Training on

Biological sample collection

ONE HEALTH IDT

Protecting Human Health through a One Health Approach ILRI/IFPRI/IWMI/WorldFish

October 2022

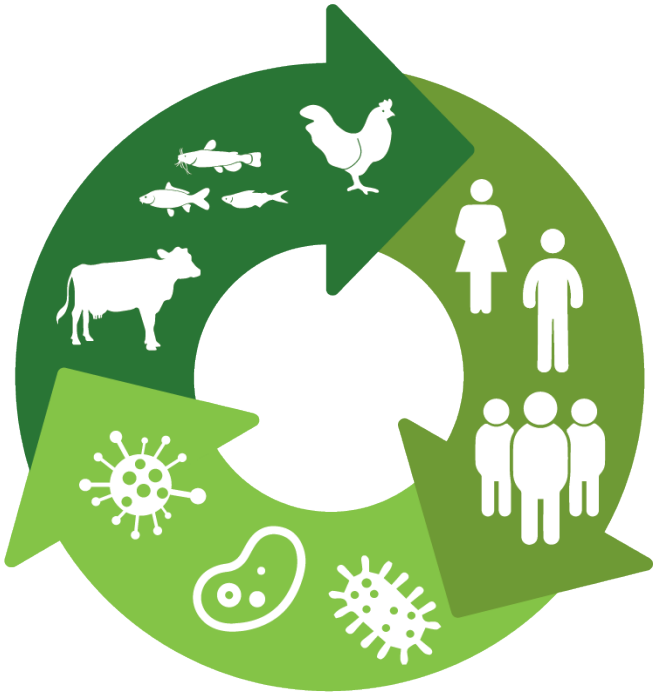
WorldFish

CGIAR

Objectives

Overall Objective

Development of improved antibiotic residues (AR) and antimicrobial resistance (AMR) surveillance in the aquaculture sector in Bangladesh



Specific Objective

Enhance skill and knowledge of enumerators on biological samples collection for fish, water, organic fertilizer and sediment. Samples will be used for antibiotic residues testing and for Antimicrobial Susceptibility Testing (AST) of key bacterial isolates recovered from those samples

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Aquatic food system and AMR

Nature of the problem

AMR can negatively affect the structure and sustainability of food production systems.

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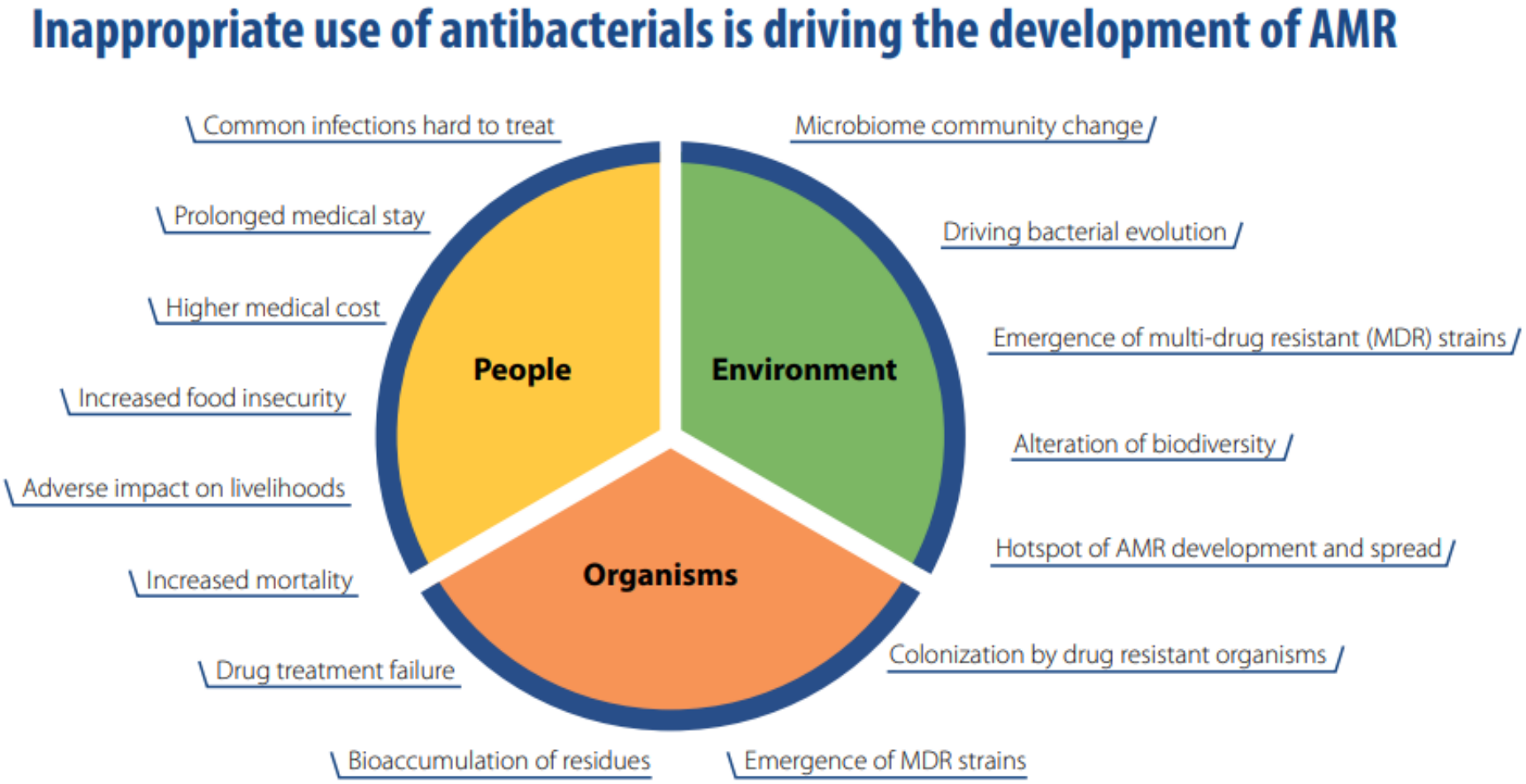
Aquatic food system and AMR



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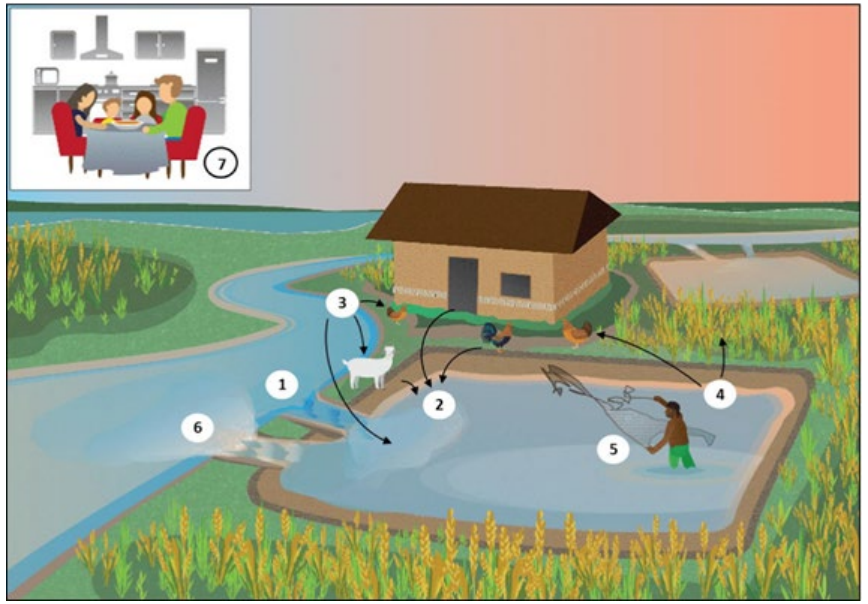
Aquatic food system and AMR

AMR spread from aquaculture

1. ABs from source water
2. Animal waste used as fertilizer can contain ARs and AMR pathogens
3. ABs can be added directly into the pond
4. ARs and AMR pathogens in pond sludge is used as fertilizer for chicken feed and crops
5. Humans have direct contact with pond environment
6. Wastewater is released into local water source
7. Fish along with AMR pathogens and ARs is consumed by humans



Source: Thornber et al. 2019.
https://doi.org/10.1111/raq.12367

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Biological sample collection for AR and AST

Sample collection

Collect biological samples from aquaculture farm including fish tissues, water effluent, organic fertilizer and sediment

For live animals, follow standard operating protocol for euthanasia

Farm pond

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Sample type & Analyses

Sample type	Analysis
Water effluent	Total bacterial load AST: E. coli; Salmonella
Organic fertilizer / Fish liver / Sediment	Antibiotic residues
Fish skin/gills	Bacterial isolation + AST: E. coli; Salmonella Genomic
Fish gut	Bacterial isolation + AST: E. coli; Salmonella Genomic

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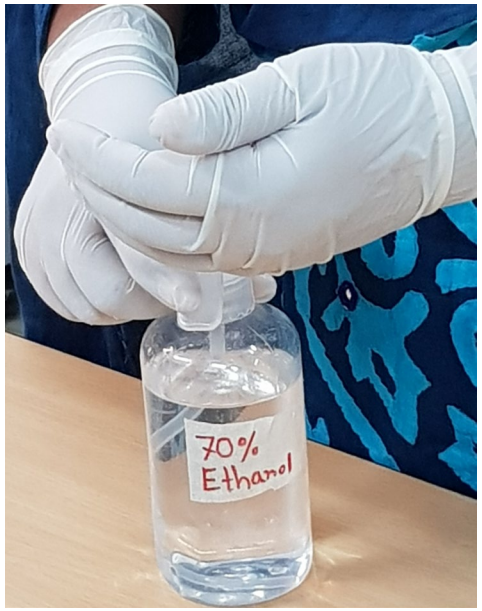
Aseptic techniques & cold chain

Disinfect hands and instruments before and after every sample collection



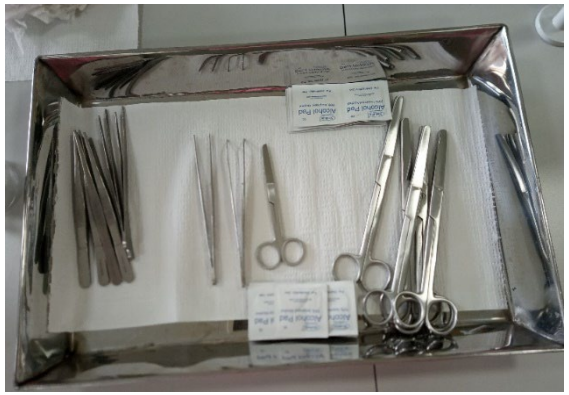
Keep samples in the ice box and transport to the lab with sample identification form

Fish necropsy for tissue samples collection

Sample processing on farm: Tilapia

Clean the dissecting tray with freshwater and disinfect with 70% ethanol.

Bring the fish on the dissecting tray



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Fish skin swab

Skin swab

Open a sterile cotton tipped swab by pulling out the stick part.

Collect skin surface swab by rubbing the cotton swab on different location of skin.

Put the swab stick in a test tube containing sterile Trypticase Soy Broth, vortex well and incubate at 37^0C for 24 hours.

Do not touch the swab tip prior to or after sampling

Centre for Environment Fisheries & Aquaculture Science

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Fish and shrimp necropsy for AST profiling

Body parts: skin, muscle and gills

Wipe mucus or debris from fish using a sterile tissue paper and disinfect the outer surface of fish by wiping properly using gauze pad soaked with 70% ethanol.

Place the fish on the dissection table so that the right side of the fish remains in contact with the table surface.

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Fish and shrimp necropsy for AST profiling

Using scalpel and forceps, carefully remove the skin of body part between dorsal and caudal fin (above the lateral line) of left side of the fish.

Collect approximately 50 g of muscle and keep in a container

Collect gills from both sides of head and keep in another container

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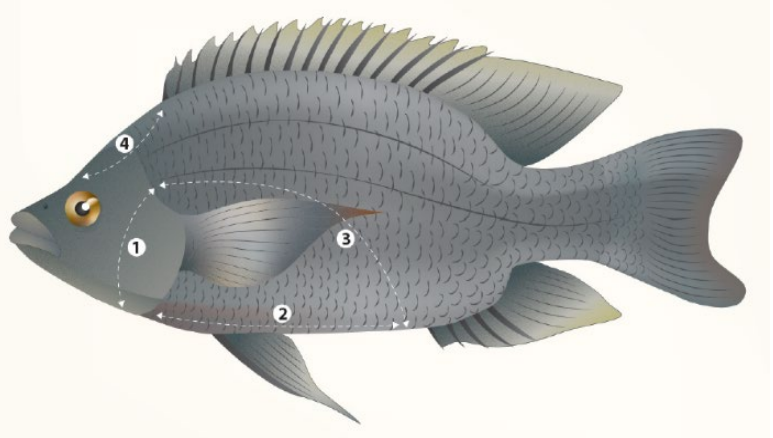
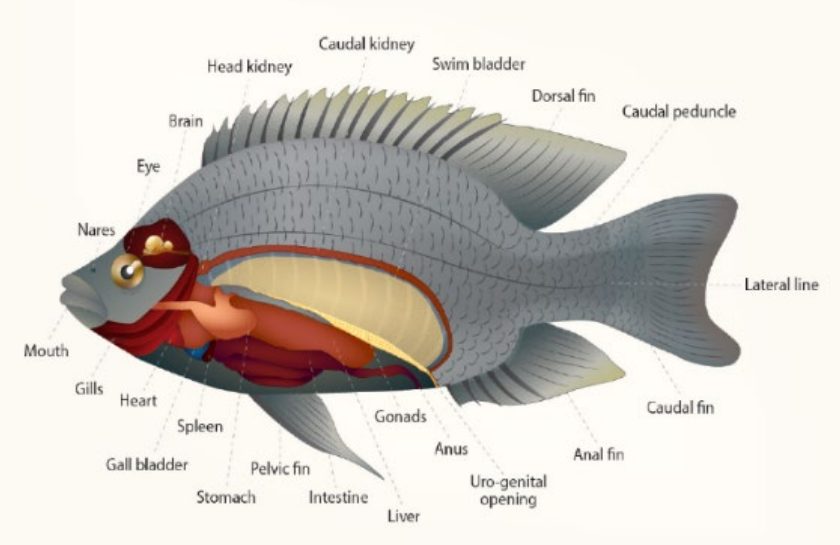
Fish and shrimp necropsy for AST profiling

Body parts: intestine, liver and other internal parts

Cut straight line of the belly of the fish from mouth to anus using sterile scissors or scalpel

Remove the liver and other organs carefully





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Fish and shrimp necropsy for AST profiling

Pull back the gut using sterile forceps.

Cut both ends (mouth and anus part) of the gut and collect it in another container

Do not cut or pierce the GI-tract

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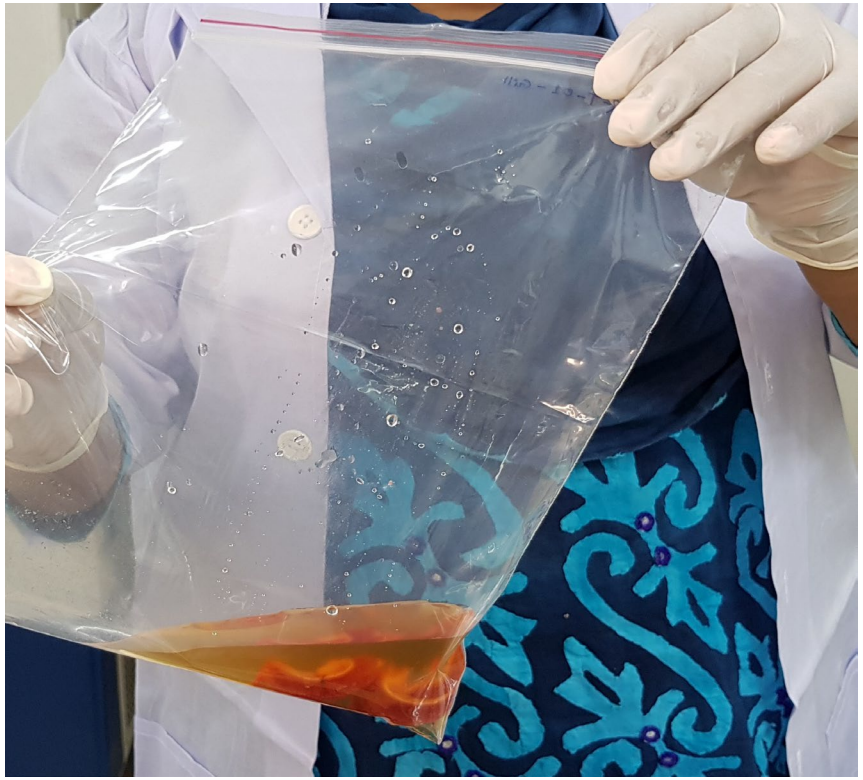
Enrichment, isolation and identification

Enrichment

- Add 25 g sample (e.g., tissue, gills, gut, meat of fish or shrimp) to 225 mL sterile Trypticase soy broth in a stomacher bag
- Homogenize using stomacher for 1-2 minutes
- Hold 60 minutes at room temperature, incubate at 35-37^{0}C for 18 hours aerobically



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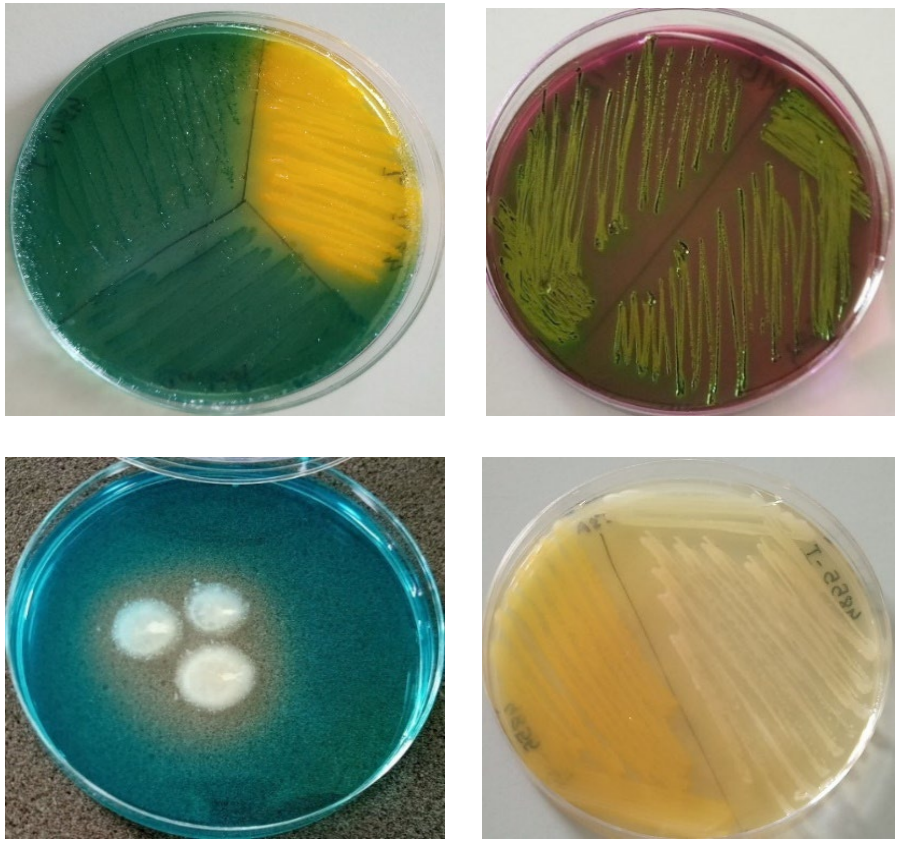
Enrichment, isolation and identification

Isolation and identification

Targeted Bacteria

- *Vibrio parahaemoliticus* (VP)
- *Escherichia coli*
- Group B *Streptococcus*
- *Salmonella enterica*



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Enrichment, isolation and identification

Targeted Bacteria	Selective Media	Incubation Condition	Colony Characteristics
Vibrio parahaemoliticus	Thiosulfate-citrate-bile salts-sucrose (TCBS) agar	35-37°C for 18-24 hours, aerobically	*V. Parahaemolyticus*: green *E.coli* : translucent
E. coli	MacConkey agar and Eosin Methylene Blue (EMB) agar	35-37°C for 18-24 hours, aerobically	On MacConkey Agar- *E.coli* : small pink *Klebsiella spp*. : large gummy whitish pink On EMB- *E.coli* : metallic green color
Group B *Streptococcus*	Blood Agar, TSA	35-37°C for 48 hours, anaerobically	On Blood Agar: beta hemolysis (clear zone around the colony) On TSA: small white colonies
Salmonella enterica	Xylose Lysine Deoxycholate agar (XLD)	35-37°C for 18-24 hours, aerobically	*Salmonella spp*: lightly transparent red halo with a black center surrounded by a pink-red zone *E. coli* : yellow

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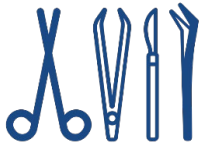
Checklist: Table of sampling materials by protocol

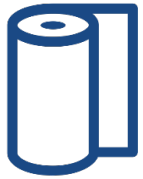
Bacteriology

Aluminium fold



Sterile dissection kit

Anaesthetic

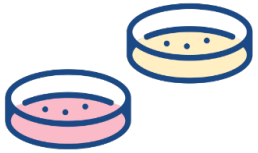


Paper towels

Normal storage and transportation

Portable Bunsen burner

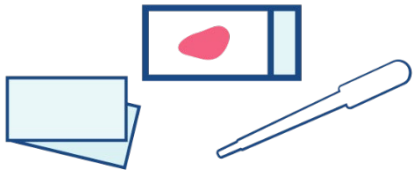
70% ethanol (normal grade)



Trypticase soy agar (TSA), blood agar, etc

Loops and cotton swab*

Bacteriology transport swab**



Microscope slides, cover slips and plastic pasteur pipette

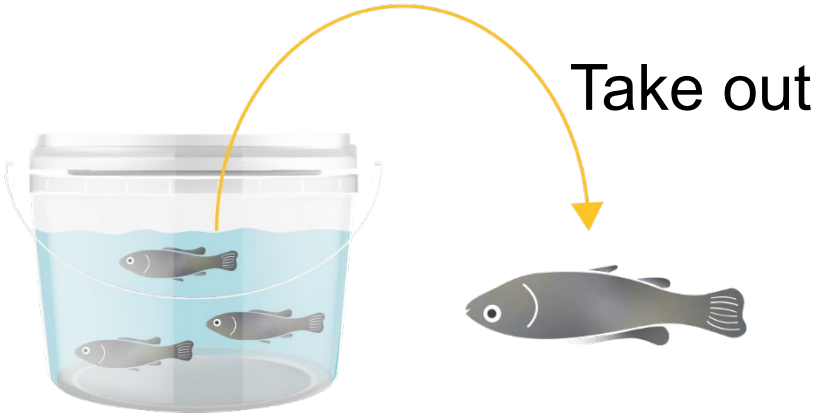
Gram stain reagents

Bacteriology sampling guide

Bacteriology is the culture and identification of bacteria growing under specific conditions. Standard bacteriology is a swab taken from the caudal or anterior kidney and inoculated onto agar (e.g. Tryptone soya agar). Additional swabs may be included skin swab or from external or internal lesions/ulcers (e.g., eye, liver, spleen, brain).

Bacteriology sampling guide

1. Euthanize fish according to standard operating procedure.

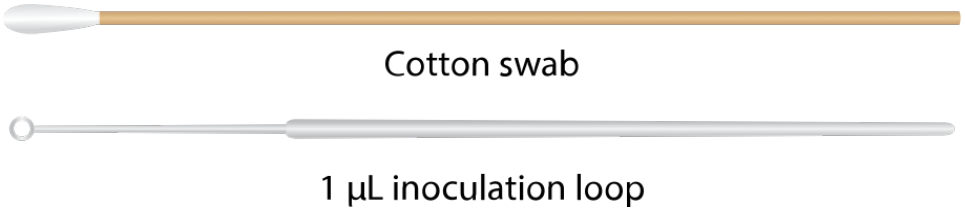


2. Place fish on a clean surface and spray with 70% ethanol. Leave to dry.

Bacteriology sampling guide

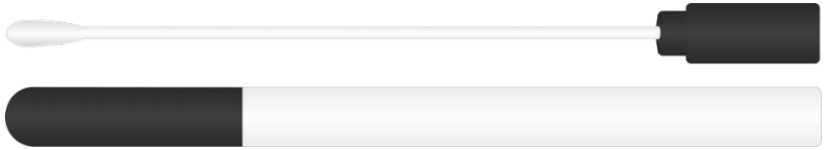
3A Sampling from ulcer:

1. Clean surface of the ulcer with an ethanol wipe.
2. Select one ulcer per fish. Expose edge of ulcer by removing scales or sharp incision.
3. Insert sterile cotton swab or 1 µL inoculation loop directly into and behind the ulcer.
4. Inoculate biological materials from cotton swab onto selective or non-selective agar plate (e.g. Tryptic soy agar TSA).



Alternatively, sampling can be done using bacteriology transportation swab(s)

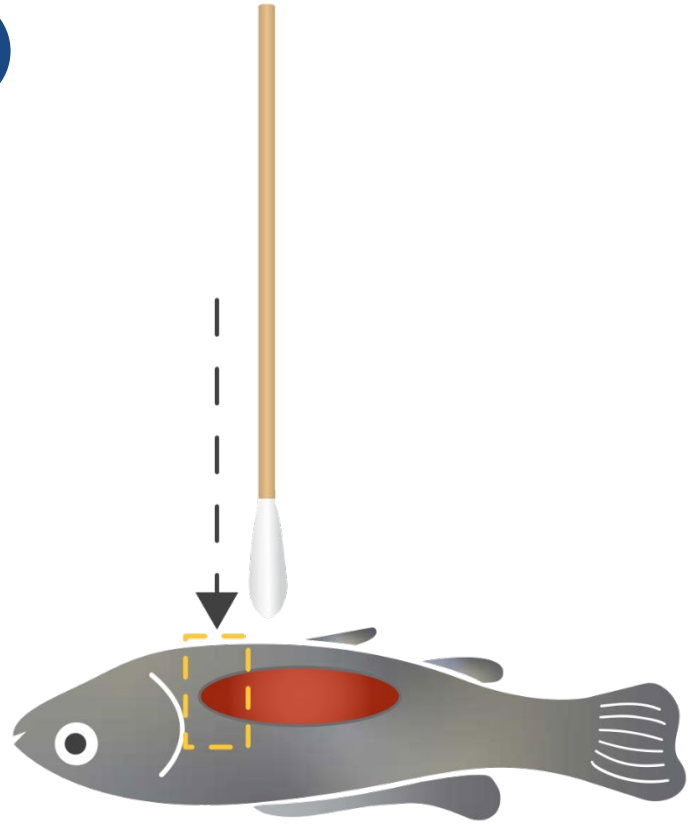
(Transwabs) using the same technique as described in 3a. Transwabs need to be transported chilled and plated onto non-selective or selective media within 48 hours.



Charcoal absorbs toxins and is better for fastidious organisms.

Bacteriology sampling guide

1



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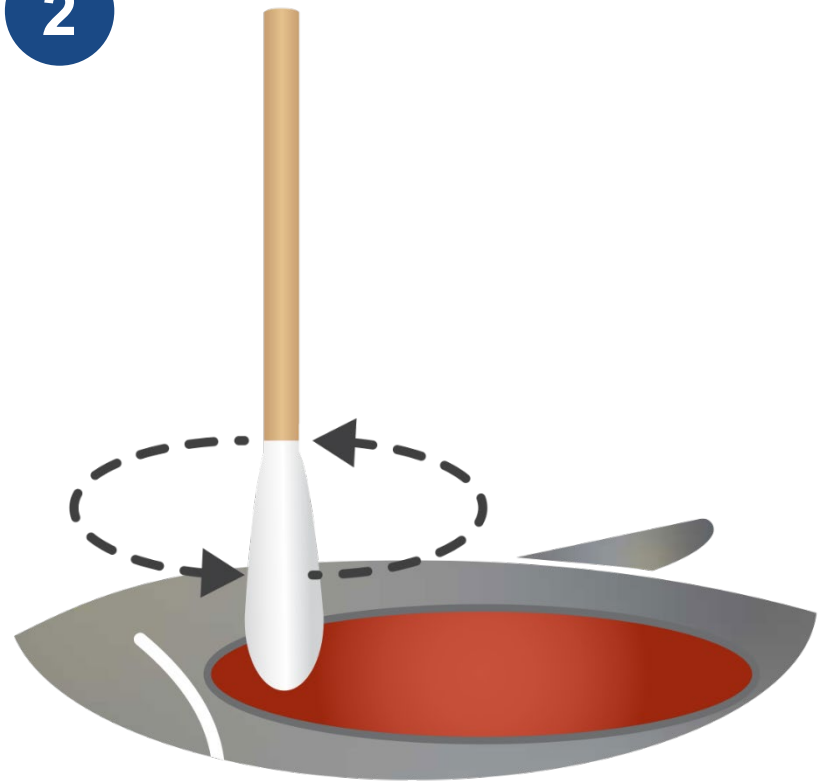
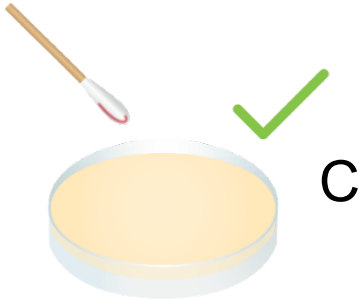
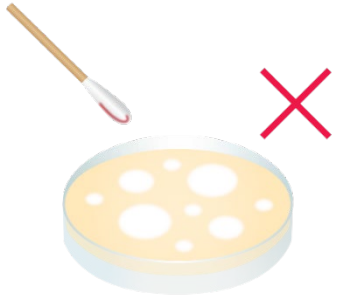


Bacteriology sampling guide

3



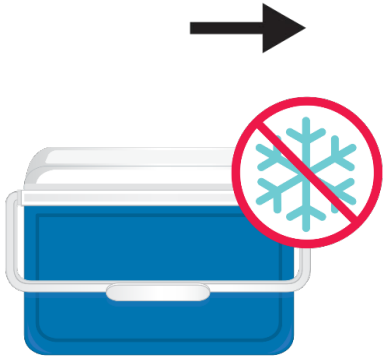
Clean TSA plate



Contaminated TSA plate

Prepare smear slide from target tissue or lesion/ulcer.

Ulcer being scraped with scalpel blade.



Scraped material being placed on slide then air dry 3–5 min.

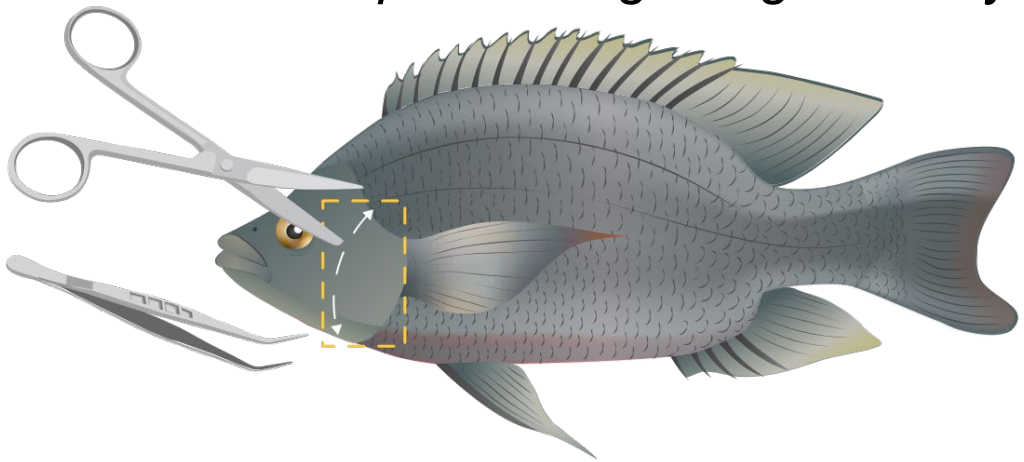
Pack and transport without cool pads.

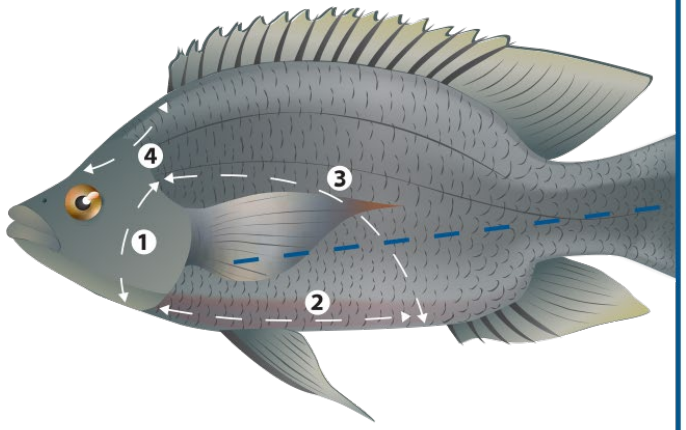
Bacteriology sampling guide

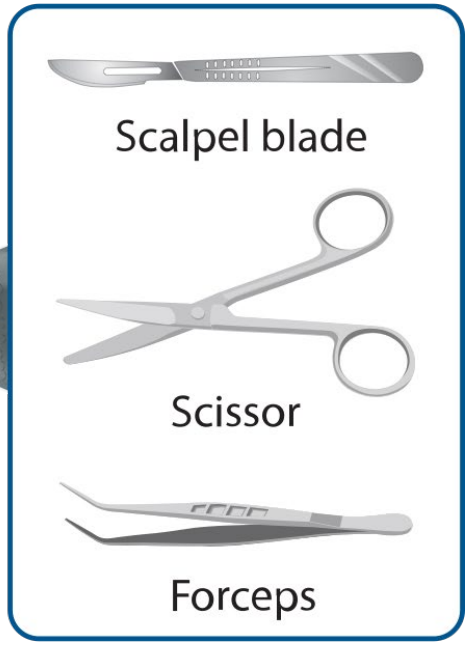
3B Sampling from kidney (head or caudal):

1. Cut away the operculum.
2. Make a ventral incision from anus toward the gills.
3. Finish the triangle opening to expose the abdominal cavity.

Caution: Avoid perforating the gut or any other organs.



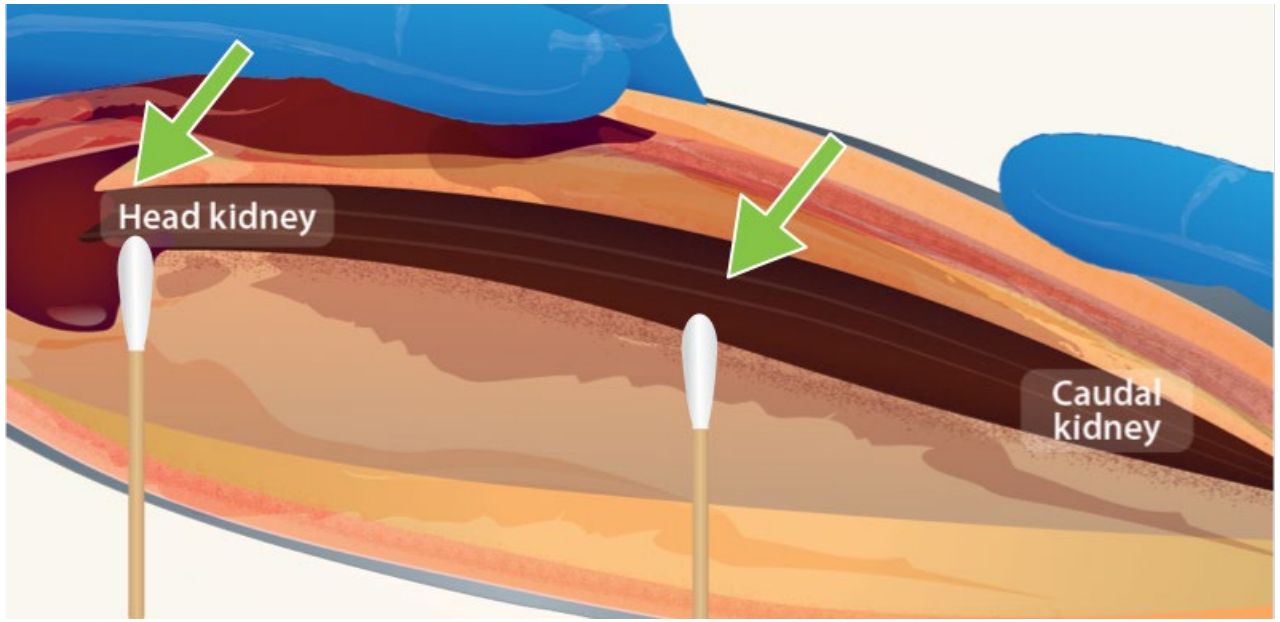




Bacteriology sampling guide

To sample caudal kidney:

- Pull out viscera with flat side of a clean scalpel.
- Puncture membrane to expose the caudal kidney using swab tip.



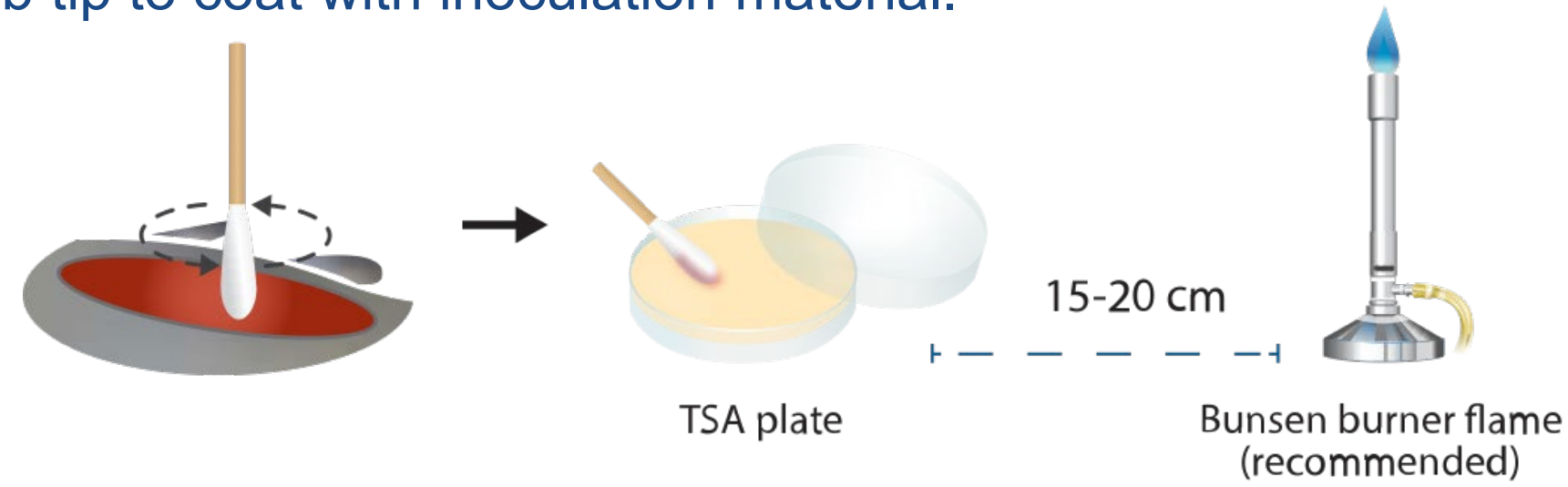
To sample head kidney:

- Remove gills or other organs using forceps, scissors or scalpel.
- Puncture membrane to expose head kidney using swab tip.

Bacteriology sampling guide

Inoculate with agar side facing down near the flame (15–20 cm). If using plates in the field, inoculate the plate in a side-to-side motion, zig-zagging down the plate. If using transwabs, when back at the lab streak plates following patterns below. On suspicion of a bacterial disease, use one plate per fish (A) or, during routine screening, one plate split in two for two fish (B). In the lab, to maximize dilution, use a new sterile loop after stroke one and two.

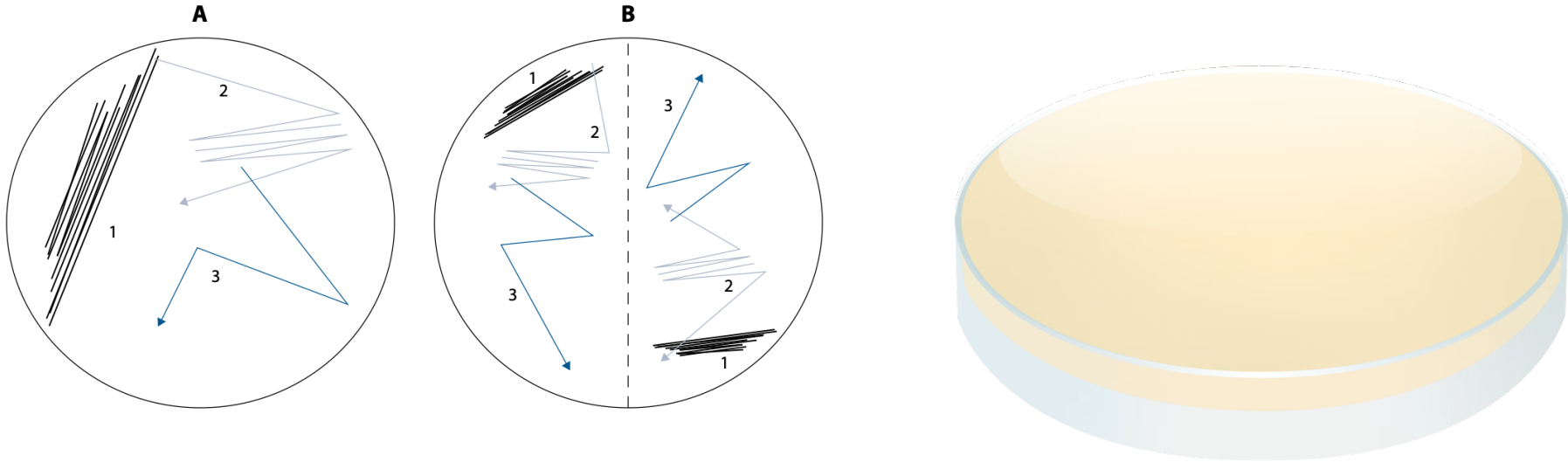
Rotate swab tip to coat with inoculation material.



Bacteriology sampling guide

Seal agar plates with parafilm. Place upside down below 30°C (as per revised protocol); return to the lab and incubate for 12–72 hours to check for bacterial colony forming units growth.

In the presence of clinical signs or depending on the targeted bacterial disease, sampling for bacteriology can be done from other organs (e.g. brain, eye, liver, heart).



Bacteriology sampling guide

4 Onsite or back in the lab, perform Gram stain from bacterial culture g 4 rowing on agar or fish tissue imprints (kidney, brain etc.). For fish <5 cm, perform Gram staining from histological material.

Suspension of bacterial culture from agar plate

Crystal violet (1–2 min), then tip off

Lugol's iodine (add for half the time of crystal violet, 30 sec–1 min)

95% ethanol or acetone

Safranin or neutral red (2 min minimum)

Dry slide with stained specimen for 5–10 min.

Examine under microscope using 100x oil immersion objective to determine morphology and color of Gram-positive (purple) and/or Gram-negative (pink) bacteria.
*With immersion oil for gram stain observation (bacteriology)

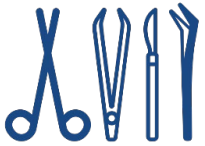
Tissue imprints

Tissue imprints can also be Gram-stained, e.g. kidney, but not ulcer smears (as almost certainly mixed bacterial species are present).

Checklist: Table of sampling materials by protocol

Molecular

Aluminium fold

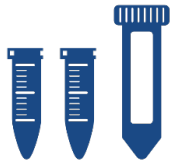


Sterile dissection kit

Anaesthetic

Paper towels

Normal storage and transportation



Sterile tubes

95%–100% ethanol (molecular grade)

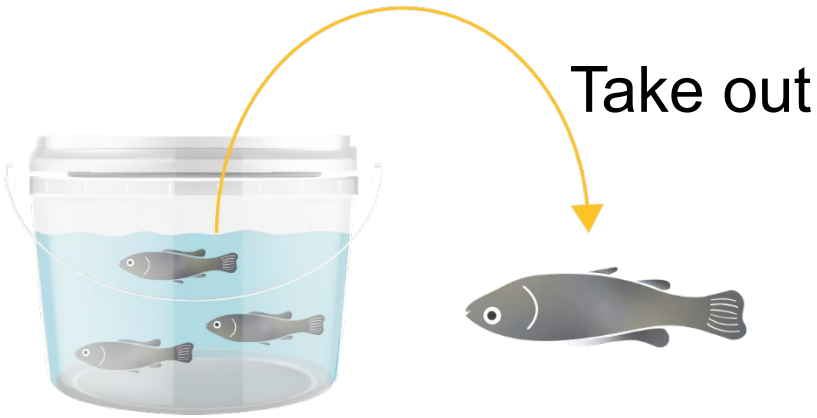
RNA stabilization solution

Molecular diagnostics sampling guide

On suspicion of an unknown disease during abnormal mortalities, it is routine for the investigators to collect clinical samples from moribund fish for molecular and virology diagnostics. Molecular diagnostics are techniques used to amplify small DNA/RNA sequence(s) that are unique to a particular pathogen to ascertain their presence or absence. Virology is a branch of microbiology that study viruses and viral diseases. Standard specimens for general molecular and virology health evaluation include kidney, liver, spleen, brain and gills. Other tissues may be collected.

Molecular diagnostics sampling guide

1. Euthanize fish according to standard operating procedures.



2. Place fish on a clean surface and spray with 70% ethanol. Leave to dry for no more than 15–30 sec, otherwise you risk degrading the internal tissues.

Molecular diagnostics sampling guide

3A

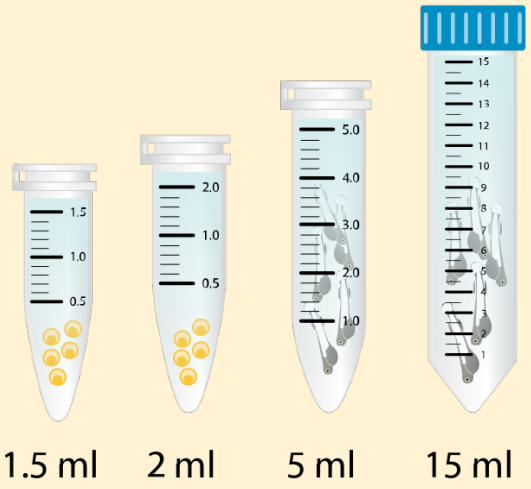
A. For eggs, sperm, fertilized eggs and swim-up fry 1–2 cm long, use whole specimens without dissection.

B. For >2 cm fry, slice open abdomen with sterile scalpel blade, and keep entire fish for diagnostic.

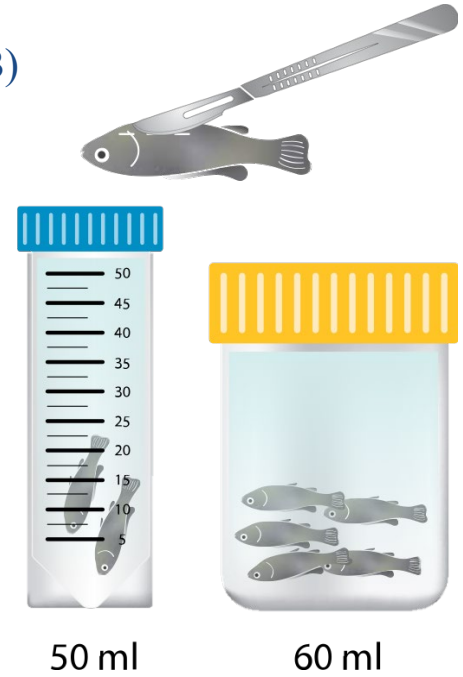
Place (A) or (B) into 100% molecular grade ethanol
(molecular diagnostic) or transport media (virology diagnostic).

(A)

For eggs/fertilized eggs or swim-up fry, place have entire specimens per tube (maintain ratio of tissues to ethanol at 1:10)



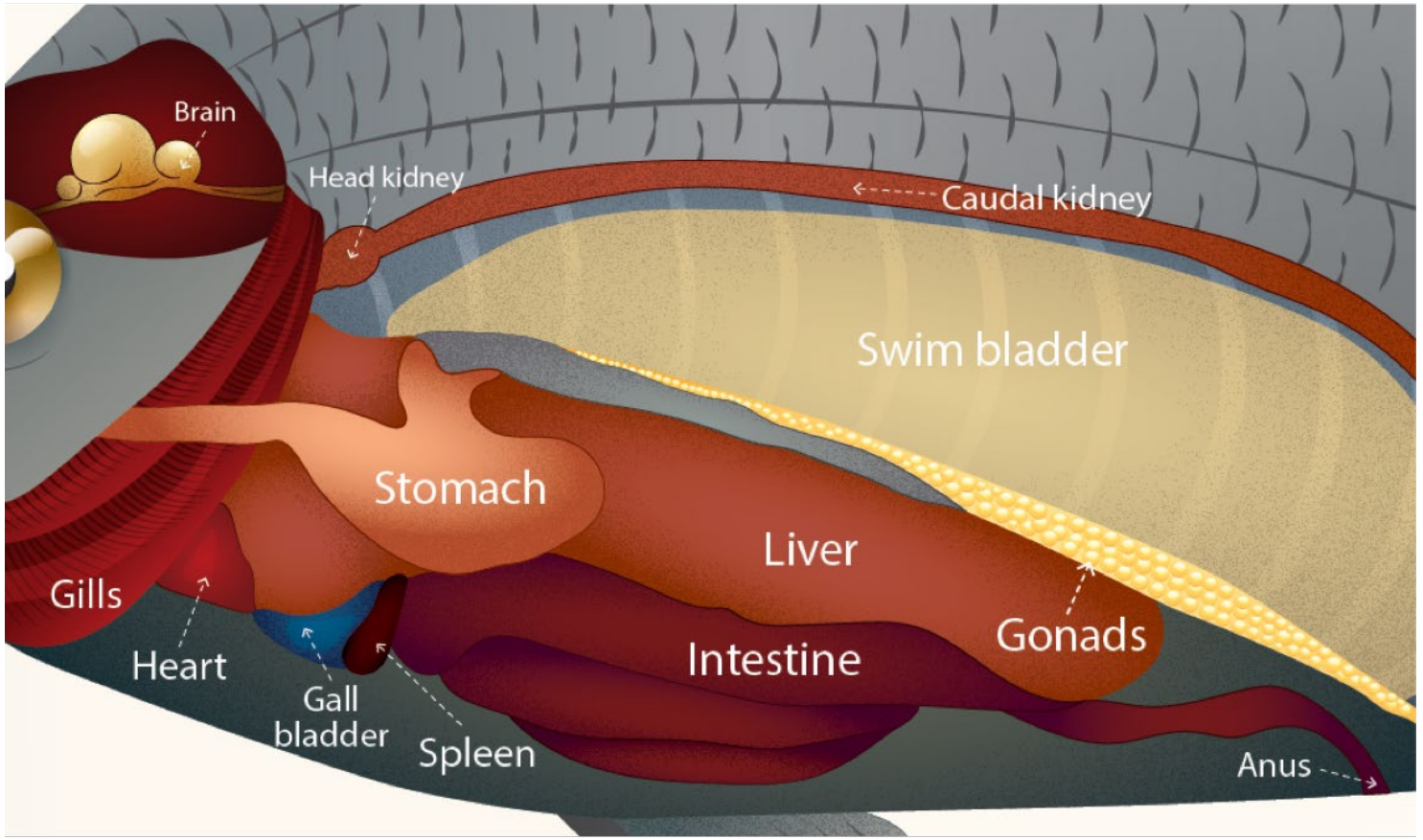
(B)



Use appropriate tube size based on amount/volume of specimens needed

Molecular diagnostics sampling guide

3B For fish under >5 cm, make an incision to expose the abdominal cavity.



Molecular diagnostics sampling guide

Dissect tissue sections (5 x 5 mm) from target organs (individually or pooled).

Molecular diagnostics sampling guide

Place specimens in 95% ethanol (molecular) or transport media (virology) at a ratio of specimens to fixative of 1:10.

Molecular samples

Pack and transport to lab without cool packs

Virology samples

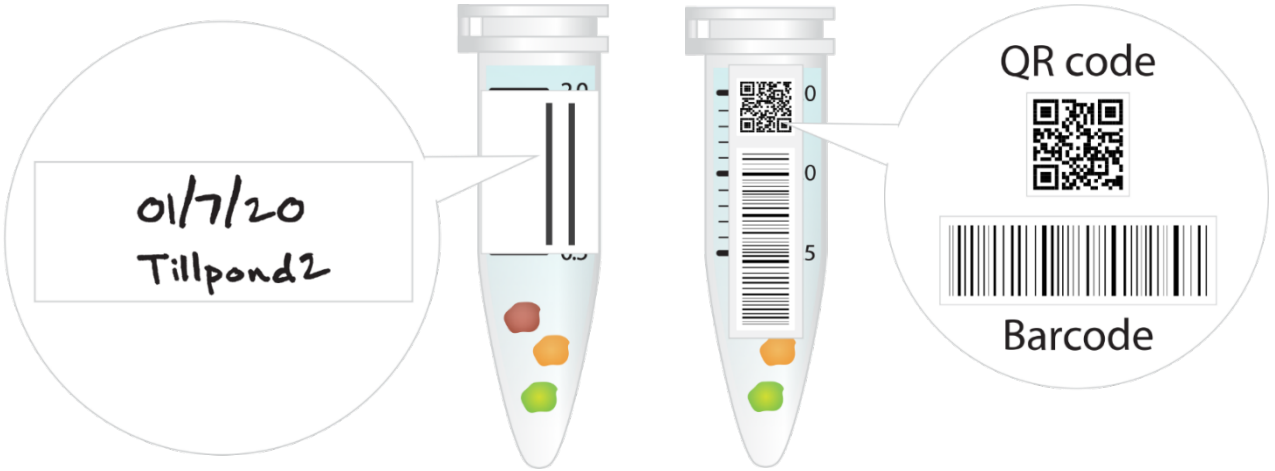
Place in insulated transport box with 3–4 cool packs (<10°C)

Molecular diagnostics sampling guide

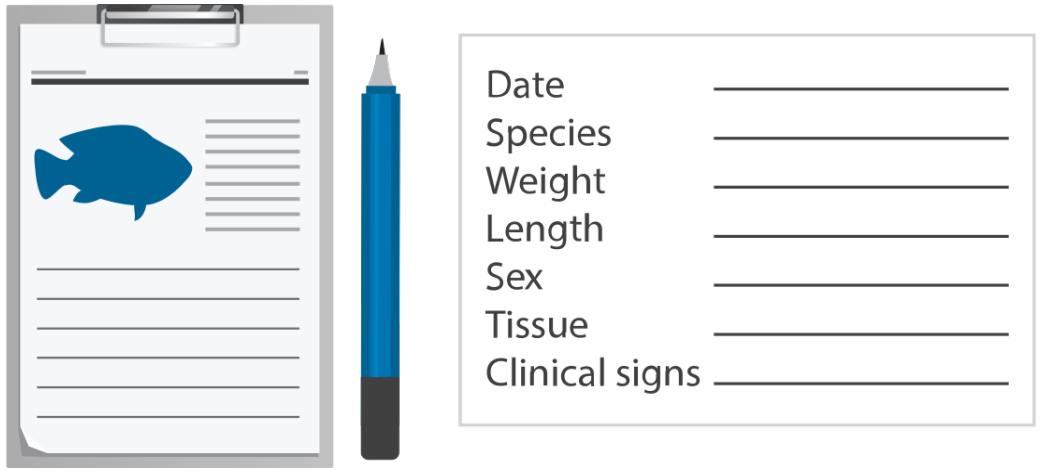
4 A. Label tubes using a solvent-resistant permanent marker pen or ideally using pre-printed barcodes.

B. Record all relevant sample details on the fish health examination and sample record form—i.e. date, species, weight, length, sex, tissues collected, clinical signs, etc.

(A)



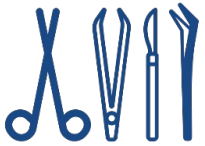
(B)



Checklist: Table of sampling materials by protocol

Histopathology

Aluminium fold

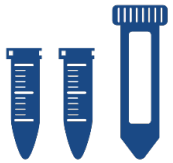


Sterile dissection kit

Anaesthetic

Paper towels

Normal storage and transportation



Sterile tubes

70% ethanol (normal grade)

10% NBF

Histology pot

Histology sampling guide

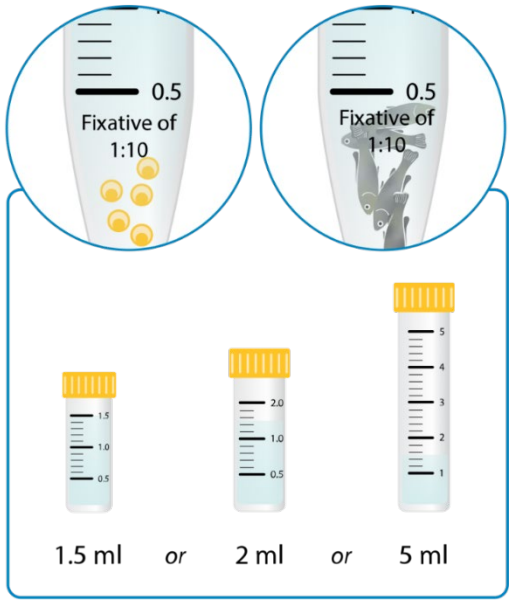
During investigation of an abnormal mortality event, fish tissues (or biopsies) for histopathology analysis are collected for disease diagnosis. Histology consists in the preparation of thin, stained tissue sections for microscopic examination to study their structure and function. Histopathology is the study of disease and disease processes by looking at the change in the anatomy or anomalies from cells, tissues and organs as seen through a microscope. WorldFish and partners developed this quick fish-sampling guide for histology. Standard biopsy specimens for histological examination consists of fixed sections of brain, gill, heart, intestine, kidney, liver and spleen. Other tissues may be collected in the presence of external lesions/ulcers (e.g., eye, skin, muscle).

Histology sampling guide

Step 1: Sample collection

1 **For fry or eggs < 1cm:**

For swim-up fry or fertilized eggs < 1 cm: Place 3–5 whole fry and/or eggs in 10% neutral buffered formalin (NBF) fixative at a ratio of fish to fixative of 1:10.

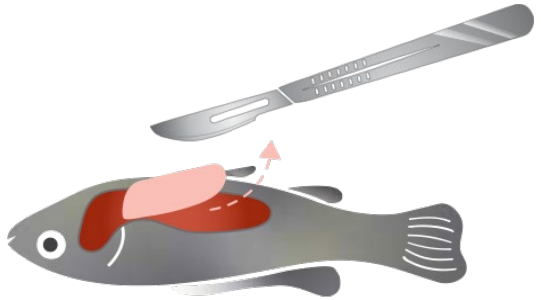


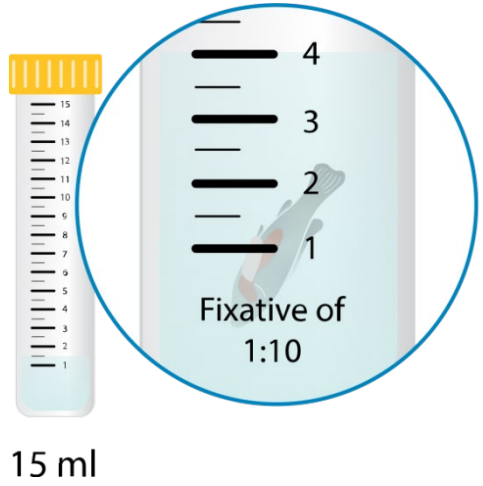
2 **For fingerlings 1–5 cm:**

Cut off gill opercula.

Open abdomen. Pull out viscera to protrude slightly from opening to expose internal organs. Place fish in 10% NBF fixative at a ratio of fish to fixative of 1:10.

Caution: Do not to cut the intestines.

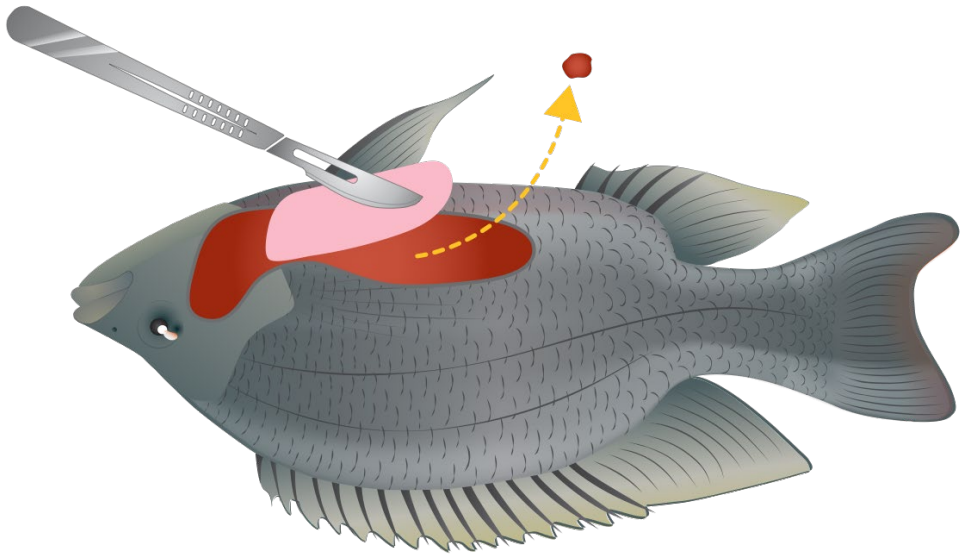


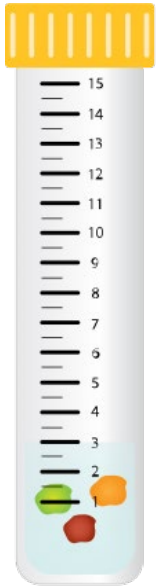


Histology sampling guide

3 **For fish > 5 cm:**

Dissect a 5 x 5 mm section (clean cuts) for each organ (take whole organ if < 5 mm thick). For standard histology, collect from brain, gill, eye, heart, pyloric caeca, stomach, intestine, spleen, liver, kidney and a representative lesion if any (see below for more details).





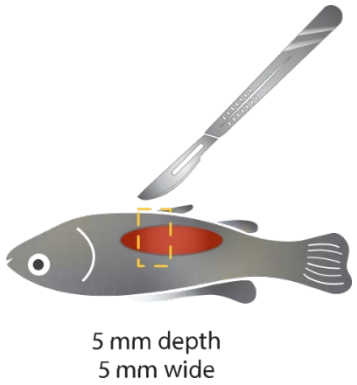
15 ml *or*

60 ml

Place organ specimens in 10% NBF fixative at a ratio of fish to fixative of 1:10.

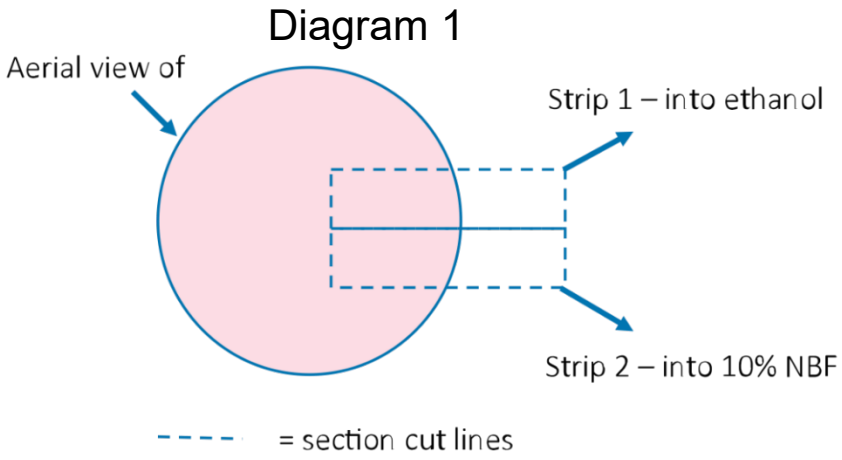
Histology sampling guide

Lesion sampling

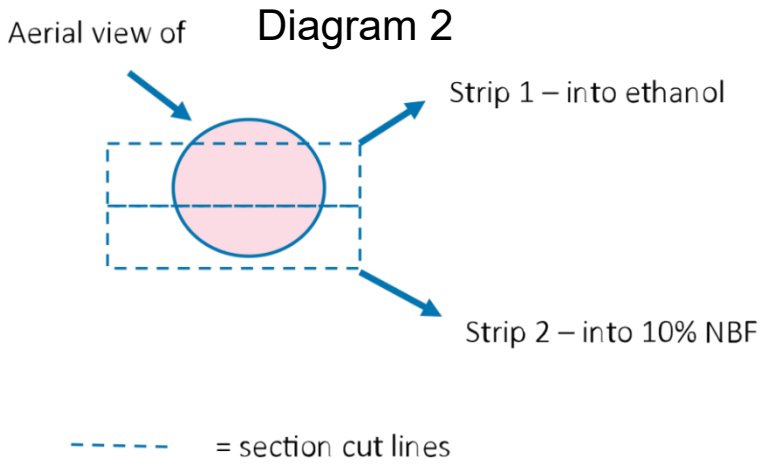


lesion sampling in addition to normal tissues

For large lesion:



For small lesion:



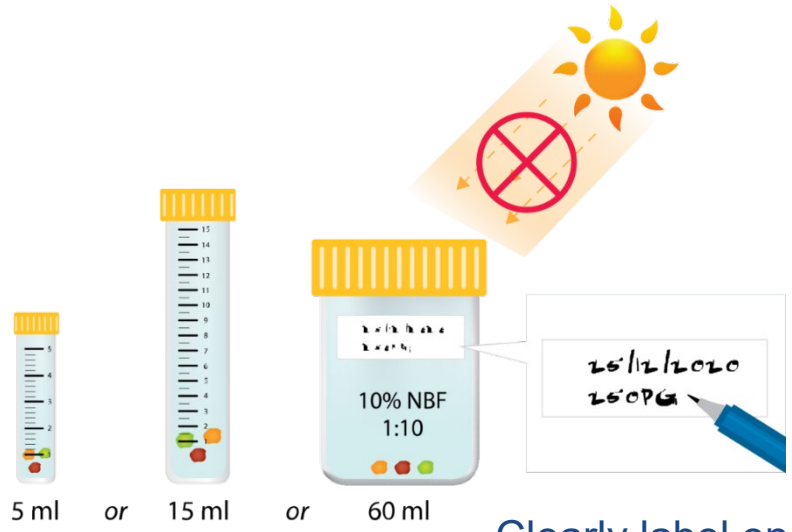
Place a strip into into 95%–100% ethanol ethanol for molecular diagnostic or in case of confirmed presence of epizootic ulcerative syndrome.

Histology sampling guide

Step 2: Fixation

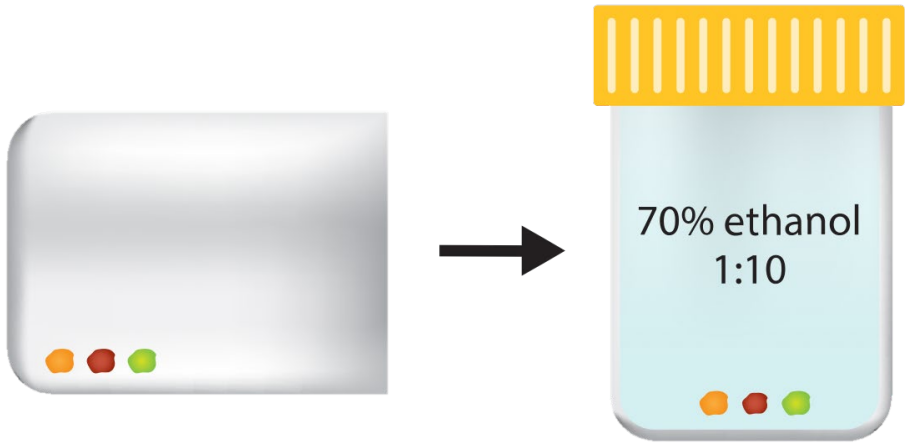
Immerse 5 x 5 mm tissue sections in 10% NBF fixative at a 1:10 ratio.

Store at room temperature away from sun for 24–48 hours.



Clearly label on the side of the tubes or pot (not the lid).

Step 3: End of fixation



Drain NBF and dispose safely

Store at 4°C

Note: Proceed with 70% ethanol for long-term storage or when doing specialist stains such as immunohistochemistry (IHC). For immediate processing, the 70% is not needed. Please note it is also harder to ship samples in alcohol, as it may require dangerous goods paperwork.

Useful references

Ali, H. et al. (2016). An assessment of chemical and biological product use in aquaculture in Bangladesh. *Aquaculture*, 454,199-209. https://doi.org/10.1016/j.aquaculture.2015.12.025

Uddin, S. A., & Kader, M. A. (2006). The use of antibiotics in shrimp hatcheries in Bangladesh. *Journal of Fisheries and Aquatic Science*, 1(1), 64-67. https://doi.org/10.3923/jfas.2006.64.67

Hinchliffe, S. et al. (2018). The AMR problem: demanding economies, biological margins, and co-producing alternative strategies. *Palgrave communications*, 4,142. https://dx.doi.org/10.1057/s41599-018-0195-4

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Lucy A. Brunton, L. A. et al. (2019). Identifying hotspots for antibiotic resistance emergence and selection, and elucidating pathways to human exposure: Application of a systems-thinking approach to aquaculture systems. *Science of the Total Environment*, 687, 1344–1356. https://doi.org/10.1016/j.scitotenv.2019.06.134

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Other WorldFish and partners AMR related outputs

- 2018: Tackling AMR in Bangladesh – a One Health approach. FAO. https://bit.ly/3Ct3AJb
- 2018: AMR in the Matrix – an irresponsible, irresistible parody. BARA Bangladesh AMR Response Alliance. https://bit.ly/3oBYqpo
- 2018: Unpacking factors influencing antimicrobial use in global aquaculture and their implication for management: a review from a systems perspective. Sustainability Science. https://doi.org/10.1007/s11625-017-0511-8
- 2018: An assessment of health management practices and occupational health hazards in tiger shrimp (*Penaeus monodon*) and freshwater prawn (*Macrobrachium rosenbergii*) aquaculture in Bangladesh. Veterinary and Animal Science. https://doi.org/10.1016/j.vas.2018.01.002
- 2018: Trade-offs related to agricultural use of antimicrobials and synergies emanating from efforts to mitigate antimicrobial resistance. Science Forum 2018 Case Study. Rome, Italy: CGIAR Independent Science and Partnership Council. https://bit.ly/3DzPNBX
- 2018: The role of infectious disease impact in informing decision-making for animal health management in aquaculture systems in Bangladesh. Preventive Veterinary Medicine. https://doi.org/10.1016/j.prevetmed.2018.03.004
- 2019: Rapid genomic detection of aquaculture pathogens (poster): https://hdl.handle.net/20.500.12348/3826
- 2019 Poster: AMFORA: Applying a One Health systems modelling to formulate strategies for mitigating the risks to human health of ABR in aquaculture. Royal Veterinary College University of London. https://hdl.handle.net/20.500.12348/2660
- 2019: WorldFish joins new research partnership to tackle global problem of antimicrobial resistance. WorldFish website. https://bit.ly/30ICKjt
- 2019: AMR Blog Story. CGIAR AMR hub website. https://bit.ly/3kQhPli
- 2019: Production without medicalization; https://hdl.handle.net/20.500.12348/3135
- 2019: Reducing antibiotic use in Bangladesh fish farming – securing our future through better practices. Developed by SAF at the University of Exeter in collaboration with WorldFish and FAO. Bangla version: https://bit.ly/3Ggc09c; and English version: https://bit.ly/303rIVC
- 2019: Why Antimicrobial Resistance (AMR) in aquaculture matters for the One Health approach, YouTube video abstract: https://bit.ly/3rHF31n; and poster: https://hdl.handle.net/20.500.12348/2753

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Other WorldFish and partners AMR related outputs

- 2020: Evidence for action: a One Health learning platform on interventions to tackle antimicrobial resistance. The Lancet Infectious Diseases; https://dx.doi.org/20.500.12348/4339
- 2020: Raising awareness of antimicrobial resistance in rural aquaculture practice in Bangladesh through digital communications: a pilot study; https://doi.org/10.1080/16549716.2020.1734735
- 2020: A New concept for Rapid Genomic detection of fish disease: https://www.youtube.com/watch?v=iFxbodO6Fos
- 2020: Combatting AMR in Aquaculture. Q&A in Nature Outlook. https://hdl.handle.net/20.500.12348/4570
- 2020: Evaluating antimicrobial resistance in the global shrimp industry. Reviews in Aquaculture. https://doi.org/10.1111/raq.12367
- 2020: Coevolutionary goverance of antibiotic and pesticide resistance. Trends in Ecology & Evolution. https://doi.org/10.1016/j.tree.2020.01.011
- 2020: Sustainable aquaculture through the One Health lens. Nature food; https://www.nature.com/articles/s43016-020-0127-5
- 2021: Bangladesh safe and sustainable aquatic food project – Embedding One Health to support aquatic food production (poster); https://hdl.handle.net/20.500.12348/4977
- 2021: Quick protocol for antimicrobial susceptibility testing (AST) in aquatic animal species from aquaculture and fisheries; https://hdl.handle.net/20.500.12348/4862
- 2021: Autogenous vaccination in aquaculture: A locally enabled solution towards reduction of the global antimicrobial resistance problem: https://doi.org/10.1111/raq.12633
- 2021: Planetary boundaries and Veterinary Services. World Animal Health Organization (OIE). https://hdl.handle.net/20.500.12348/4873
- 2021: Reducing disease risks in fish through better detection, management and prevention. WorldFish FISH CRP Program Brief. https://hdl.handle.net/20.500.12348/4833
- 2021: Characterizing antibiotics in LCA—a review of current practices and proposed novel approaches for including resistance. The International Journal of Life Cycle Assessment. https://doi.org/10.1007/s11367-021-01908-y
- 2021: System-thinking approach to identify and assess feasibility of potential interventions to reduce antibiotic use in tilapia farming in Egypt. Aquaculture; https://doi.org/10.1016/j.aquaculture.2021.736735

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Other WorldFish and partners outputs

- Sampling materials for fish disease diagnostics; https://hdl.handle.net/20.500.12348/4836
- Bacteriology sampling guide; https://hdl.handle.net/20.500.12348/4840
- Wet mount sampling guide (for ectoparasites & fungi) https://hdl.handle.net/20.500.12348/4837
- Microbiome sampling guide https://hdl.handle.net/20.500.12348/4838
- Blood sampling guide https://hdl.handle.net/20.500.12348/4839
- Molecular diagnostics sampling guide https://hdl.handle.net/20.500.12348/4841
- Histology sampling guide https://hdl.handle.net/20.500.12348/4842

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