2.1 Sampling Introduction

This section describes procedures for the collection of samples for both lot and facility inspections. These guidelines represent the **minimum acceptable standard** and may be superceded by applicable State, Tribal, interjurisdictional, and foreign aquatic animal health protection guidelines, policies, and /or regulations.

Size and age group classifications, lot definitions, sample size, general sampling considerations, and a recommended protocol for sample collection are also included. Screening and confirmatory tests to be run on the samples collected are provided, in detail, for each pathogen in subsequent chapters of this handbook (see table of contents).

The following necropsy and sample collection protocol is for four bacteria (*Aeromonas salmonicida, Yersinia ruckeri, Edwardsiella ictaluri*, and *Renibacterium salmonarium*), eight viruses (Infectious Hematopoietic Necrosis Virus, Infectious Pancreatic Necrosis Virus, Infectious Salmon Anemia Virus, Largemouth Bass Virus, *Oncorhynchus masou* Virus, Spring Viremia of Carp Virus, Viral Hemorrhagic Septicemia Virus, and White Sturgeon Herpesvirus), and four parasites (*Myxobolus cerebralis, Ceratomyxa shasta, Tetracapsula bryosalmonae,* and *Bothriocephalus acheilognathi*). A brief description of clinical signs of the diseases caused by each of these pathogens is included in this handbook. However, references such as the AFS/FHS Blue Book (Thoesen 1994) and others listed herein should be consulted for more detailed descriptions and information.

It is the responsibility of the inspector to evaluate the facility and select the appropriate fish from which to collect samples, check the policies and regulations of the jurisdictions requiring the inspection, and coordinate sample submission requirements with the laboratory that will be performing the assays. This will ensure recognition of the inspection results by the competent authorities. A guideline for inspection preparation is provided in 2.2.A "Guidelines for Preparation of a Fish Health Inspection."

The inspection criteria outlined in this chapter is based on the testing of a statistically valid number of individuals to assess the health of the entire population and is intended only to provide information for risk management involving the movement of fish from one location to another. The lack of detection of a particular pathogen resulting from an inspection indicates only that the pathogen was not isolated by the methods used on the individual fish examined on the date of sampling. It does not indicate that every fish in that population is free of that pathogen or will be free of that pathogen at any time in the future.

DISCLAIMER: Mention of specific brands or manufacturers does not warrant endorsement by the U. S. Fish and Wildlife Service and/or the United States government. Any comparable instrument, laboratory supply, or reagent may be substituted in this protocol if operation and performance are deemed comparable to the items specified.

2.2 Sampling

A. Guidelines for Preparation of a Fish Health Inspection

It is the responsibility of the inspector to obtain the appropriate information from the facility manager, receiving jurisdiction, and testing laboratory in order to assess all appropriate samples to collect from each lot at the facility. The following is a checklist of information to consider when planning and preparing for an inspection.

1. Facility Information

a. Facility water source(s) – do any of the sources contain live fish?

b. Facility water temperature regime.

c. Identification of all lots of fish present at the facility at the time of inspection and during the previous 12 months (see 2.2.C "Lot and Inspection Definitions" for definition of lot).

d. Origin and history of each lot: strain information, transfer information, and previous inspection history.

e. Location of fish in each lot at the facility (by both water source and rearing unit).

f. General health history, including any therapies administered to fish during the previous 12 months.

2. Receiving Jurisdiction Information

a. Name(s) of state contacts.

b. State regulations that apply.

c. Regional policy that may apply.

d. Pathogens listed by regulation or policy.

e. Sampling and laboratory analysis methods required by regulation or policy.

f. Does testing laboratory meet all requirements of receiving jurisdiction(s)?

Listed in Table 2.1 are the target fish species, size/age group and tissue to be selected for inspection for each pathogen. To the extent that inspection requirements allow for it, sampling efforts will be directed at the most susceptible species, age, temperature, and rearing units for that pathogen. For some species, strain susceptibility or resistance, as well as other performance factors can be obtained and reviewed from the National Fish Strain Registry (http://159.189.37.201/).

All samples shall be processed as soon as possible after collection. If the collected animals are not maintained alive before processing, samples shall be stored chilled (0 to 4°C) but not frozen and shall

be processed as soon as possible after collection. All samples for virology must be inoculated onto cell cultures within 72 hours post-collection. Fish selected should be representative of the lot being inspected and shall include fish with lesions and moribund fish when present.

Samples are collected by, submitted by, and accepted from a federally accredited veterinarian, a state or federal animal health official, or an American Fisheries Society (AFS) certified inspector or pathologist. Contact the laboratory that will be performing the assays to coordinate sampling.

3. Sample information will include the following information at a minimum:

    a. Name and address of owner.

    b. Location of sample collection.

    c. Type of water source (well, spring, surface).

    d. Whether a water source is fish-free.

    e. Name and address of the submitting individual.

    f. Age, species, origin of fish (fish or eggs).

    g. Number of fish present in each lot.

    h. Number of fish sampled in each lot.

    i. For *M. cerebralis* sampling, age should be given in temperature degree-days. If continuous temperature data is not available, report age in months. It should be indicated how long the fish have resided in the water supply.

B. Size/Age Groups

Environmental and species differences can markedly affect the growth rate of fish. In addition, some pathogens are most readily detected when fish are a certain size, whereas others are most readily detected when fish are a certain age. For the purpose of fish health inspections, fish are assigned to one of four groups based on either size or age depending on the pathogen of interest. The following table provides a general reference for these classifications. These classifications may not fit all species.

Table 2.1. Target fish species, size/age group, and tissue to be selected for inspection for each pathogen.

ORGANISM	COMMON NAME OF DISEASE	KNOWN SUSCEPTIBLE SPECIES	TISSUE FOR SAMPLING	PRIMARY (SCREENING TECHNIQUE)	CONFIRMATORY TECHNIQUE	COMMENTS
Bacterial Pathogens						
Aeromonas salmonicida	Furunculosis	Any freshwater fish	Kidney	Bacterial culture of kidney on TSA or BHIA media	Fluorescent Antibody Test (FAT)	May be isolated from many species of fish, birds, and protozoan parasites
Yersinia ruckeri	Enteric Red Mouth (ERM)	Any freshwater fish	Kidney	Bacterial culture of kidney on TSA or BHIA media	FAT	May be isolated from many species of fish and birds
Edwardsiella ictaluri	Enteric Septicemia of Catfish (ESC)	Ictalurids	Kidney	Bacterial culture of kidney on TSA or BHIA media	FAT	
Renibacterium salmoninarum	Bacterial Kidney Disease (BKD)	Salmonids	Kidney, ovarian fluid	Direct fluorescent antibody test on kidney smear or ovarian fluids	Bacterial culture using SKDM-2 media for a total of 6 weeks *or* nested Polymerase Chain Reaction (PCR) technique	
Piscirickettsia salmonis		Salmonids, freshwater, and marine fish	Kidney/Spleen/Liver/Blood	Cell culture on CHSE for 28 days. Hold for an additional 14 days. Or tissue impression stained with Giemsa.	IFAT, Immuno-histochemistry or PCR	Use antibiotic-free media in cell cultures.
Viral Pathogens						
Infectious Hematopoietic Necrosis Virus	IHN	Salmonids	Whole fry, viscera, or kidney/spleen - depending on size, ovarian fluid	Cell culture on EPC cells for 14 days at 15°C. Followed by a 14-day blind pass.	Serum neutralization or nested PCR or IFAT	Target tissues should be kidney/spleen from larger fish and ovarian fluid from spawning broodstock.
Infectious Pancreatic Necrosis Virus	IPN	Wide variety of freshwater and saltwater fish and shellfish	Whole fry, viscera, or kidney/spleen - depending on size, ovarian fluid	Cell culture on CHSE-214 cells for 14 days at 15°C. Followed by a 14-day blind pass.	Serum neutralization or nested PCR or IFAT	Target tissues should be kidney/spleen from larger fish and ovarian fluid from spawning broodstock. May be isolated from many species of aquatic organisms
Infectious Salmon Anemia Virus	ISA	Salmonids and Atlantic herring	Whole fry, viscera, or kidney/spleen - depending on size; ovarian fluids	Cell culture on SHK-1 cells for 14 days at 15° C. Followed by a 14-day blind pass.	PCR or IFAT	In addition to sampling kidney spleen, when available sample ovarian fluid from spawning broodstock. Most mortality occurs in saltwater with fluctuating temperatures
Oncorhynchus masou Virus	OMV	Salmonids	Viscera, ovarian fluids	Cell culture on CHSE-214 cells for 14 days at 15° C. Followed by a 14-day blind pass.	PCR technique or send to reference lab for confirmation	Target tissues should be kidney/spleen from larger fish and ovarian fluid from spawning broodstock. Only known to occur in Japan

Table 2.1. (Continued) Target fish species, size/age group, and tissue to be selected for inspection for each pathogen.

ORGANISM	COMMON NAME OF DISEASE	KNOWN SUSCEPTIBLE SPECIES	TISSUE FOR SAMPLING	PRIMARY (SCREENING TECHNIQUE)	CONFIRMATORY TECHNIQUE	COMMENTS
Viral Hemorrhagic Septicemia Virus	VHS	Salmonids, pike, turbot, herring, pilchard	Kidney/spleen	Cell culture on EPC cells for 14 days at 15° C. Followed by a 14-day blind pass.	PCR	In addition to sampling kidney spleen, when available sample ovarian fluid from spawning broodstock.
White Sturgeon Herpesvirus	WSHV	White sturgeon, possibly shortnose sturgeon	Kidney/spleen, ovarian fluids	Cell culture on WSS-2 cells for 14 days at 20° C. Followed by a 14-day blind pass.	Send to reference lab for confirmation	
Largemouth Bass Virus	LMBV	Centrarchids and ecocids	Kidney/spleen/swim bladder	Cell culture on FHM or BF-2 cells for 14 days at 20 to 25° C. Followed by a 14-day blind pass.	PCR	
Spring Viremia of Carp Virus	Infectious carp dropsy	Cyprinids, also brown trout, pike, shrimp and copepods	Kidney/spleen	Cell culture on EPC cells for 14 days at 20 to 25° C. Followed by a 14-day blind pass.	Serum neutralization or PCR	Most easily isolated in the spring during and for several weeks after epizootics.
Parasite Pathogens						
Myxobolus cerebralis	Whirling Disease	Salmonids	Cranial cartilage (entire head or wedge/core sample from larger fish	Pepsin-trypsin digest	Histological observation of spores/lesions consistent with infection in cranial cartilage or nested PCR	For a facility inspection only one lot of the most susceptible species on each water source need be inspected. When possible select fish that have been on that water supply, while at a susceptible age, for a minimum of 1800 degree-days C or for six (6) months.
Ceratomyxa shasta	Ceratomyxosis	Salmonids	Intestine (posterior)	Wet mounts of intestinal scraping	Detection of spores or PCR	When possible select fish 1) in earth ponds or ponds receiving untreated surface water, 2) that have been on that water supply for a minimum of six (6) months and 3) that are moribund or lethargic.
Tetracapsula bryosalmonae	Proliferative Kidney Disease (PKD)	Salmonids	Kidney	Smears of kidney stained with Leishman-Giemsa or Lectin	Histology	When possible: 1) select fish from earth ponds or raceways receiving untreated surface water, 2) sample moribund fish and 3) conduct sampling during summer or early fall months.
Bothriocephalus acheilognathi	Asian Tapeworm	Cyprinids, silurids, poeciliids, percids, centrarchids, gobiids, cyprinodontids	Intestine (anterior one third)	Visualization of cestode with pyramidal scolex in the semi-contracted state	Positive identification by use of a key	Late summer and fall sampling optimal for detection.

Table 2.2. Suggested categories for grouping fish for sample collection.

Designation	Total length	or	Age
Fry	< 4 cm		0–3 months of age
Fingerlings	4 – 6 cm		4-12 months age
Yearlings/Adults	> 6 cm		Non-brood fishes greater than 12 months of age
Broodstock	> 6 cm		Sexually mature fish greater than 12 months of age and used as broodstock

C. Lot and Inspection Definitions

Refer to Table 2.1 and to the pathogen specific sections of this handbook for detailed information on what species of fish are susceptible to each pathogen and the conditions under which it is most readily detected.

1. **Lot of Non-Broodstock Fish**
   A group of non-brood fish of the same species and age group (see definitions of age group in Table 2.2) that have continuously shared a common water source throughout their life history. A representative sample of all strains and rearing units containing this lot shall be included.

2. **Lot of Broodstock Fish**
   A group of sexually mature fish of the same species that share a common water source. The sample must be representative of all age groups (e.g. three-, four-, and five-year-old brood fish) and strains present at the facility.

3. **Lot Inspection**
   The collection and examination of a statistically valid number of the appropriate samples from a specific lot of a susceptible species for any pathogen listed in this handbook. Moribund fish will be included when present. Unless otherwise stated in the policies and/or regulations of the jurisdictions involved, sampling for the required pathogens will be performed at the 5% APPL with a 95% confidence level. See Table 2.3 for further explanation of the number of samples required.

   a. Exception
      In broodstock lots where there is access to ovarian fluid, sampling for the required viral pathogens will be performed at the 5% APPL with a 95% confidence level in both kidney/spleen tissues and ovarian (coelomic) fluid. Kidney/spleen and ovarian fluid samples must come from different individuals.

      ***Example*:** In a broodstock population consisting of 2500 individuals, kidney/spleen samples will be collected from 60 fish (males and/or females) and ovarian fluid will be collected from an additional 60 females for a lot inspection requiring IHNV testing.

b. Exception
A lot of anadromous salmon regularly monitored for *Renibacterium salmoninarum* through ELISA or quantitative PCR techniques may be considered positive for this pathogen without additional testing. Results of the monitoring must be provided to the jurisdictions involved when requested.

4. Facility Inspection
Lot inspection of each and every susceptible lot of fish held on the facility for any of the bacterial, viral, and parasitic pathogens listed in this handbook.

a. Exception
For *Myxobolus cerebralis*, only one lot of the most susceptible species on each water source at the facility needs to be inspected. It is essential that the lot chosen has had sufficient exposure to create a detectable infection

b. Inspection Frequency
Most regulating jurisdictions require that a history of annual inspections be submitted with the inspection report prior to permitting the importation, stocking, and/or transfer of aquatic animals. It is, therefore, recommended that a program of annual facility inspections be encouraged for any facility participating in intrastate, interstate, and/ or international commerce of their animals.

D. Sample Number

Unless otherwise dictated by the receiving jurisdiction, the number of fish to be collected from each lot must be in accordance with a plan that provides 95% confidence that at least one infected fish will be collected if the minimum assumed pathogen prevalence level (APPL) of infection equals or exceeds 5%. Examples of the number of fish to sample for various population sizes are listed in Table 2.3. Table 2.3 also includes examples of the number of fish to sample if a 2% or 10% APPL is required by the requesting authority. If the population size is estimated to be between two grouping levels, the sample is taken from the next higher population class (Amos 1985; OIE 2000; Ossiander and Wedemeyer 1973; Thoesen 1994).

Table 2.3. Sample number based on an assumed pathogen prevalence level (APPL) in the population of 10%, 5 %, or 2%.

Lot Size	Number of Fish Required for Sample		
(number of fish)	10% APPL	5% APPL	2% APPL
50	20	35	50
100	23	45	75
250	25	50	110
500	26	55	130
2000	27	60	145
>100,000	30	60	150

E. Sample Collection

The order in which tissues are collected will vary depending on the tests to be run. What tissues and fluids are collected, will vary depending on the size of the fish, age of the fish, purpose for which the inspection is being performed, and the requirements of the assays used by the receiving laboratory.

1. Necropsy

A detailed necropsy procedure can be found in "Fish Disease: Diagnosis and Treatment" (Noga 1996).

a. Examine and note presence of gross external lesions. If lesions are collected for histological examination, it must be done in a manner that will not compromise the aseptic collection of samples for bacteriology and virology.

b. Collected fish are humanely euthanized immediately prior to sample collection.

Note: If confirmation of *M. cerebralis* infection is to be done histologically, fish should not be killed by a blow to the head as this may compromise the integrity of skeletal elements.

c. Fry are generally only examined for viruses. Fingerling, yearling, and adult sized fish may be examined for bacteria, virus, and/or parasites.

d. The instruments used during sample collection are at a minimum cleaned between sample pools and disinfected between lots.

e. The body cavity is opened being careful not to compromise the target sample tissues with contents from the intestinal tract.

f. If it blocks access to the kidney, the swim bladder is moved.

2. Collection of Kidney Cultures for the Detection of *Aeromonas salmonicida, Edwardsiella ictaluri, Yersinia ruckeri, and/or Renibacterium salmoninarum*

Samples for bacteriology should always be taken first with proper aseptic technique to minimize contamination. These cultures are not usually collected from fry.

a. A sterile swab or inoculating loop is inserted into the posterior kidney and streaked on a plate or slant (2.3.B "Media Preparation") of the appropriate media. Streaks from up to four fish in the same lot may be made on a single culture plate. When multiple cultures will be made from one fish, reinsert the sterile inoculation loop or swab into the kidney before each plate or slant is streaked. If fish are large enough, a piece of kidney tissue may be excised and used to streak the media.

i. *Aeromonas salmonicida, Edwardsiella ictaluri, and Yersinia ruckeri:* Brain Heart Infusion Agar (BHIA) or Trypticase Soy Agar (TSA) (2.3.A.1 "Growth Media")

ii. *Renibacterium salmoninarum*: SKDM-2 (2.3.A.2 "Growth Media") if the receiving laboratory will be using bacterial culture for the confirmation of R. salmoninarum.

b. Sample any organs with visible lesions.

c. Incubate media and identify pathogens by the methods described in Chapter 3 Bacteriology.

d. A smear for the *R. salmoninarum* FAT (3.5.A "Summary of Screening Test") is made from the posterior kidney on a microscope slide. The slides will be screened by FAT as described in 3.5.A "Summary of Screening Test."

e. If the polymerase chain reaction technique (PCR) (3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA") will be used to confirm a positive *R. salmoninarum* FAT slide, a kidney sample is collected after sampling of the kidney is completed for bacteriology and virology.

Note: Approximately 25 mg of kidney is collected into a sterile vial and frozen. Tissues collected for PCR archiving should be labeled so that those tissues can be identified individually if corresponding FAT slides are found positive for *R. salmoninarum*.

f. Ovarian fluid, when available, may be collected from spawning female broodstock for detection of *R. salmoninarum*. The ovarian fluid sample may be obtained from an aliquot of the sample collected for viral analysis (2.2.E.3.e) and processed at the laboratory as described in 3.5.A.1.b "Ovarian Fluid Pellet Smear."

3. Collection of Tissues for the Detection of Viral Agents

During collection, transport, and storage prior to processing, samples should be kept chilled (0 to 4°C). **Do not freeze.** During processing, samples should be kept on ice and at no time exceed 15°C or virus viability may be compromised. Tissues for viral testing may be collected and stored in a viral transport media such as Hank's Buffered Salt Solution (HBSS) with or without antibiotics (2.3.C.2 "Hanks Balanced Salt Solution (HBSS)"). The pH should be maintained within the 7.2 to 7.6 range. The samples must be processed and inoculated onto cell cultures within 72 hours of collection and 48 hours or less is recommended.

a. From fry, the entire fish is collected and placed into sterile containers; when present, yolk sacs should be removed to reduce toxicity in cell culture and muscle tissue may be trimmed off as needed to maintain a reasonable sample volume.

b. From fingerling-sized fish, the visceral mass including the kidney is collected and placed into sterile containers. If the stomach is filled with feed, it may be removed.

c. From yearling/adult fish, approximately equal amounts of the spleen and kidney are obtained using aseptic technique and placed into sterile containers. Tissues from up to five fish may be pooled in the same container with approximately an equal amount of tissue from each fish. Total sample volume should not exceed 1.5 grams of tissue.

d. From broodstock fish, approximately equal amounts of the spleen and kidney are obtained using aseptic technique and placed into sterile containers. Tissues from up to five fish may be pooled in the same container with approximately an equal amount of tissue from each fish. Total sample volume should not exceed 1.5 grams of tissue.

e. From female broodstock at spawning, ovarian fluid is collected into an appropriately sized sterile container. Approximately equal volumes (1 mL per fish) of ovarian fluid from up to five fish may be pooled in the same container.

4. Collection of Specimens for the Detection of *Myxobolus cerebralis* Evaluation

Selection of appropriate species and age should be made using Table 2.2 and information in 5.2 *Myxobolus cerebralis* (Whirling Disease) (Lorz and Amandi 1994; Meyers 1997).

a. Fish from the same lot may be processed in pools of up to five fish by pepsin-trypsin digest (PTD). For confirmation by PCR or histology, head/core samples are processed individually. Therefore, in order to track positive pools, all corresponding samples must be labeled appropriately.

b. From fingerling and yearling fish (less than 15 cm), the entire head, including opercles, is severed from the body.

c. For larger fish where size makes collecting the entire head impractical, a wedge or core samples may be taken. Include gill arches for more resistant species (5.2 *Myxobolus cerebralis* (Whirling Disease)).

    i. A triangle-shaped wedge is cut posterior to the orbit at the dorsal surface almost to the ventral edge of the opercula. The top (dorsal) portion of the wedge should measure 1.5 cm (Figure 2.1).

    ii. A core sample is taken by inserting a biopsy or boring tool (at least 19mm diameter; boring drill bit or sharpened pipe fitted to a drill work well) into the dorsal surface of the head just posterior to the eyes and forcing it ventrally until it penetrates into the mouth (Figure 2.2).

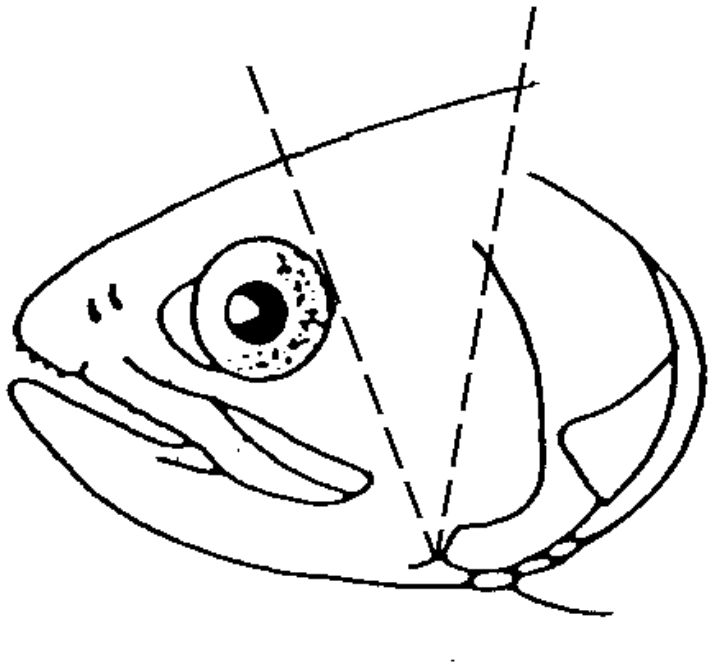

Figure 2.1. Diagram of location of wedge sample from adult fish.

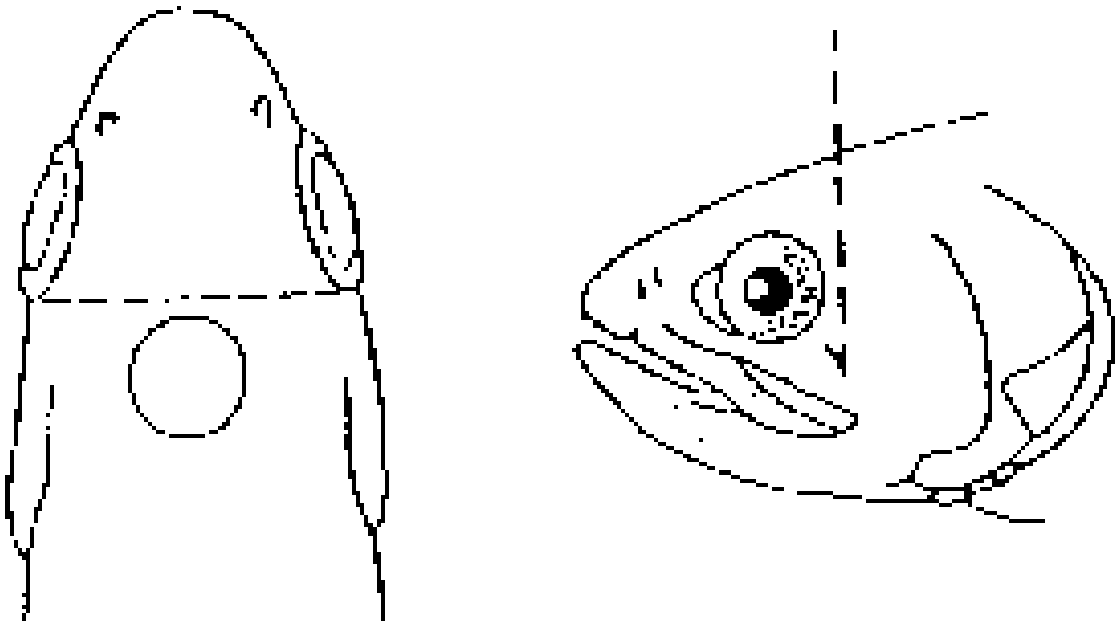

Figure 2.2. Diagram of location of core sample from adult fish.

d. Each head, wedge, or core sample should be split such that each piece contains all the tissue layers. The tissue that is to be processed by PTD for screening should be placed in a plastic bag and transported to the laboratory on ice. Tissues representing up to five fish may be combined for PTD assay.

e. The other half-head, wedge, or core is preserved in a manner suitable for confirmatory testing as follows:

   i. For PCR confirmation, at the time of collection, refrigerate, place on ice or freeze. Upon receipt by the laboratory, samples may be frozen at -20°C.

      Note: extreme care should be taken in the collection of samples in which PCR confirmation may be used. Refer to Chapter 6 for appropriate precautions.

   ii. For histological confirmation, at the time of collection place tissue in a fixative suitable for histology, such as 10% neutral buffered formalin (2.3.C.3 "10% Neutral Buffered Formalin (10% NBF)") or Davidson's (2.3.C.4 "Davidson's Fixative") fixative. Use a 10:1 (volume/volume) volume of fixative to sample.

f. Number individual samples to correspond to the tissue pool to be analyzed by PTD.

5. Collection of Tissues for the Detection of *Ceratomyxa shasta* (Bartholomew 2001)

a. For detection of *C. shasta,* fish are sampled individually and tissues are not pooled for examination.

b. Wet mounts are prepared from intestinal scrapings from the posterior intestine and from any lesions. When possible, fish should be examined immediately after death, but whole fish or intestines can be shipped on ice and examined within 24 hours.

c. For PCR confirmation of *C. shasta*, excise a small portion of the lower intestine (about 2 to 5 mm) and transfer to a vial with 500 µL DNA extraction buffer (5.6.G "DNA Extraction Buffer"). Alternatively, the sample may be frozen or fixed in 100% ethanol (EtOH). Samples in ethanol may be stored at room temperature; those in extraction buffer should be refrigerated or frozen for long-term storage.

Note: extreme care should be taken in the collection of samples in which PCR confirmation may be used. Refer to Chapter 6 Polymerase Chain Reaction (PCR) for appropriate precautions.

6. Collection of Tissues for the Detection of *Bothriocephalus acheilognathi*

a. Collected fish are best processed shortly after euthanasia, but may be transported on ice for up to 24 hours. Fixed specimens are not acceptable because of recovery and identification problems. This is especially true for small tapeworms.

b. If fish larger than 20 cm are to be examined, the anterior third of intestinal tracts can be removed and placed in bags on ice to avoid transporting whole large fish.

c. To remove the intestine, cut at the anus and just posterior to the stomach. Unravel intestines gently with fingers and cut off the anterior third and place in a bag on ice. Discard the lower two-thirds of the intestines.

d. Fish smaller than 20 cm are best transported to the laboratory alive but may be shipped whole on ice in plastic bags.

7. Collection of Samples for the Detection of *Tetracapsula bryosalmonae* (Klontz and Chacko 1983; Hedrick et al. 1986; Kent 1994)

a. For *T. bryosamonae* fish are sampled individually and tissues are not pooled for examination. However, impressions of more that one fish can be made on a single microscope slide.

b. Impressions of kidney tissue of each fish are made by excising a small ($5mm^2$) piece of tissue, blotting the tissue on paper to remove the excess blood, and making serial impressions on an alcohol-cleaned slide. Tissue is fixed in either absolute (100%) methanol (MeOH) for five minutes for Leishman-Giemsa staining or in acetone-ethanol (60:40) at -20°C for 10 minutes for lectin staining. The slide is labeled appropriately (location, reference) and stored in a slide box. For prolonged storage of slides for lectin staining, they should be stored dessicated at -70°C.

c. A small piece of kidney from each fish is saved in 10% neutral buffered formalin (2.3.C.3 "10% Neutral Buffered Formalin (10% NBF)") or other suitable tissue fixative (2.3.C.4 "Davidson's Fixative") for confirmation of the presence of *T. bryosalmonae* by histopathology.

d. For PCR confirmation of *T. bryosalmonae*, excise a small portion of the kidney (about 2 to 5 mm^2) and transfer to a vial with 500 µL DNA extraction buffer (5.6.G "DNA Extraction

Buffer"). Alternatively, the sample may be frozen or fixed in 100% ethanol (EtOH). Samples in ethanol may be stored at room temperature; those in extraction buffer should be refrigerated or frozen for long-term storage.

Note: extreme care should be taken in the collection of samples in which PCR confirmation may be used. Refer to Chapter 6 Polymerase Chain Reaction (PCR) for appropriate precautions.

2.3 Reagents, Media, and Media Preparation

A. Growth Media

Most of these media are commercially available as pre-made formulas or as bases, which can be easily made in the laboratory. These commercial products are entirely acceptable and should be made and stored according to the manufacturer's recommendations.

1. **Brain Heart Infusion Agar (BHIA) and Tryptic Soy Agar (TSA)**
   These two basic agars are interchangeable for bacterial cultures obtained during an inspection. They are both commercially available.

2. **Selective Kidney Disease Medium-2 (SKDM-2) (Austin, et.al.1983)**
   Used for the selective isolation of *Renibacterium salmoninarum.*

Peptone	10 g
Yeast extract	0.5 g
L-Cysteine HCL	1 g
Agar	15 g
Distilled water	to 1000 mL

Adjust pH to 6.5 before adding agar. Autoclave for 15 minutes at 121°C. Cool to ~ 50°C and add:

Fetal Bovine Serum	200.0 mL

The following antibiotics can also be added to the SKDM-2 to reduce overgrowth from other bacterial organisms (Austin et. al. 1983).

4.0 mL Cyclohexamide (1.2 g Cyclohexamide in 96 mL dH_2O)
1.0 mL D-Cycloserine (0.3 g D-Cycloserine in 24 mL of dH_2O)
2.0 mL Polymyxin B-sulfate (0.3 g Polymyxin B-sulfate in 24 mL of distilled H_2O)
1.0 mL Oxolinic Acid (0.06 g Oxolinic Acid in 24 mL of 5% NaOH)

B. Media Preparation

1. **Plate Media**

   a. Prepare media in stainless steel beakers or clean glassware according to manufacturer instructions. Check pH and adjust if necessary. Media must be boiled for one minute to completely suspend agar. Use of a stir bar will facilitate mixing of agar.

   b. Cover beaker with foil or pour into clean bottles being sure to leave lids loose. Sterilize according to manufacturer's instructions when given or at 121°C for 15 minutes at 15 pounds pressure.

   c. Cool media to 50°C.

d. Alternatively, media can be autoclaved and cooled to room temperature and refrigerated for later use. Store bottles labeled with media type, date, and initials. When media is needed, boil, microwave, or use a water bath to completely melt the agar. Cool to 50°C. Avoid reheating media multiple times before use.

e. Before pouring media, disinfect hood or counter thoroughly and place sterile petri dishes on the disinfected surface. Aseptically mix any antibiotic solutions, sheep's blood, or Fetal Bovine Serum into the media at this temperature.

f. Label the bottom of each plate with medium type and date prepared.

g. Remove bottle cap and pour plates or dispense with a sterile Cornwall pipette, lifting each petri dish lid as you go. Pour approximately 15 to 20 mL per petri dish. Replace lids as soon as the plate is poured.

h. Immediately wash medium bottle, cap, and pipette in hot water to remove agar and clean up any spilled agar.

i. Invert plates when the media has cooled completely (~ 30 to 60 minutes) to prevent excessive moisture and subsequent condensation on the plate lid.

j. Allow plates to sit overnight at room temperature. Store plates upside down in the refrigerator in a tightly sealed plastic bag or plate storage tin.

k. Follow manufacturer's recommendation for storage period of prepared media. Each batch should be labeled with date of preparation and/or an expiration date.

2. Tube Media

a. Prepare media in stainless steel beakers or clean glassware according to manufacturer's instructions. If the medium contains agar, boil for one minute to completely suspend the agar. Use of a stir bar will facilitate mixing of agar.

b. Media with indicators must be pH adjusted. This can be done when medium is at room temperature; otherwise, compensation for temperature needs to be made.

c. Arrange test tubes in racks. Disposable screw cap tubes can be used for all tube media.

d. Use an automatic pipetter or Pipette-aid™ to dispense the medium. If using the Brewer or Cornwall pipette, prime with deionized water and then pump the water out of the syringe prior to pipetting. Discard the first few tubes of media that are dispensed. Dispense approximately 5 to 10 mL media in 16 x 125mm or 20 x 125mm tubes. Close caps loosely.

e. Immediately after use, rinse the automatic pipetter in hot tap water followed by distilled water to remove all media and prevent clogging of the instrument.

f. Follow manufacturer's recommendation for autoclave time and temperature.

g. If making slants, put tubes in slant racks after autoclaving. Adjust the slant angle to achieve the desired slant angle and butt length (i.e. long butt and short slant for TSI or a standard slant over ¾ of the tube length for TSA or BHIA).

h. Cool completely to room temperature in the slanted position. Tighten caps.

i. Label the tubes or the tube rack with type of medium and date made.

j. Store at 2 to 8°C, following manufacturer's recommendation for long-term storage.

C. Reagents

1. 70% Ethanol (EtOH)

Ethanol (95%)	737 mL
Distilled water	to 1000 mL

2. Hanks Balanced Salt Solution (HBSS)

10X HBSS	100.0 mL
Cell culture grade water	895.0 mL
$NaHCO_3$ (7.5%)	5.0 mL

If antibiotics are used, subtract 320 mL of water and add in its place:

Penicillin/Streptomycin (16%)	160.0 mL
Penicillin G (10,000 units/mL)	
Streptomycin sulfate (10,000 ug/mL)	
Fungizone	160.0 mL
250 ug/mL Amphotericin B	
205 ug/mL Desoxycholate	

Mix. Filter with 0.22 um filter. Store at 4°C.

3. 10% Neutral Buffered Formalin (10% NBF)

Formalin (37% formaldehyde)	100 mL
Distilled water	900 mL
Sodium phosphate (monobasic)	4 g
Sodium phosphate (dibasic)	6.5 g

Store at room temperature.

4. Davidson's Fixative

95% Ethanol	600 mL
Formalin (37% formaldehyde)	400 mL
Acetic acid (glacial)	200 mL
Distilled water	600 mL

Store at room temperature.

2.4 Glossary

Anterior kidney - portion of the kidney containing hematopoietic tissues with little or no urinary function. Generally it is the portion of the kidney closest to the head.

Aseptic technique - prevention of contact with microorganisms not contained in the target tissue.

Disinfect - to free from pathogenic organisms or render those organisms non-infectious.

ELISA - Enzyme-Linked Immunosorbent Assay.

Inspector - a federally accredited veterinarian, a state or federal animal health official, or an American Fisheries Society (AFS) certified inspector or pathologist.

Health history - past events pertaining to infectious or noninfectious agents found during routine monitoring or diagnostic testing.

Life history -all life stages including eggs.

PCR - Polymerase Chain Reaction.

Posterior kidney - portion of the kidney containing excretory elements and responsible for performing urinary function.

Statistically valid number - the number of tissue or fluid samples sufficient to assess the risk of a particular pathogen being present in the population of interest. Selection of this sample number involves specifying both a minimum assumed prevalence level of the pathogen in the population and the minimum acceptable confidence interval for the detection of that pathogen.

Sterile - free from living microorganisms.

Strain - a fish population that exhibits reproducible physiological, morphological, or cultural performance characteristics that are significantly different from other fish populations or a broodstock derived from such a population and maintained therafter as a pure breeding population.

Strain resistance - the development of disease resistance by a particular strain of fish either through natural or artificial selection.

Susceptible species - any species capable of becoming infected with a particular pathogenic organism.

Therapies - the use of any drugs and/or chemicals for the treatment of disease.

Visceral mass - contents of the abdomen of the fish that includes the liver, spleen, stomach, pyloric caeca, intestine, and kidney.

Water source - a spring, lake, river, stream, creek, or aquifer. Multiple wells from the same aquifer or multiple inlets from the same surface water supply constitute a single water source for a facility. Wells in different aquifers or with demonstrably different water chemistry must be considered

different water supplies even if present on a single facility. Unless they are known to come from the same aquifer, every well should be considered a separate source.

2.5 References

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