Utility (general):*

- Culture techniques allow for preliminary identification of the organism often to the family level, eg. Herpesvirus. Additional analysis, however, is needed for specific identification.

*Advantage (general):*

- Isolation of the organism allows for additional analysis that is required for specific identification to a species and subspecies level and for characterization of novel agents.

*Disadvantage (general):*

- False negatives often can occur with more fastidious or less hardy organisms, especially if overgrown by concurrent infection or contamination. False positives if environmental or cross-contamination occurs at sampling or in the lab.

### 1.4.1. Bacterial culture

*Utility:* Ability to evaluate the current infection status with respect to bacteria in a clinically relevant fresh sample.

*Method:* Involves inoculation of bacterial culture media to grow the bacteria. Culture media used depend on the type of bacteria that are being cultured:

- Aerobic bacteria: The standard medium is blood agar plates, which is useful for non-enteric species. Some labs also use a MacConkey agar plate, which is useful for enteric species.
- Anaerobic bacteria: Blood agar plates are used that are prepared specially by ridding of oxygen and its products as much as possible. The plates are cultured in a CO<sub>2</sub> or directly anaerobic environment.

- More fastidious bacteria require specific culture methods:
  - *Brucella* spp. are grown on serum dextrose, tryptose, and brucella (Albimi) agars and cultures should be incubated for a minimum of 10 and up to 21 days.
  - Spiral bacteria (*Helicobacter* spp., *Campylobacter* spp.) should be grown on moist plates with antibiotic selective media.
  - Spirochetes: *Leptospira* spp. are often slow growing and may require Ellinghausen, McCullough, Johnson and Harris (EMJH) medium; *Brachyspira* spp. should be grown on blood agar plates with spectinomycin.
- Bacterial culture often is followed by antibiotic resistance testing, which is useful to monitor for the presence of and changes in drug resistance within a population and also helps to ensure that an individual is treated with the most effective antibiotic.

*Samples:* May include tissues, feces, CSF, and blood from infected animals or clinical samples such as swabs of secretions, wounds or abscesses. Note: with effusive lesions, tissue samples usually have a higher yield than fluid, because fluid often dilutes microbes or contains non-viable microbes.

*Advantage:* Detects the presence of bacterial organisms and is therefore very specific.

*Disadvantage:* Can be fairly time consuming. May require a fair amount of manipulation in order to grow more fastidious organisms, e.g. *Brucella* spp.

*General Reference:*

Hirsh DC, MacLachlan NJ and Walker RL (2004) Laboratory Diagnosis. In: Veterinary Microbiology. 2<sup>nd</sup> edition. Blackwell Publishing, Ames, Iowa. Pp. 15-25

### 1.4.2. Protozoal culture

*Utility:* Ability to evaluate the current infection status with respect to protozoa in a clinically relevant sample.

*Method:* Protozoa are cultured on cell monolayers of various cell lines (*Toxoplasma* and *Neospora* spp. have been grown on Vero cells and bovine monocytes). These cultures are maintained in culture supplemented with fetal bovine serum. Since bacterial contamination is often a problem with protozoal culture, antibiotics, such as penicillin and streptomycin, are added. Merozoites can be harvested 5-6 days post-infection.

*Samples:* Fresh feces, blood aspirates, tissues - best to place tissues in antibiotic saline (to help prevent bacterial overgrowth) and refrigerate, ship to diagnostic lab on wet ice within 24 hours.

*Advantage:* Detects the presence of protozoal organisms and is therefore very specific.

*Disadvantage:* Can be fairly time consuming and may require a fair amount of manipulation to maintain cultures. Fresh samples are required that need to be sent to the diagnostic lab within 24 hours after collection.

### 1.4.3. Virus culture

*Utility:* To aid in the diagnosis of an active viral infection.

Viruses can be classified according to morphology, means of replication, and type of genome (DNA versus RNA, and single-stranded versus double-stranded). Since virus isolation almost always will be combined with PCR, categorization by genome is best for sampling and diagnostic purposes.

1. DNA viruses have a genome that is entirely made up of DNA. They include those in families: Adenoviridae, Hepadnaviridae, Herpesviridae, Iridoviridae, Papovaviridae (papilloma, polyoma), Parvoviridae, and Poxviridae.
2. RNA viruses have a genome that is entirely made up of RNA. They include those in families: Arenaviridae, Birnaviridae, Bunyaviridae, Caliciviridae, Coronaviridae, Orthomyxoviridae (ex: influenza virus), Paramyxoviridae (ex: morbilli and distemper viruses), Picornaviridae, Rhabdoviridae (ex: rabies virus), and Togaviridae (ex: West Nile Virus, EEE, SLE).

*Samples:*

- Samples should be collected as early as possible following disease onset. Viruses may no longer be present as early as 2 days following onset of clinical signs.
- Always indicate on the submission form to your laboratory: specimen type, suspected viruses, and time of sample relative to disease onset.
- Any swab types (cotton, rayon, Dacron) are acceptable. Swabs with a plastic shaft are superior to those with a wooden shaft.
- Recommendations for sample collection:
  - General: Tissue or excretions always are superior to swabs. Sterile collection is required.
  - Throat, nasopharyngeal swabs or aspirates.
  - Bronchial, bronchoalveolar: wash and lavage fluids, e.g. collected during bronchoscopy, are excellent for isolation of viruses that infect the lower respiratory tract.
  - Rectal swabs, stool specimens: stool specimens are superior, although many gastroenteritic viruses do not grow well in cell culture and require TEM for detection. Collect 5-10 ml of freshly passed diarrheal stool.
  - Sterile body fluids: CSF, urine, effusions
  - Blood (e.g. intracellular agents): collect 5-10 mL of anti-coagulated (preferably citrated) blood. Clotted specimens are unacceptable.
  - Tissue specimens: collect at edge of active lesions.
- Sample transport and storage: requirements vary for each virus type, so contact your laboratory. General guidelines are:
  - Ask your laboratory about appropriate, perhaps critical transport and storage media (contains protein and antimicrobials).
  - Place samples on ice and transport to the laboratory immediately (within 12 to 24 hrs).
  - If the sample ( $\pm$  in media) cannot be transported immediately, the sample should be refrigerated, not frozen. For storage up to 5 days, store at 4°C. For storage for 6 or more days, store at -70°C (except blood).
  - Blood must be kept at 4°C until processing.

*Method:* Viruses are strict intracellular parasitic organisms, so living suitable host cells are required to detect a virus. These host cells, referred to as cell cultures (immortalized or primary), are grown to confluency inside a Petri dish or flask. Upon the arrival in the laboratory, the samples may be homogenized to release the virus from the cells. Sample extracts then will be placed on appropriate cell cultures. For optimal recovery, an array of cell cultures should be used. Cultures typically will be incubated at either 30-32°C (fish, reptiles) or 37°C (mammals). Successive blind passages may be needed before reporting the culture negative. Cultures should be examined periodically for cytopathic effect during incubation.

*Interpretation of results:*

- In general, the isolation of a virus from host tissues, CSF, or blood is significant and is a very good indication of a viral etiology. Viruses, however, also can contribute to or predispose to disease, without being the main causative agent of the disease presented.
- It is important to show the presence of the virus within the lesion or disease process. Many viruses can be commensals, contaminants, or only predispose to but not cause the disease process, and thus may be an incidental or indirect finding.
- To establish a firm diagnosis, always try to confirm the etiologic role of the virus using one or more of the following in-situ techniques: cytology, histology and when possible complemented by in-situ hybridization, IHC, IFC, LCM, or TEM.
- No conclusions can be drawn from the failure to isolate a virus from a lesion. Certain viruses are very refractory to *in vitro* culture (Norwalk, sea turtle fibropapillomavirus, all papillomaviruses, and others).
- Virus isolation from a diarrheal stool sample should be interpreted cautiously.
- PCR protocols are available for the identification of most known viruses. PCR protocols exist for screening and identification of new viruses. An unambiguous causal relationship to disease, however, only rarely can be made based on PCR results alone.

*General reference:*

Forbes BA, Sahm DF and Weissfeld AS (2002) Laboratory methods in basic virology. In: Diagnostic Microbiology. 11<sup>th</sup> edition. (Forbes BA, Sahm DF and Weissfeld AS, eds.). Mosby Inc., St Louis, Missouri. Pp. 799-863.

#### **1.4.4. Fungal culture**

*Utility:* To aid in the diagnosis of an active infection with fungus, including yeast forms. For sampling and diagnostic purposes, fungal organisms can be categorized best into four groups of clinical relevance:

1. Superficial or cutaneous mycoses: infections of all keratinized tissues (hair, skin, nails) without invasion of the deeper tissues. The fungi in this category include the dermatophytes (e.g. ringworm) and water molds.
2. Subcutaneous mycoses: infections that are contained to the subcutaneous tissues without dissemination to distant sites (e.g. chromoblastomycosis).
3. Systemic mycoses: infections that are widely disseminated and can include any organ system (e.g. *Blastomyces*, *Coccidioides*, *Histoplasma*).
4. Opportunistic mycoses: infections occur primarily in immunocompromised hosts (underlying disease processes, immunosuppressive agents, stress). In these cases, fungi

with low pathogenicity may be the cause of localized or systemic infections (e.g. *Aspergillus*, *Zygomycetes*, *Candida*, *Cryptococcus*)

#### *Samples:*

- The diagnosis of mycoses and identification of the fungus are dependent entirely on the selection and collection of an appropriate clinical specimen for culture.
- Many systemic and opportunistic mycoses have a primary focus in the respiratory tract, so respiratory secretions should be sampled for culture when possible. Once dissemination has occurred, fungi also may be recovered from other organs.
- Recommendations for sample collection:
  - Respiratory tract secretions (sputum, bronchial washings, bronchoalveolar lavage, tracheal aspirations): consult with your laboratory about the addition of antibiotics or antifungals to prevent overgrowth by bacteria or rapidly growing molds during transportation.
  - Cerebrospinal fluid: ideally 1.0 ml, no antibacterial or antifungal agents should be added. CSF specimens should be processed promptly. If prompt processing is not possible the samples should be kept at room temperature, or placed in a 30°C incubator.
  - Blood: blood cultures are an excellent method for diagnosing disseminated fungal infections. Submit whole blood, because red and white blood cells are most likely to contain the organisms.
  - Hair, skin, and nail scrapings: for diagnosis of superficial mycoses, the skin or nails can be scraped with a scalpel blade or microscope slide. Infected hairs can be collected by plucking deep to the root. All samples should be placed in a sterile container before culturing. They should not be refrigerated.
  - Urine: samples should be processed as soon as possible after collection. Consult with your laboratory about the addition of antibiotics or antifungals to prevent overgrowth by bacteria or rapidly growing molds during transportation.
  - Tissue samples: the sample should be placed in a sterile container for shipment. Do not mince tissues samples. Process as soon as possible.

*Method:* Upon arrival in the laboratory, the samples may be minced, ground and filtered.

Sample extracts subsequently will be placed on appropriate culture media. For optimal recovery, an array of media should be used. Cultures will be incubated at room temperature, or preferably at 30°C, for 30 days before reporting the culture negative. Cultures should be examined at least 3 times weekly during incubation. Ideally, the species of all fungal isolates should be identified.

#### *Interpretation of results:*

- It is common for many types of yeast (e.g. *Candida*) to be recovered from healthy tissues. Try to rule out environmental contamination – consult with your microbiologist. Generally, monocultures are clinically much more meaningful than mixed cultures.

Recent reviews of fungal infections and their clinical significance are available

- Marine mammals: Reidarson TH et al. (2001) Mycotic Diseases. In: CRC Handbook of Marine Mammal Medicine, 2<sup>nd</sup> Ed. (Dierauf LA and Gulland MD, eds.), CRC Press, Boca Raton, Florida. Pp.337-355.
- Marine and freshwater fish: Yanong RPE (2003) Fungal diseases of fish. *Vet Clin Exot Anim* 6: 377-400.

- o Manual of Reptiles. 2<sup>nd</sup> Edition. Eds: SJ Girling and P Raiti. BSAVA, Gloucester, England, 2004. Pp. 383.
  - o Amphibian Medicine and Captive Husbandry. Eds: KM Wright and BR Whitaker. Krieger Publishing Company, Malabar, Florida, 2001. Pp. 499.
- Minimum incubation time: all fungal cultures, especially dermatophyte cultures (superficial mycosis), should be incubated for a minimum of 30 days before they can be reported as negative.
  - Cytological examination of clinical specimens: examine a smear of the clinical specimens with light microscopy (special stains like PAS or GMS can facilitate visualization), or ask your microbiology lab to do this for you. Cytology often can provide the first, rapid and accurate proof of a fungal infection.
  - Where possible, try to confirm the etiologic role of a fungus using histopathology.
  - Additional tests to confirm the clinical significance and identity of an isolated fungus:
    - o Immunodiffusion assays: available for the identification of some common systemic mycoses. These assays, however, have been replaced largely by PCR.
    - o PCR: Nucleic acid testing is not used routinely for the diagnosis of fungal infections. These assays, however, are sometimes available in research settings.
    - o Serology: Fungal serologic tests are available to detect exposure to or infection with some of the common systemic mycoses.
  - Antimicrobial susceptibility tests are available. They will help you chose the appropriate antifungal agent for treating a specific infection. Check with your microbiologist about the availability.

*General reference:*

Forbes BA, Sahn DF and Weissfeld AS (2002) Laboratory methods in basic mycology. In: Diagnostic Microbiology. 11<sup>th</sup> edition. (Forbes BA, Sahn DF and Weissfeld AS, eds.). Mosby Inc., St Louis, Missouri. Pp. 711-797.

## **2. SEROLOGIC TECHNIQUES**

- Serologic methods are generally antibody based and are used to detect viral or bacterial proteins or the host response to the virus, bacteria, or parasite in serum samples.
- The most important point about serologic methods is that they are useful for determining whether animals have been previously exposed to a disease agent; however, they provide little information regarding the presence of a current infection in an animal or population (unless paired samples are tested).
- It is also important to note which immunoglobulin the test is detecting, as this will impact the interpretation of the result (IgM = detected earlier in the response or during a first exposure; IgG = detected later in the response; IgE = hypersensitivity response).

*Serologic reagents*

- Monoclonal antibodies: an antibody that is mass produced in the laboratory from a single clone and that recognizes only one antigen. Useful diagnostic agents in assays to detect specific antigens (ie. less prone to cross-react between agents and host species).
- Polyclonal antibodies: antibodies that are derived from different cell lines. These antibodies have specificity for different epitopes. Useful for recognizing many proteins produced by a

disease agent, but less specific reagent than monoclonal antibodies (ie. more prone to cross-react between agents and host species).

- Cross reactive reagents: recognize antibodies from many different species. Not very specific, but useful in species for which specific reagents do not exist. Examples include protein A and protein G.

*General Reference:*

Kuby J (1997) Antigen - Antibody Interactions. In: Immunology. 3<sup>rd</sup> edition. W.H. Freeman and Company, New York, New York. Pp. 143-164.

## **2.1. Serum / Virus neutralization**

*Utility:* Test for exposure to viral agents, by measuring biologically relevant antibody responses.

*Method:* Serum samples are incubated with infectious virus in tissue culture and monitored for their ability to prevent viral infection in the cells. Results are reported as titres.

*Samples:* Serum, plasma.

*Advantage:* These assays can be very specific and are considered the “gold standard” serologic test.

*Disadvantage:* Virus neutralization can be time consuming, and results are often not available for 5 to 7 days.

## **2.2. Enzyme linked immunosorbent assay (ELISA)**

*Utility:* Tests for exposure to a variety of disease agents (bacteria, viruses, parasites). Agents are purified, and the concentration of antibodies (ie. titer) against the antigen of the agent or the agent itself of interest can be quantified.

*Samples:* Serum, plasma.

*Method:* The concentration of antibodies against the disease agent of interest is measured based on a standard curve of a known concentration. Results often are reported as concentrations of antibody, but sometimes can be reported as titers.

Basic ELISA designs include indirect, direct, and competitive ELISA, but these ELISA designs can be combined:

- Indirect: antigen of interest is purified, and the presence or concentration of antibodies against it is detected or measured.
- Direct or antigen capture: presence or concentration of the antigen of interest is detected or measured.
- Competitive: inhibition type assay, in which the presence or concentration of antibody is detected or measured.
- Sandwich: presence or concentration of antibodies is detected or measured.

*Advantage:* These assays are very sensitive and convenient. Large numbers of samples can be tested at one time, and results can be obtained in a few hours; therefore, it often is a good screening test.

*Disadvantage:* Reagents such as monoclonal or polyclonal antibodies are necessary (ie. have to be developed) to run the assay.

### **2.3. Fluorescent antibody testing (FA/IFA)**

*Utility:* Immunofluorescent assays are used to detect antigens of specific disease agents inside infected tissues or cells. Allows for visualization of the antigen in the cell when it binds to a fluorescent-labeled antibody (similar to IHC).

*Samples:* Tissues, cell cultures, serum; can be frozen, dry, fixed/unfixed.

*Method:* Immunofluorescence can be detected either by direct or indirect procedures and occurs when both the antigen and antibody are present.

- Direct test (detects antigen i.e. infection) requires an antigen-specific antibody labeled with a fluorochrome (fluorescein, rhodamine)
- Indirect test (detects antibody i.e. exposure) requires a fluorescent labeled anti-serum to an agent-specific immunoglobulin.

*Advantage:* Sensitive and rapid method for detecting and identifying disease agents in smears, tissues, or cultures.

*Disadvantage:* Requires development of fluorescent-labeled antibody or anti-serum. Results can be subjective (ie. dependent on the experience of the reader), causing false positive or negative results. Cross-reaction with another antigen may occur; the antigen against which the antibody is made determines the assay's specificity.

### **2.4. Immunodiffusion (ID)**

*Utility:* Immunodiffusion is a simple assay that detects the presence of antigens from agents in serum, can be qualitative or quantitative.

*Samples:* Serum, plasma.

*Method:* The sample is placed in a well and allowed to diffuse into a gel containing the antibody. A visible precipitate forms when the antigen of interest and antibody forms a complex in the gel. Two different methods are available, radial immunodiffusion and double immunodiffusion.

*Advantage:* Relatively simple and specific assay.

*Disadvantage:* The antigen of interest must be soluble so it can diffuse into the gel. Not a highly sensitive assay.

### **2.5. Agglutination assays**

*Utility:* Tests for exposure to agents, measures the interaction between an antibody and

particulate antigen.

- Hemagglutination inhibition (HI): measures exposure to a virus that contains a hemagglutination protein.
- Bacterial agglutination: measures serum antibodies produced against a bacterial infection.
- Agglutination inhibition: detects small quantities of antibody against the agent of interest.

*Samples:* Serum, plasma.

*Method:* Visible clumping is visualized in a tube between the antibody in the serum sample and the disease agent of interest. Can be qualitative or quantitative (reported as titres).

*Advantage:* Relatively simple and specific assay.

*Disadvantage:* The disease antigen of interest must be a particulate. Not a highly sensitive assay.

## **2.6. Plaque reduction neutralization test**

*Utility:* For quantization of anti-viral antibodies.

*Samples:* Serum, plasma.

*Method:* Serum samples are incubated with infectious virus in tissue culture then overlaid with cellulose. The cellulose layer prevents free movement of virus in the culture media and thus allows for cell-to-cell spread of the virus only. This cell-to-cell spread causes the formation of a “plaque”. Theoretically, one virion is one plaque forming unit. The monolayers then are stained, and the viral plaques are counted visually. The serum titer is the dilution required to reduce the number of plaque forming units (pfu) by 50%.

*Advantage:* These assays are very specific.

*Disadvantage:* Virus neutralization can be time consuming, and results often are not available for 5 to 7 days.

## **2.7. Western blot**

*Utility:* Western blotting can be used to detect antibodies to a full range of the disease agent’s proteins. Primarily used to detect viral proteins.

*Samples:* Serum, plasma.

*Method:* Proteins are revealed as discrete bands by running sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a nitrocellulose membrane. When serum is applied, antibodies in the serum bind to the proteins on the membrane.

*Advantage:* The most specific viral diagnostic test available, as it provides a full antibody profile against the disease agent.

*Disadvantage:* Reagents, such as monoclonal or polyclonal antibodies, are necessary to run the assay.

### 3. MOLECULAR TECHNIQUES

- These methods can be used to identify the presence of disease agents by detecting DNA or RNA, rather than just exposure in an animal or population. They can be used to probe archived tissues to detect infection, as well for analysis of ante- and post-mortem samples to be collected in the future. Small amounts of DNA from cells or tissues are needed for analysis.
- It is important to note that the presence of infection does not mean the presence of disease and establishing a link between the agent and resulting lesions still is needed.

#### 3.1. Polymerase chain reaction (PCR)

*Utility (general):* The PCR reaction is ideal for detecting the presence of bacterial, viral, parasitic (protozoal or metazoal), or fungal pathogens. The principle of detection is to attempt PCR with primers that are complementary to a part of the genome of the pathogen, but not of the host.

For sampling and diagnostic purposes, it is important to distinguish between conventional PCR, Real-time Taqman® PCR, and rtPCR:

1. Conventional PCR protocols are for the detection and identification of DNA sequences (DNA viruses, fungi).
2. Real-time Taqman® PCR is a variation of conventional PCR. This type of PCR can be used to detect and quantify the load of an infectious agent in clinical samples. It can detect RNA or DNA sequences.
3. reverse transcriptase PCR (rtPCR) protocols are for the detection and identification of RNA sequences (RNA viruses, bacteria).

*Method (general):*

- In the laboratory, either DNA or RNA will be extracted from the samples. An aliquot of the DNA/RNA will be included in a PCR reaction mix. This mix also will contain primers (specific or degenerate). During the PCR reaction, the primers will bind to the complementary segment of the genome of the pathogen. Sufficient copies of the genome segment (e.g. 600 nucleotides) between the primers will be made to yield a visible amount of DNA (ie. cDNA). The resulting PCR mix will be analyzed using electrophoresis on a gel, to see if a band of the expected size (in this example 600 nucleotides) is present. If DNA copies of the expected size are present, these copies will be sequenced. The resulting sequence then will be compared to other known sequences to establish the identity of the sequence and its original host (the microbe).

- Specific primers versus degenerate (also called consensus or generic) primers:

*Primers* are synthetic DNA molecules that are designed to be complementary to a target DNA/RNA sequence of the pathogen. Primers always are made in pairs. Each primer within a pair binds at opposite ends of the target sequence. In most applications of PCR, the primers are designed to match a known target sequence precisely (= specific primers). In some situations, however, such as the screening for a novel pathogen, primers are targeted to unknown sequences. In such cases, primers usually are designed based on the alignment of multiple related sequences and to allow for some mismatching of nucleotides (= degenerate primers). These often are group specific primers (eg. pan-herpesvirus primers).

- Controls: Where applicable, the following controls should be included in every PCR reaction to help ensure the validity of each test:
  - a. Without DNA/RNA: contains all components of the reaction, except the template DNA.
  - b. Open top: contains all components of the reaction, except the template DNA. The reaction tube should be kept open at all times.
  - c. Host genomic DNA/mRNA: ensures that the sample has amplifiable nucleic acid (ie. the sample was not too autolyzed and was taken, handled, and transported properly) and that the primer pair does not bind with the host genome.
  - d. Positive control: contains all components of the reaction, including template DNA that is complementary to the primers.

- Phylogenetic trees:

Once the identity of the amplified pathogen sequence has been obtained, a phylogenetic tree can be created. Examination of a phylogenetic tree allows you to determine how closely your pathogen is related to other pathogens with known functional or clinical roles.

Phylogenetic trees can be constructed using different methods (algorithms), but regardless of the method, a phylogenetic tree is akin to a family tree. The tree depicts the relationship of the pathogen to other known pathogens, based on the amplified sequence fragment and is ideally based on the entire genomic sequence. Because the entire genomic sequence is not currently available for aquatic animal pathogens, phylogenetic trees of aquatic animal pathogens will contain some error. This error is inversely proportional to the length of the sequence on which the tree is based. The shape of the tree represents the order in which the different sequences diverge. The bootstrap value for each branch of the tree (i.e. the numbers along the branches) is a statistical estimate of the certainty of these groupings. Tree branches with a bootstrap value below 50% should be ignored.

*Interpretation of results:*

- Cross-contamination: PCR results should always be interpreted with caution. Cross-contamination is a common problem in running PCR reactions, even with the most experienced PCR operator. Because PCR is highly sensitivity, minute amounts such as from aerosols that are formed when opening vials can cause detectable contamination. Due to the method of detection, Taq-man PCR has greater sensitivity than other types of PCR. When interpreting PCR results, one should always
  - o Inquire which controls were used (see above).
  - o Ask for the result of sequence comparison analysis (BLAST or other analysis).
  - o Inquire whether samples were run in the same assay that may have contained the same pathogen.
  - o Use in-situ techniques for confirmation (cytology, histology, IHC, IFC, LCM, TEM).

*General References:*

- Eeles RA and Stamps AC (1993) Polymerase Chain Reaction (PCR). The technique and its applications, 3<sup>rd</sup> ed. (Eeles RA and Stamps AC, eds.). RG Landes Company, Austin, Texas. Pp.107.
- Flint SJ, Enquist LW, Krug RM, Skalka AM and Racaniello VR (2000) Virus cultivation, Detection, and Genetics. In: Principles of Virology: Molecular Biology, Pathogenesis and Control. Flint SJ (ed). ASM Press, Washington D.C. Pp. 24-55.
- Leutenegger CM (2001) The Real-Time TaqMan PCR and Applications in Veterinary Medicine. Veterinary Sciences Tomorrow 1: 1-15.

- Leonard DGB (2003) Major Problems in Pathology (Volume 41). Diagnostic Molecular Pathology. (Leonard DGB, ed.). Saunders, Philadelphia, Pennsylvania. Pp.208

### 3.1.1. Conventional Polymerase chain reaction (PCR)

*Utility:* Currently one of the most sensitive techniques for detecting the presence of DNA viruses (eg. herpesvirus, poxvirus), bacteria, fungi, and parasites.

*Samples:*

- Samples may include diseased tissues, swabs, feces, blood, secretions.
- Sample storage: DNA is a very stable molecule. Samples for PCR can be kept fresh or frozen (-20°C or -70°C).
- When needed, PCR can be attempted on archived paraffinized tissues; however, the failure to amplify a DNA sequence will not be meaningful.
- Formalin cross-links and degrades DNA. DNA that has been exposed to formalin for over 48 hrs can no longer be amplified reliably, and the failure to amplify a DNA sequence will not be meaningful.

*Advantage:*

- Theoretically, as little as one copy of DNA will be amplified and detected, so this technique can be used to detect very small amounts of pathogens.
- DNA is a rather stable molecule, so PCR still can be performed on non-fresh samples that can no longer be used for viral, bacterial, or fungal culture.
- PCR is much faster than culture.
- PCR can be performed on very small samples (max. 25 mg of sample per reaction).

*Disadvantage:*

- Risk of cross-contamination: high sensitivity, operator error, aerosol.
- Need for second independent diagnostic method to confirm etiologic diagnosis.

### 3.1.2. Reverse transcriptase (rt) PCR

*Utility:* Most sensitive technique for mRNA detection and quantitation currently available. Useful for detecting the presence of RNA viruses (eg. cetacean morbillivirus, influenzaviruses) or RNA genes from bacteria (eg. 16s and 28s).

*Samples:* May include tissues, feces, and blood from infected animals or clinical samples, such as swabs of secretions, wounds, or abscesses.

*Note:* RNA degrades rapidly so submerging the sample in RNAlater® (Ambion, Inc) will help to stabilize and protect the RNA. The use of this product will enhance the ability to obtain RNA from the sample for analysis and can be stored at room temperature for 6 months or ≤ -20°C indefinitely. Samples should be placed in 5-10 volumes of RNAlater®.

*Method:* RNA is extracted from the sample and then converted to cDNA. The PCR reaction then is performed as described for detection of DNA viral sequences.

*Advantage:* Sensitive and specific tool for detecting the presence of RNA viral infection or bacterial infection based on 16s or 18s gene sequences.

*Disadvantage:* RNA degrades quickly; therefore, unless samples have been collected in a timely manner or stored in chemicals such as RNAlater®, RNA extraction is difficult.

### **3.1.3. Realtime Taqman® PCR**

*Utility:* Real-time Taqman® PCR is a variation of conventional PCR. This type of PCR can be used to detect and quantify the load of an agent in clinical samples.

*Samples:* May include tissues, feces, and blood from infected animals or clinical samples such as swabs of secretions, wounds, or abscesses.

*Method:* Based on the 5'-3' exonuclease activity of the DNA polymerase, which results in cleavage of fluorescent dye-labeled probes during the PCR reaction. The intensity of fluorescence then is measured and correlates to the amount of DNA from the disease agent present in the sample.

*Advantage:* This is a very sensitive and specific method. Allows for quantification of the virus load of an agent. Results can be obtained rapidly (24-48hr).

*Disadvantage:* Can be expensive. Requires time and expertise to design the system.

### **3.2. Restriction fragment length polymorphism (RFLP)/DNA fingerprinting**

*Utility:* Used to identify a change in the genetic sequence. Can be used to trace inheritance patterns, identify specific mutations, and identify bacterial strains.

*Samples:* May include tissues, feces, and blood from infected animals; clinical samples such as swabs of secretions, wounds, or abscesses; parasites, bacteria, protozoa, or fungi.

*Method:* The restriction enzyme is added to the DNA being analyzed and incubated for several hours, allowing the restriction enzyme to cut at its recognition sites. The DNA then is run through a gel, which separates the DNA fragments according to size. The size of the DNA fragments then are visualized.

*Advantage:* Highly specific and reliable (ie. repeatable) method.

*Disadvantage:* Time consuming (10 to 14 days to produce results).

#### *General References:*

- Yeary TJ (2004) A primer on Diagnostic Virology: Direct and Molecular-Based Detection of Viral Pathogenesis. Compendium, September 2004, Pp. 730-740.
- Watson JD, Gilman M, Witkowski J and Zoller M (1998) DNA-Based Diagnosis of Genetic Disease. In: Recombinant DNA. 2<sup>nd</sup> Edition. W.H. Freeman and Company, New York, New York. Pp. 545-547.

### **3.3. Microarray analysis**

*Utility:* Used to detect gene expression. It assesses the expression or activity of a specific gene of an organism by quantifying the amount of mRNA the gene produces. Microarray analysis is

an assay for the detection of gene expression and not commonly for the detection of infectious agents.

*Method:* Microarray analysis is a modification of conventional rtPCR, in which multiple rtPCR reactions, each for a different target sequence, can be run simultaneously. The principles of microarray are therefore the same as for rtPCR.

*Samples:* Host tissues. Refer to rtPCR for sample storage.

*Interpretation of results:* Microarray typically is used to evaluate the response of genes to a stimulus (e.g. environmental contaminants such as endocrine disruptants, cancer, etc.). The effect of an agent on the activity of several hundred genes of an organism can be evaluated; however, the function of most of these genes, especially in aquatic animals, is unknown.

*Advantage:* This is a very rapid method that allows for the simultaneous evaluation of the activity of several hundred genes of an organism.

*Disadvantage:* The function of most of these genes, especially of aquatic animals, is unknown.

*General reference:*

Shimkets RA (2004) Methods in Molecular Biology (Volume 258) Gene expression profiling. Methods and protocols. (Shimkets RA, ed.). Humana Press, Totowa, New Jersey. Pp.7-12.

### **3.4. In-situ hybridization (ISH)**

*Utility:* Detects presence and location of nucleic acids within lesions in order to establish a link between infection and disease.

*Samples:* Formalin fixed paraffin embedded tissues.

*Method:* Involves using labeled nucleic acid probes to detect nucleic acid sequences within infected tissues and cells. Commonly used on the same formalin fixed paraffin embedded tissues that are used for histopathologic examination.

*Advantage:* In-situ hybridization can be a very powerful method to provide a link between the presence of infection and its association with lesions and disease.

*Disadvantage:* In-situ hybridization can be technically difficult, requires expertise to set up, and may not work on tissues that were stored in formalin for extended lengths of time prior to embedding in paraffin.

*General Reference:*

Southern SA and Herrington CS (1998) In-situ hybridization. In: PCR In-Situ hybridization: A practical approach. Herrington CS and O'Leary JJ (eds). Oxford University Press, Oxford, UK. Pp. 26.

## **Appendix 1: Illinois Veterinary Diagnostic Laboratories - Laboratory User's Manual - Parasitology**

Fecal samples should be mailed in plastic bags or other water – tight containers. If the samples reach the laboratory in three days, no preservatives need to be used.

However, some of the ova may hatch during this time unless air is excluded from the container. The recommended method is to place the fecal sample in 10 – 15 volumes of 10% formalin (*the same concentration as for submitting histopathology samples*) or, if coccidia are suspected, place the feces in 2.5% potassium dichromate solution. If special procedures such as the Baermann technique to find *Strongyloides* spp. or lungworm larvae are needed, they should be requested. Fresh fecal samples without preservatives or fixatives are required for the Baermann technique.

If examination for intestinal protozoa other than coccidia (*particularly Giardia spp. or amoebae*) is requested, the sample should be fixed and shipped using sodium acetate – acetic acid – formalin (SAF) fixative. The fixative also preserves helminth eggs. The SAF solution is prepared as follows:

Sodium acetate 1.5 g

Acetic acid, glacial 2.0 ml

Formaldehyde solution (40%) 4.0 ml

Water 92.5 ml

**TOTAL 100.0 ml SAF fixative.**

Mix one volume of feces with at least three volumes of SAF fixative. Shake container well to ensure complete dispersal of specimen. Vials containing SAF fixative are available from the Parasitology Laboratory. Please call or write to receive pre – loaded specimen vials.

### **Parasite Specimens:**

Gross specimens require fixation and preservation prior to mailing. Trematodes and cestodes that are recovered at necropsy or passed in the feces should be placed in tap water, kept overnight in a refrigerator, and then fixed and stored in 10% formalin. Nematodes should be fixed, preferably in 70% ethanol, but can also be fixed in 10% formalin.

Most nematodes from domestic animals can be easily identified, but if nematodes are from exotic or wild animals, special fixation is needed. The best method is to heat 70% ethanol to about 60 degrees Celsius, drop the parasites into the hot alcohol one by one, and remove them as soon as they are fixed in an extended position. They should then be placed in fresh 70% alcohol for mailing.

Blood specimens for examination for microfilaria (*Knott's test*) should be fixed in at least 10 volumes of 2% formalin. Serum (*1 ml*) should be sent for the occult heartworm antigen test.

Ectoparasites should be fixed and submitted in 70% alcohol.

Skin scrapings may be sent on slides if a coverslip is sealed to the slides with clear nail polish or similar material. Be sure there is enough oil under the coverslip so that the material will not dry out in transit. Send more than one slide if possible. In addition, submit a scraping from the same area the parasites were found. Take scrapings from the edges of lesions and try to squeeze the skin while scraping to bring mites to the surface. A good, deep scraping is indicated by the presence of red cells. Submit in 70% alcohol.

[http://www.cvm.uiuc.edu/vdl/Parasit\\_man.html](http://www.cvm.uiuc.edu/vdl/Parasit_man.html)