Food and Agriculture Organization of the United Nations

SUSTAINABLE DEVELOPMENT GOALS

VIRTUAL COURSE

26 March to 15 April 2021

Design of an Active Surveillance for Tilapia Lake Virus (TILV) Disease and Its Implementation

TCP/INT/3707: Strengthening biosecurity (policy and farm level) governance to deal with Tilapia lake virus

Food and Agriculture Organization of the United Nations

SUSTAINABLE DEVELOPMENT GOALS

CHECKLIST #6

5 April 2021

Checklist 6: TiLV Diagnostics (Level I-II)

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TCP/INT/3707: Strengthening biosecurity (policy and farm level) governance to deal with Tilapia lake virus

Learning Objectives (Checklist # 6: Diagnostic testing)

- To understand the requirements and criteria for Checklist 6

- To select appropriate diagnostic levels for TiLV active surveillance for suspected and confirmed cases based on case definition (Checklist 5) in recognition of the importance of sensitivity and specificity.

TiLV Diagnostic (Level I)

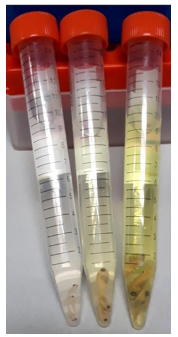
Observation of animals and environment

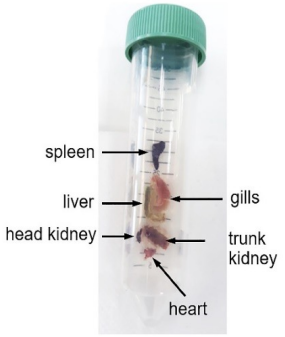
affected farm

unaffected farm

Gross signs (external and internal)

Preserve representative specimens for further optional analysis (Level II & III)





Histology/ISH

PCR TEM

Fresh/frozen (Cell culture)

Observation of Animals and Environment (Level I)

Host

- Fish species/strain/size
  - ☑ Nile tilapia (Chitralada/GIFT/hybrid)
  - ☑ Hybrid red tilapia
  - ☑ Giant gourami
  - ❑ Other ……..
- Mortality pattern
  - ☑ within 1-4 weeks after stocking
  - ☑ abnormal, non-stop for 5-7 days
  - ☑ Infectious
- Abnormal symptoms
  - ☑ Losing appetite
  - ☑ Stopped schooling
  - ☑ Stopped eating
  - ☑ Lethargy
  - ☑ Gather on water surface & corner of the pond/cage

Environment

- Water parameters
  - ❑ DO……
  - ❑ pH……
  - ❑ T^o……
  - ❑ NH_3……
  - ❑ Other……
- Weather/season

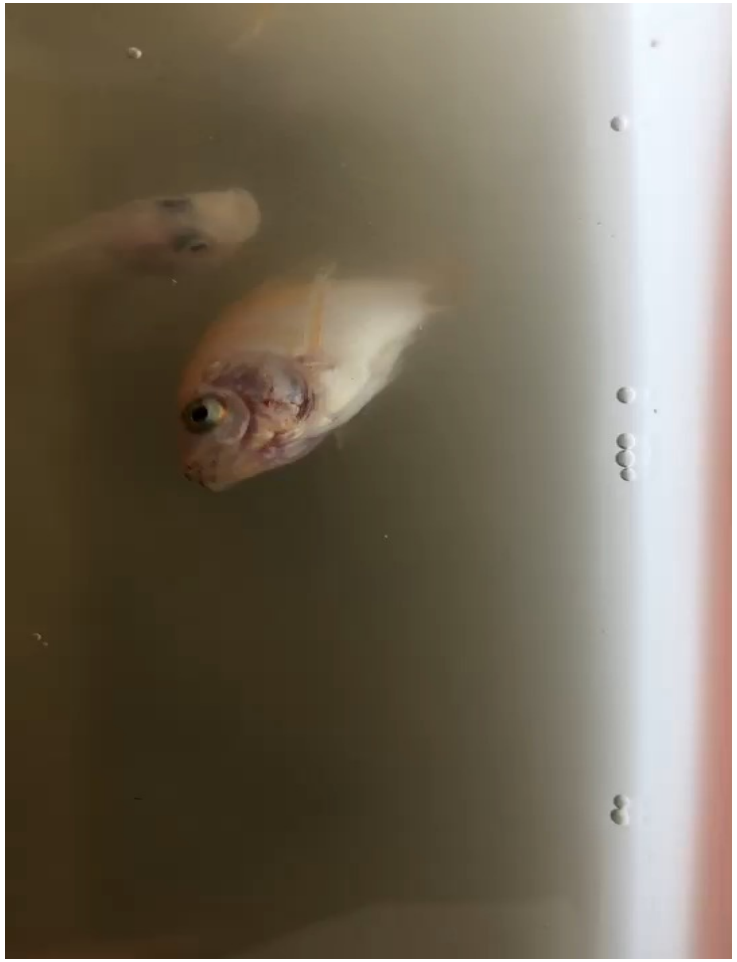
What meet the criteria of case definition in checklist 5?


Healthy population
schooling


TiLV infected population
Pale color
Pale color
stopped schooling
losing appetite


lethargy
darkening
TiLV infected population

Observation of Animals and Environment (Level I)



Dinh-Hung et al. 2021 J Fish Dis

Gross Clinical Examination (Level I)

From natural outbreaks in Ecuador and Israel

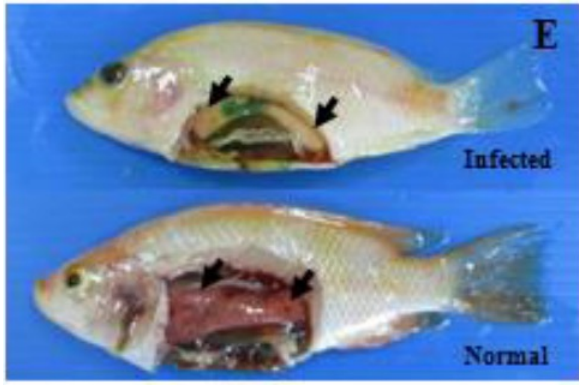
- Darkened body, abdominal distension
- Scale protrusion
- Exophthalmia
- Gill pallor
- The fluid in the abdominal cavity was watery and colourless

- Shrinkage of the eye and loss of ocular functioning
- Dermal erosions and ulcers

Ferguson et al. J Fish Dis 2014, 37, 583–589
Eyngor et al. J Clin Microbiol 2014, 52, 4137-46

Gross Clinical Examination (Level I)

From natural outbreaks in subsequent reports



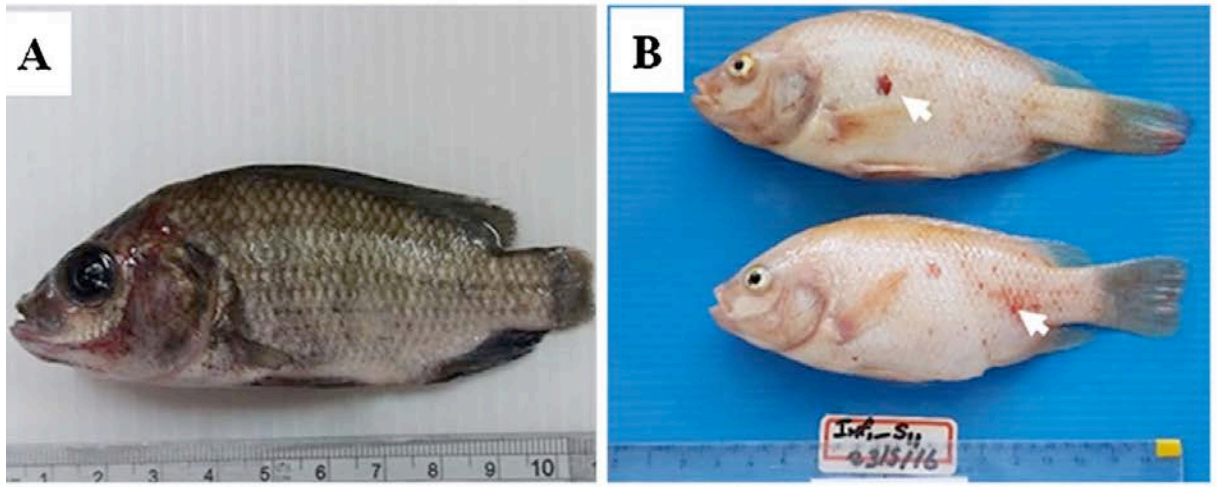
- discoloration
- loss of scales
- skin erosion
- reddish opercula
- skin hemorrhage
- congestion in the brain
- pale/green/watery liver
- abdominal distension
- Ascites
- Swollen spleen

Surachetpong et al. 2017 Emerg Infect Dis
Behera et al. 2018 Aquaculture
Amal et al., 2018 Aquaculture
Jansen et al. 2019 Review in Aquaculture
Dong et al., 2017. Aquaculture

Gross Clinical Examination (Level I)

Clinical signs from experimentally infected fish

- ✓ exophthalmia
- ✓ abdominal swelling
- ✓ scale protrusion

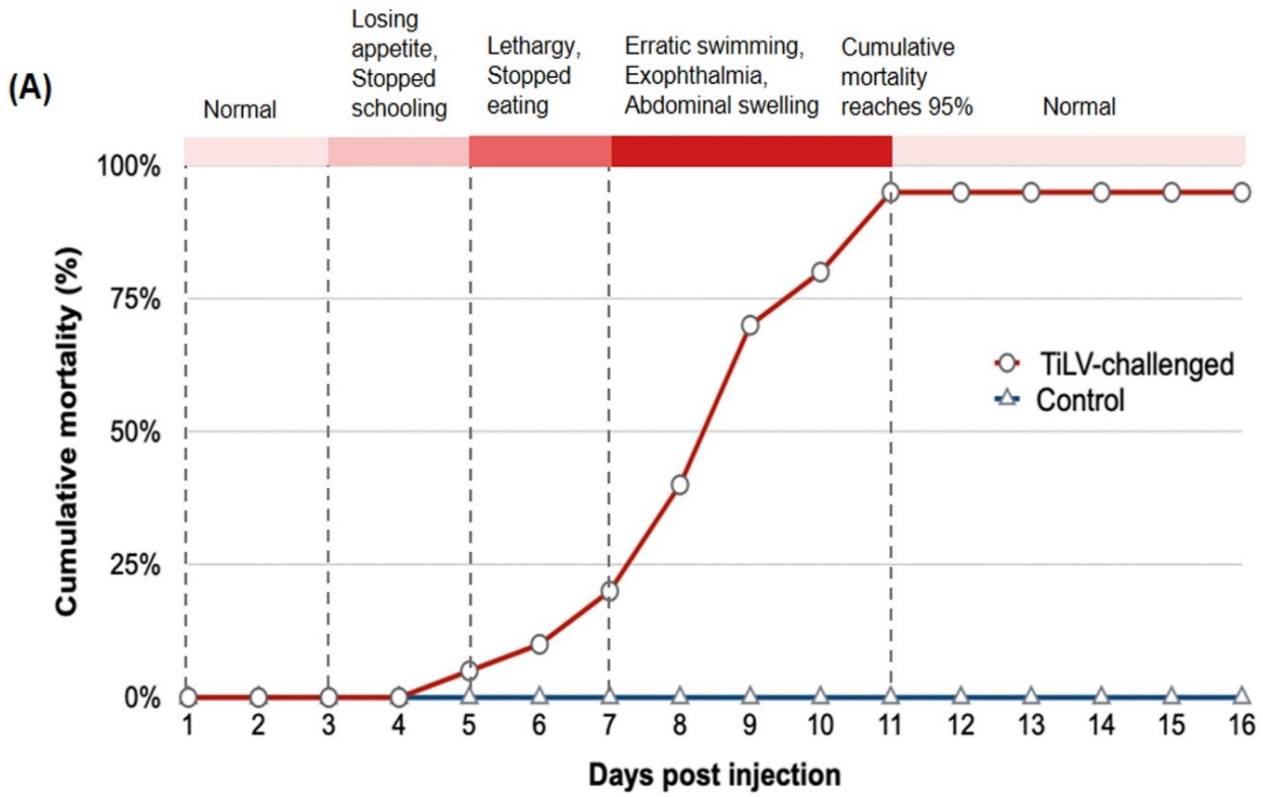


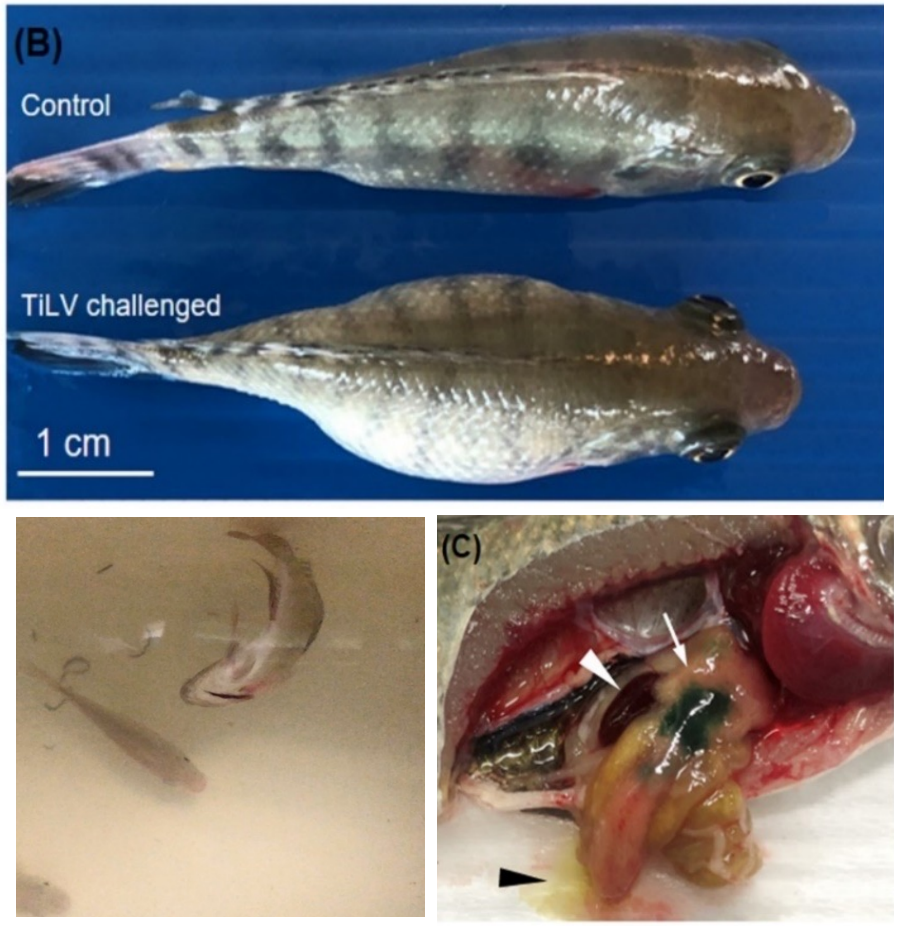
- ✓ skin erosion and hemorrhage
- ✓ skin redness
- ✓ mild exophthalmos and abdominal swelling

Tattiyapong et al. 2017 Vet Microbiol
Behera et al. 2018 Aquaculture

Gross Clinical Examination (Level I)

Clinical signs from experimentally infected fish

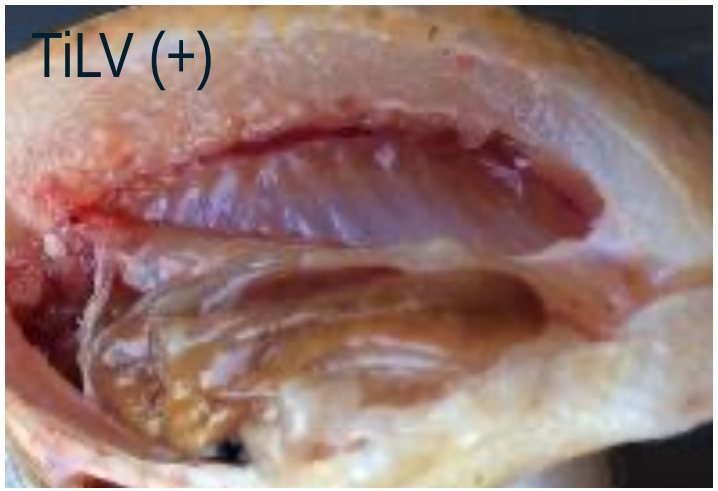


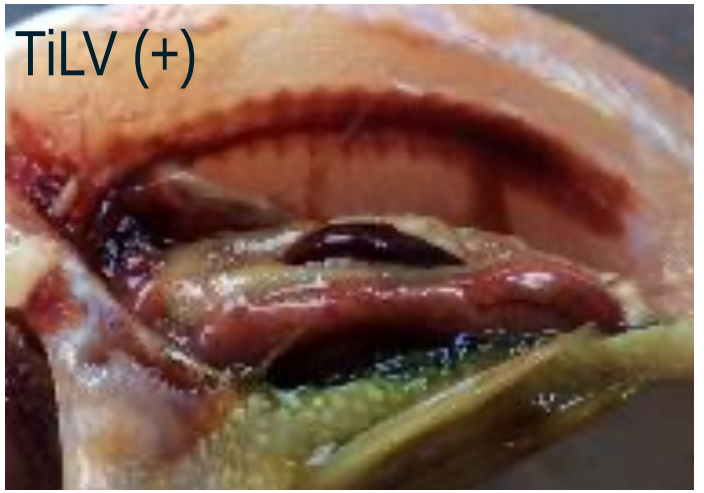


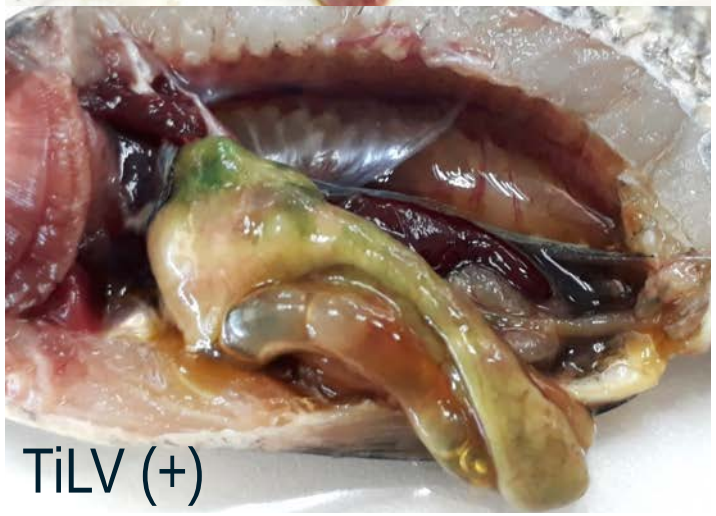
Dinh-Hung et al. 2021 J Fish Dis

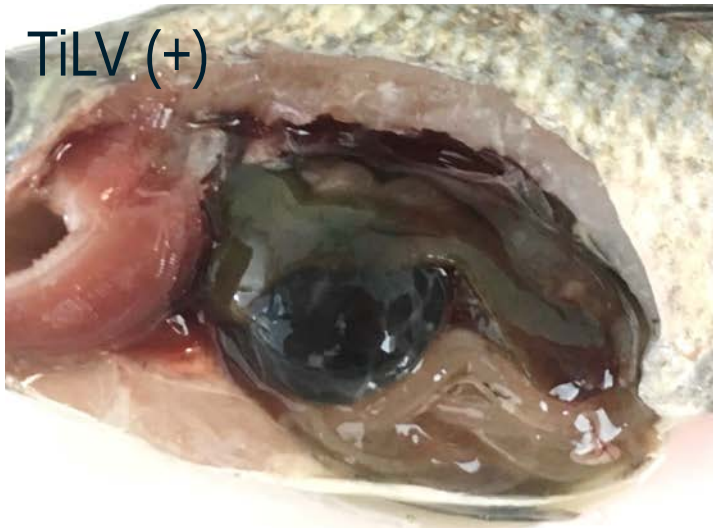
Gross Clinical Examination (Level I)

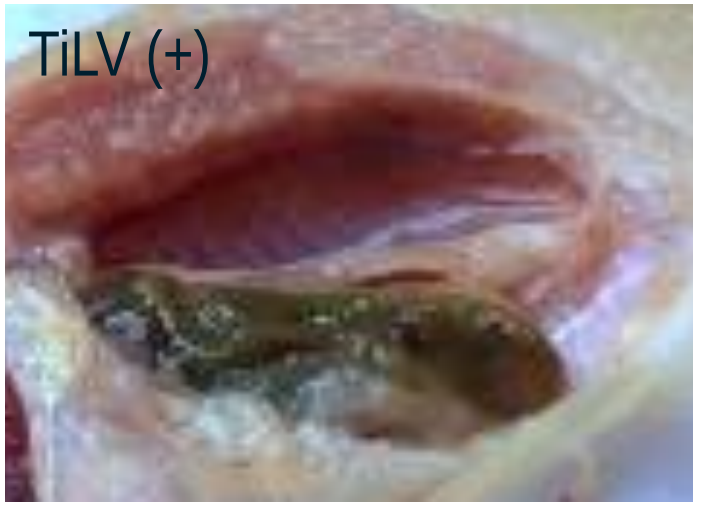
Can the fish liver tell you something about TiLV?











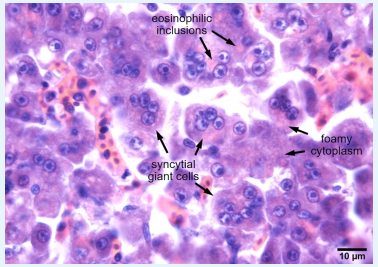
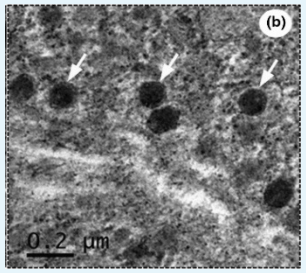
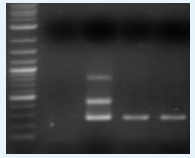
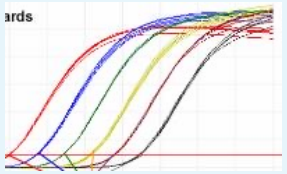
Summary gross signs reported for disease caused by TiLV

- ❑ darkening
- ☑ abdominal distension
- ☑ scale protrusion
- ❑ exophthalmia
- ❑ skin discoloration
- ❑ shrinkage of the eye(s)
- ❑ hemorrhage
- ☑ reddish opercula (red tilapia)
- ☑ liver watery
- ☑ green, pale or dark liver
- ❑ spleen swollen
- ❑ empty gut
- ❑ gallbladder swollen
- ❑ ascites

What signs meet the criteria of suspect case in case definition (checklist 5)?

Sample preservation for further analysis

(Link to checklist 7, study design and sampling)

Purpose	Procedures
Histology (Level II) 	• Fix in NBF or Bouin for 24-36h (sample: fixative, 1:10 (w/v)), then replace by alcohol 70% (sample: fixative, 1:10 (w/v)) for long-term preservation • Keep at room temperature
TEM (Level III) 	• A specimen should not be more than 4 mm thick, preserve in 2.5% glutaraldehyde, keep at 4 ^{0}C for 24 h (sample: fixative, 1:20 (w/v)) then wash with phosphate buffer, then alcohol 70% at 4 ^{0}C
Molecular analysis (PCR, RT-PCR, qPCR) (Level III)  	• Trizol (sample: fixative, 1:3 (w/v), keep at -20/-80 ^{0}C • RNA later, sample: fixative, 1:10 (w/v), keep at -20/-80 ^{0}C • Alcohol 95%, sample: fixative, 1:10 (w/v) (keep at -20 ^{0}C) • Fresh sample, frozen sample -20/-80 ^{0}C

Sample preservation for further analysis

Purpose	Procedures
Virology (Level III) 	• Fresh or frozen (whole fish or target tissues e.g. liver, brain, spleen, kidney) • -80 ^{0}C
Strip test (Level I/II) Not yet available for TiLV 	• Fresh or probably frozen

Sample preservation for further analysis

Small fish
(< 2 g)

Preserve whole fish

Large fish

Necropsy

Liver, Kidney, Spleen, Brain, Gills

Preserve

Sample preservation for further analysis

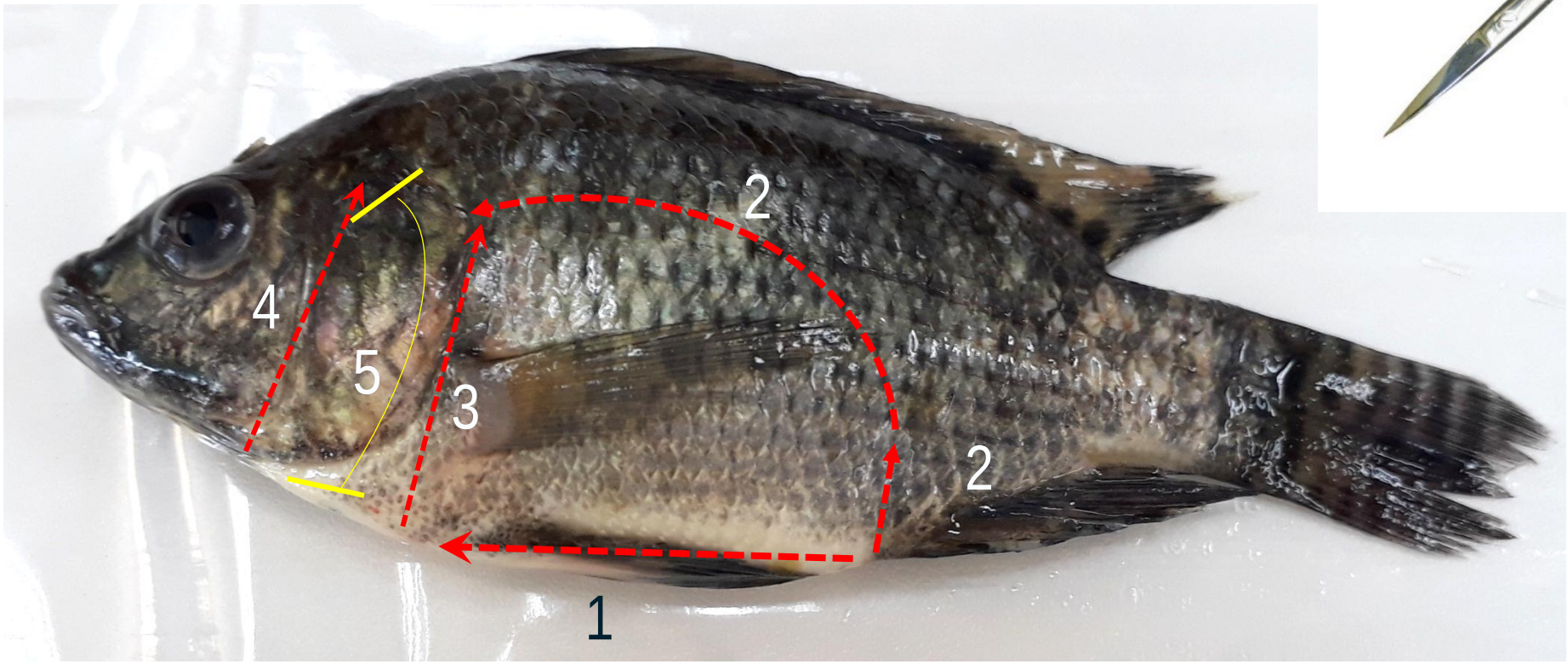
Fish Necropsy

- ❑Preparation according to the checklist
- ❑Necropsy form
- ❑Terminate the fish by an overdose of clove oil (≥200 ppm) or put on ice
- ❑Disinfect the fish body surface with alcohol 70%
- ❑Dissect the fish (see picture below)
- ❑Collect target tissues for different purpose

Histology
Molecular analysis
TEM
Virus isolation

Fish Necropsy

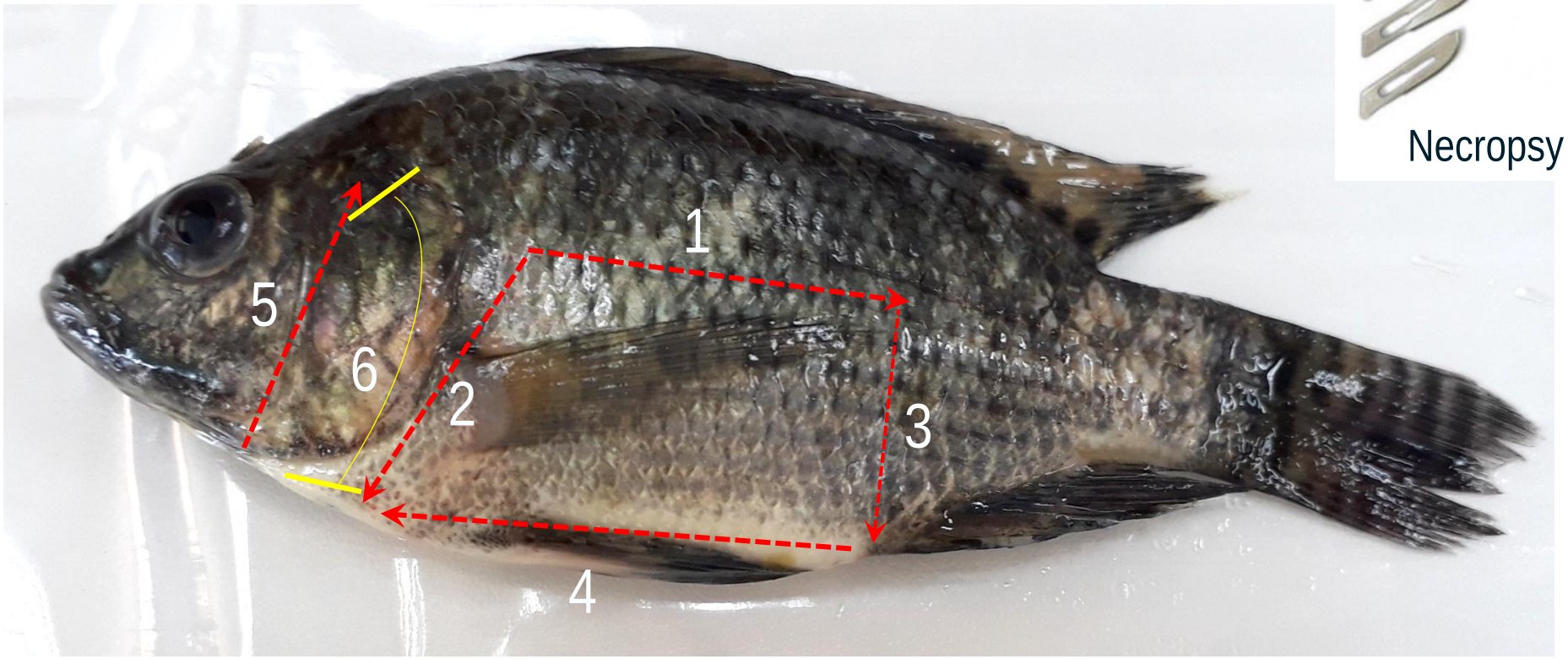
Large fish



How to dissect a fish?

Fish Necropsy

Large fish

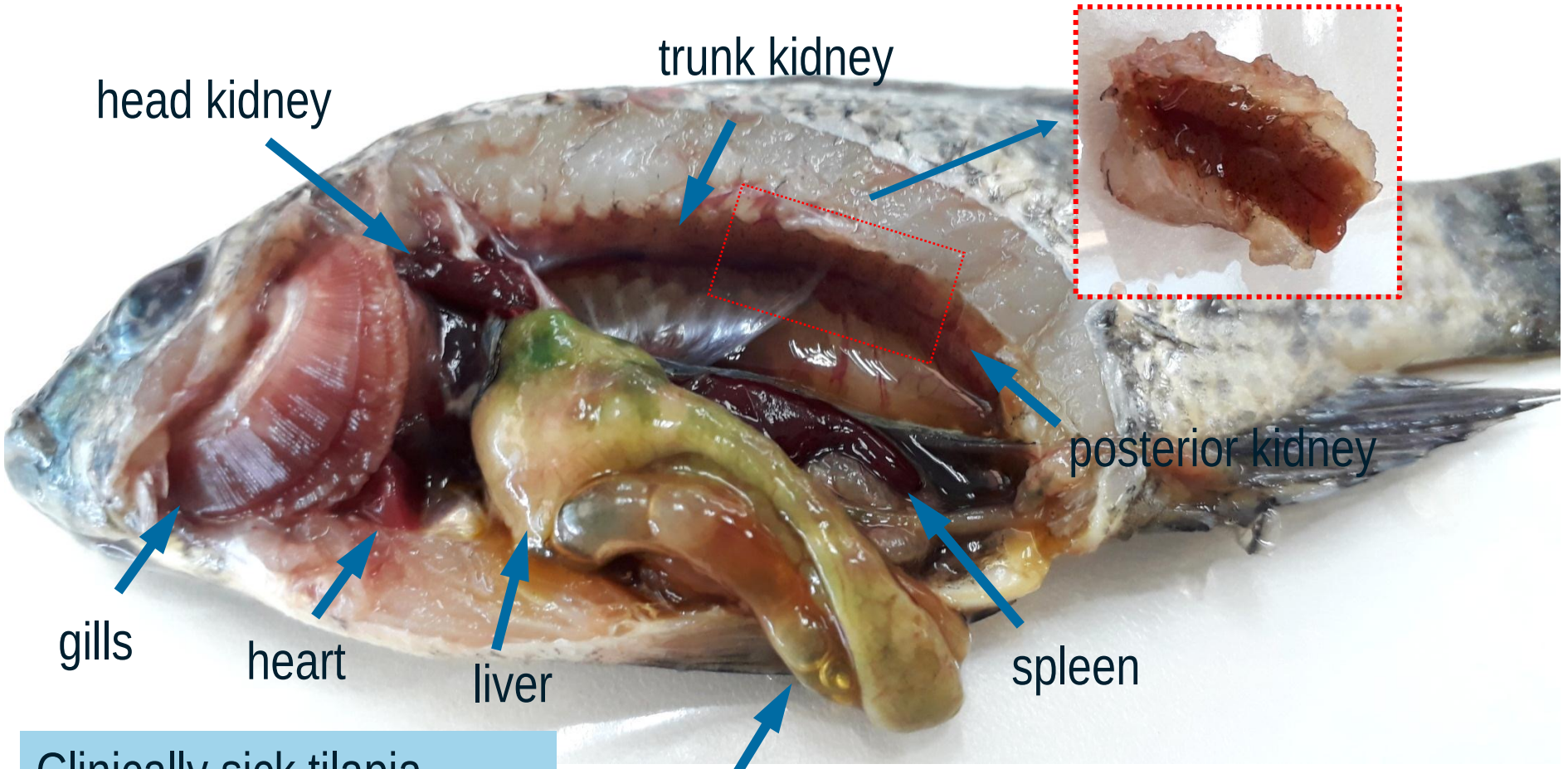


Necropsy blade

How to dissect a fish?

Fish Necropsy

How to collect kidney?



Clinically sick tilapia

Sample preservation for further analysis (Cont.)

Record Information Using Necropsy Data Sheet

Visceral cavity	normal
Liver	pale, some green areas, watery
Gall bladder	disappear/ very big (swollen)
Stomach	empty
Intestine	yellow liquid, gas accumulation
Spleen	bigger than normal
Kidney	N/A
Heart	
Brain	hemorrhage
Others	
Taking photo/video	Yes (very useful)

Sample preservation for further analysis (Cont.)

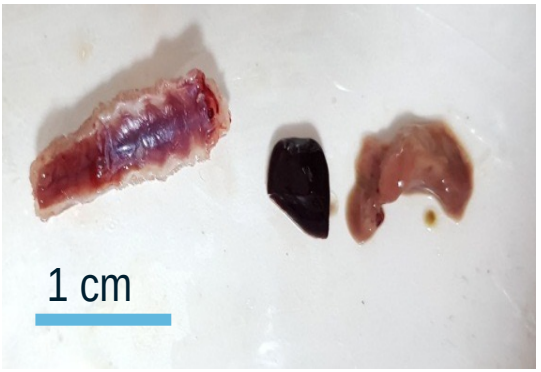
For Histopathology

- Samples should be collected from moribund fish or freshly dead fish (best within 15 min post mortem)
- Do not use frozen fish
- LIVER is the best tissue for TiLV histopathological diagnosis
- Additional organs, such as kidney, spleen, brain, gills may be useful

Sample preservation for further analysis (Cont.)

For Histopathology

- Fry and fingerlings can be preserved whole
  - Remove gill opercula
  - Open fish cavity by a cut along midline and viscera should be pulled out to allow fixative to penetrate properly into the tissues
- For bigger fish, necropsy should be performed
  - Small pieces (~3-5 mm thickness) of individual organs should be collected and preserved in fixative



good size for preservation

Sample preservation for further analysis (Cont.)

Fixation

The purpose of tissue fixation is to permanently preserve the tissues in a life-like state and to prevent autolysis and decomposition.

10% neutral buffered formalin (NBF)

- 37% Formaldehyde: 50 mL
- Distilled water: 450 mL
- Sodium phosphate, diabasic (Na_2HPO_4): 3.25 g
- Sodium phosphate, monobasic (NaH_2PO_2): 2 g

Combine all ingredients and mix well, label and date. Store at room temperature

Bouin's Fixative

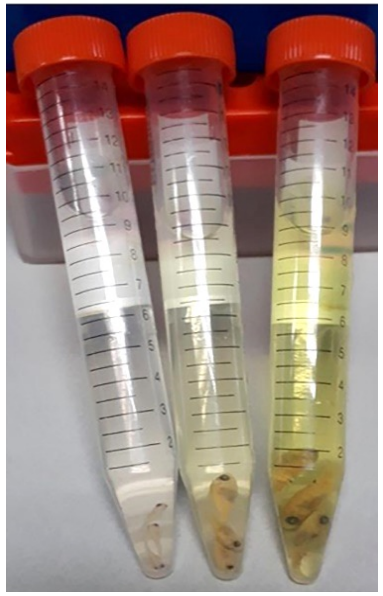
- Saturated picric acid: 3000 mL
- 37% Formaldehyde: 1000 mL
- Glacial acetic acid: 200 mL

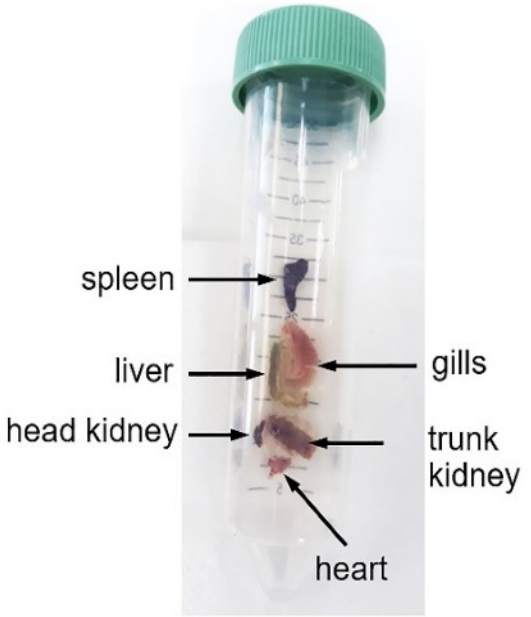
Combine all ingredients and mix well, label and date. Store at room temperature

Sample preservation for further analysis (Cont.)

Sample preservation for histology

- Ratio of sample: fixative should be 1:10 (w/v)
- After 12-24 hours, preserved tissues should be transferred to 70% ethanol, ratio 1:10 (w/v) for long-term storage
- Keep at room temperature





Sample preservation for further analysis (Cont.)

Sample preservation for histology

What to avoid?

- Dead fish → post mortem change
- Dissection takes too long time → autolysis
- Physical destruction of tissue → not good for histology
- Tissue pieces are too big → not good for penetration of fixative
- Fixative is not enough → not appropriate for histology
- Sample in formalin 10% for too long → not good for ISH

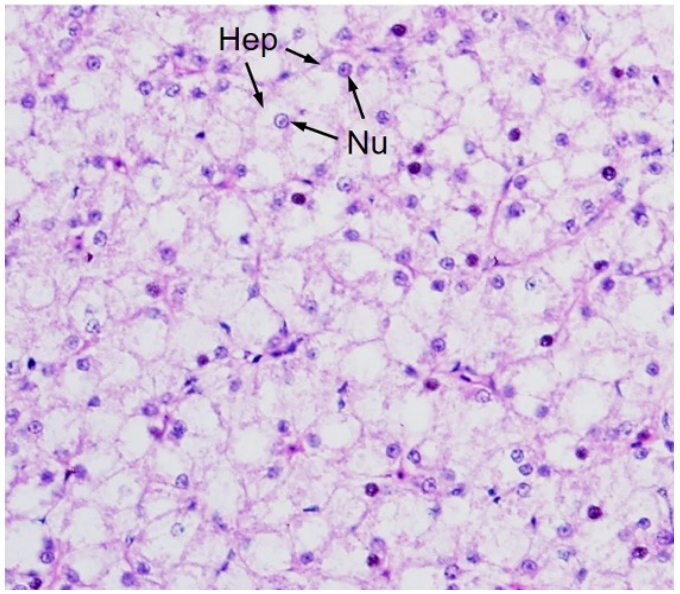
Not enough fixative

TiLV Diagnostics (Level II)

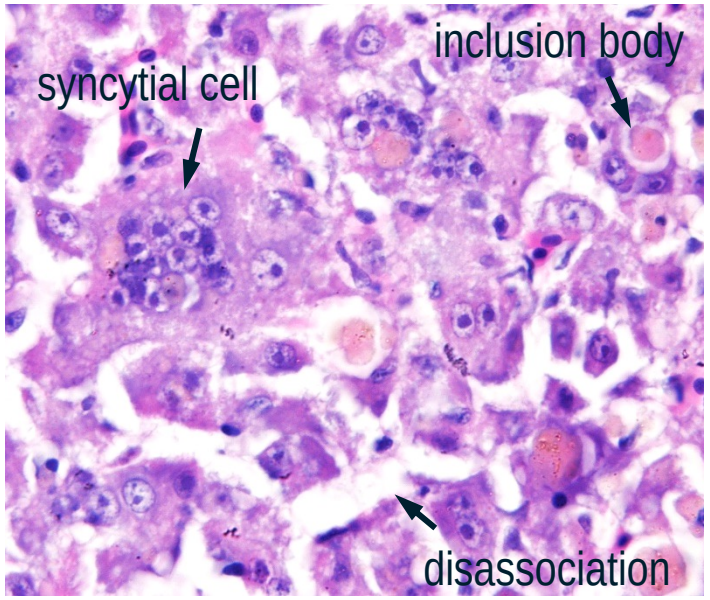
Histopathology

- The study of changes in tissues caused by disease
- Comparison between NORMAL fish and SICK fish
- Looking for pathognomonic lesion of disease caused by TiLV

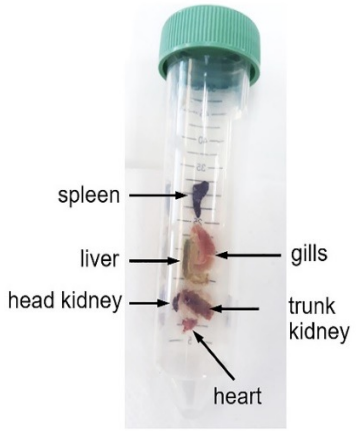
Liver of a normal fish



Liver of a sick fish

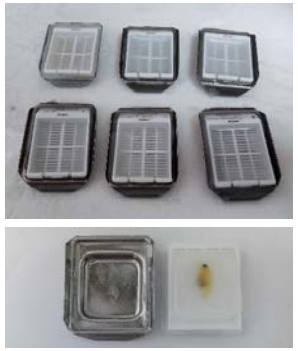


TiLV Diagnostics (Level II)



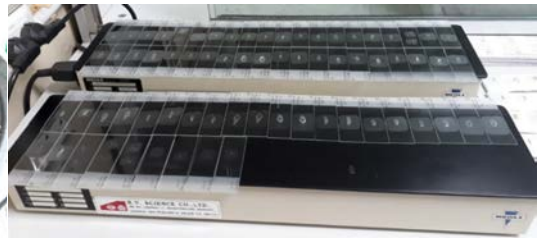
Preserved samples

Dehydration of samples in an automatic tissue processing machine



Embed samples in molten paraffin

Section embedded tissue at 4-5 µm thickness

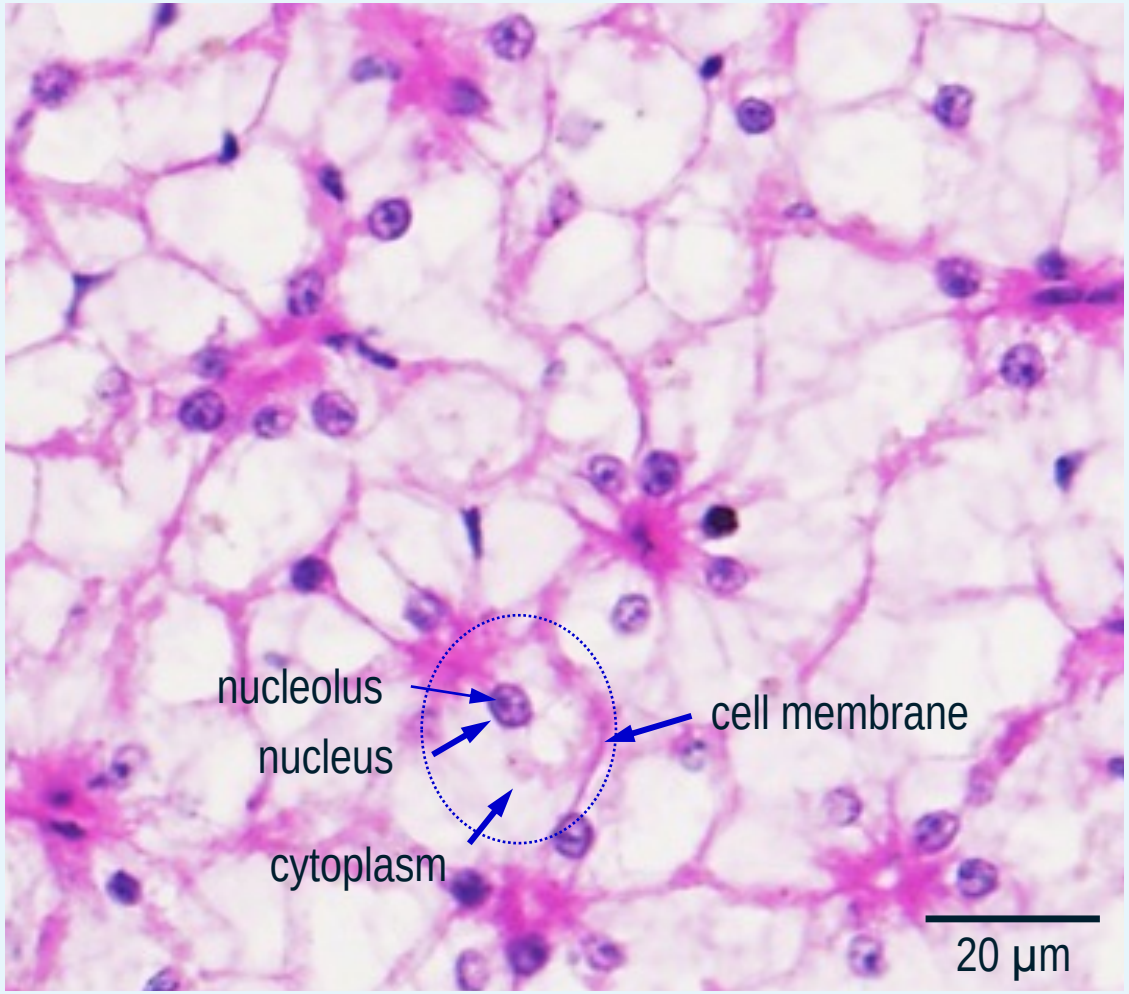


Float the tissue ribbon onto the surface of a ~45 °C water bath before placing sections onto slides

Staining of sections with hematoxylin and eosin (H&E)

Mounting slides, examination under a light microscope

TiLV Diagnostics (Level II)



polyhedral shape of tilapia hepatocytes

- Hematoxylin (Blue/Purple) stains basophilic substances in cells e.g. the nucleus and chromatin
- Eosin (Pink) stains all eosinophilic substances in cells not stained by hematoxylin e.g. cytoplasm, collagen, muscle fibers

Food and Agriculture Organization of the United Nations

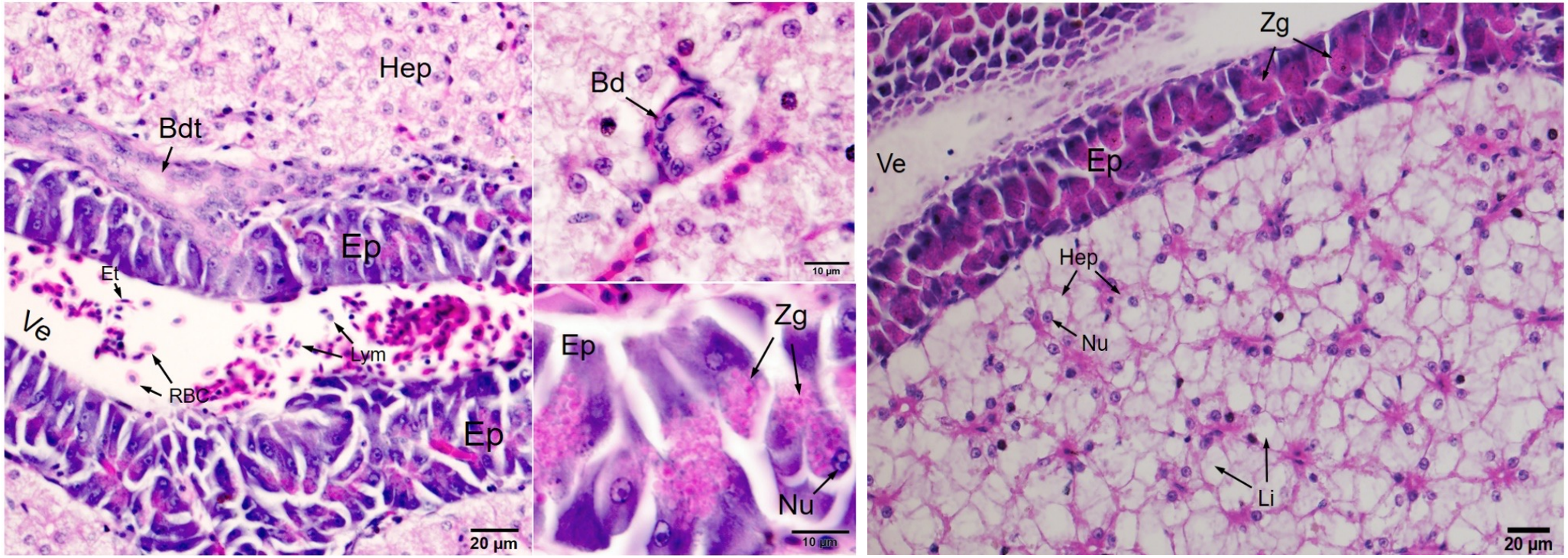
SUSTAINABLE DEVELOPMENT GOALS

Normal Histology of Liver (Hepatopancreas)

Normal Liver

Macroscopic examination

Microscopic examination



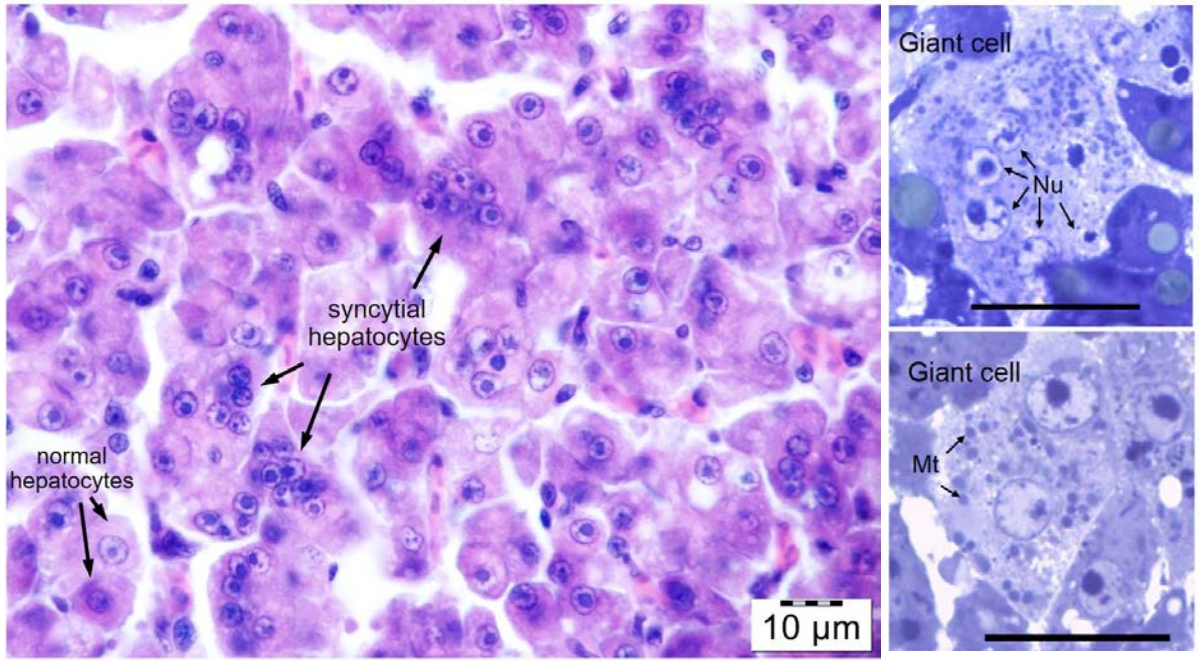
Photomicrographs of the H&E stained normal liver of Nile tilapia juvenile. Normal liver cells have polyhedral shape. Bdt, bile ductile; Ep, exocrine pancreas; Hep, hepatocyte; Li, lipid droplets; Nu, nucleus; RBC, red blood cells; Si, sinusoid; Ve, vein; Zg, zymogen granules

Histopathological lesion of TiLV infection (Level II)

- The disease was named Syncytial Hepatitis of Tilapia (SHT) by Ferguson et al. 2014

- Syncytial hepatitis is pathognomonic histopathological feature found in TiLV outbreaks

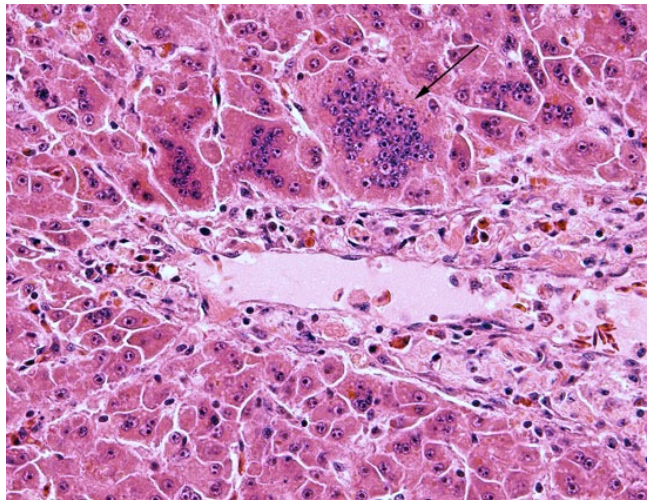
Macroscopic examination (Level I)



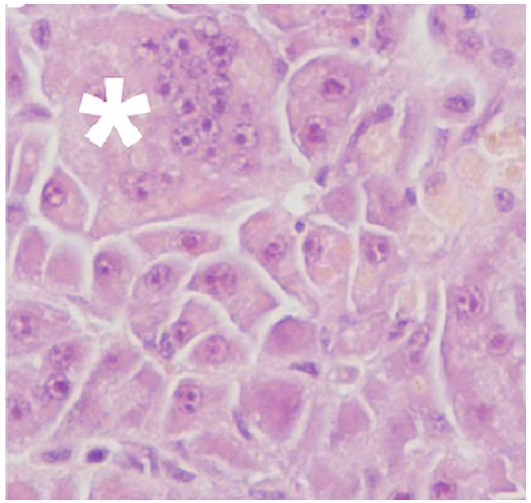
Microscopic examination (Level II)

Confirmed case – Case definition (Checklist 5)

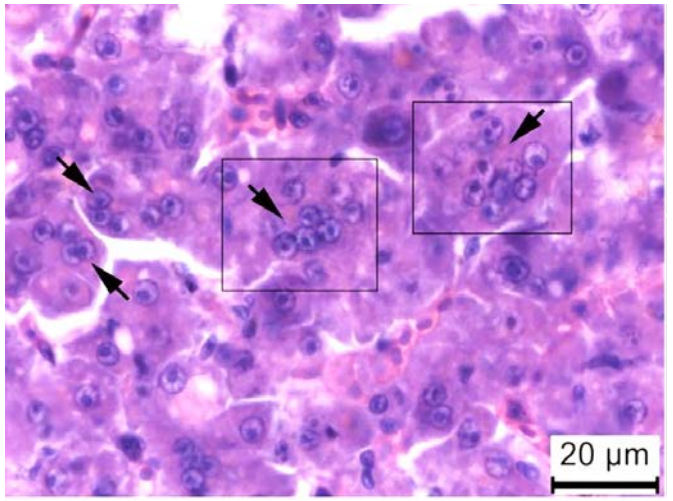
Histopathological lesion of TiLV infection



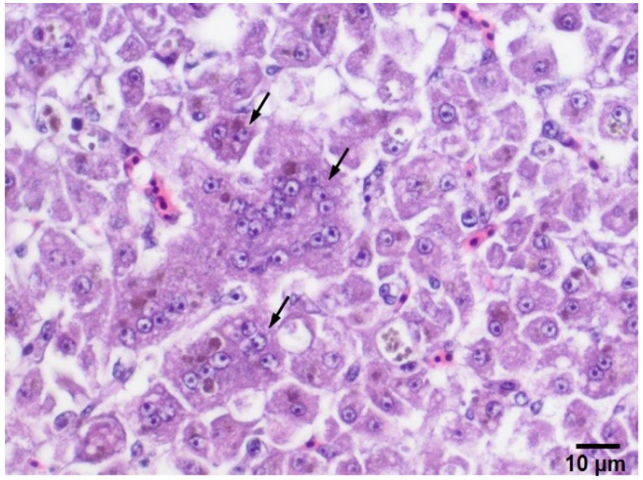
Ecuador (Ferguson et al. 2014)



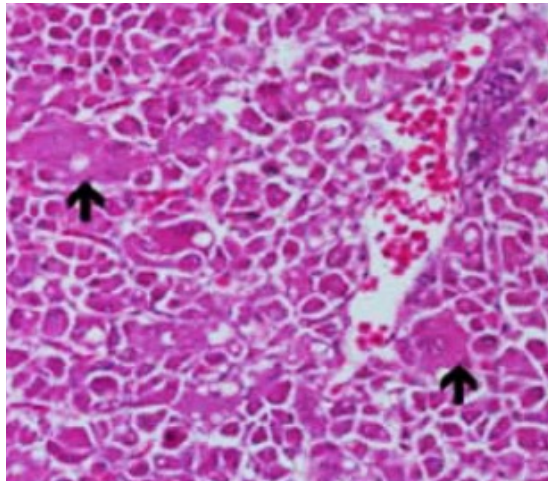
Israel (Bacharach et al. 2016)



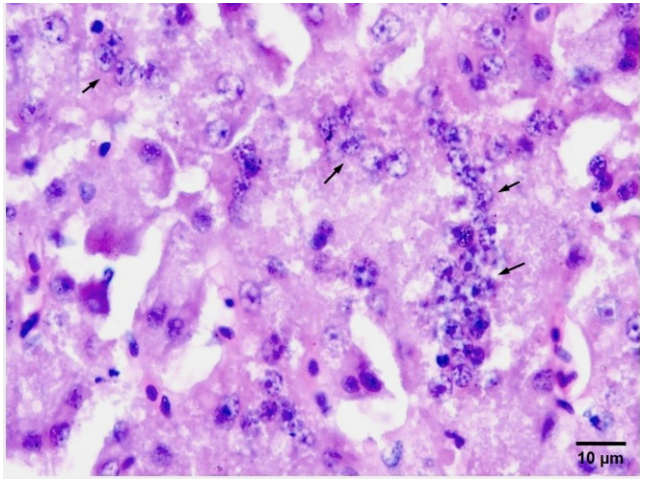
Thailand (Dong et al. 2017)



India (Behera et al. 2018)

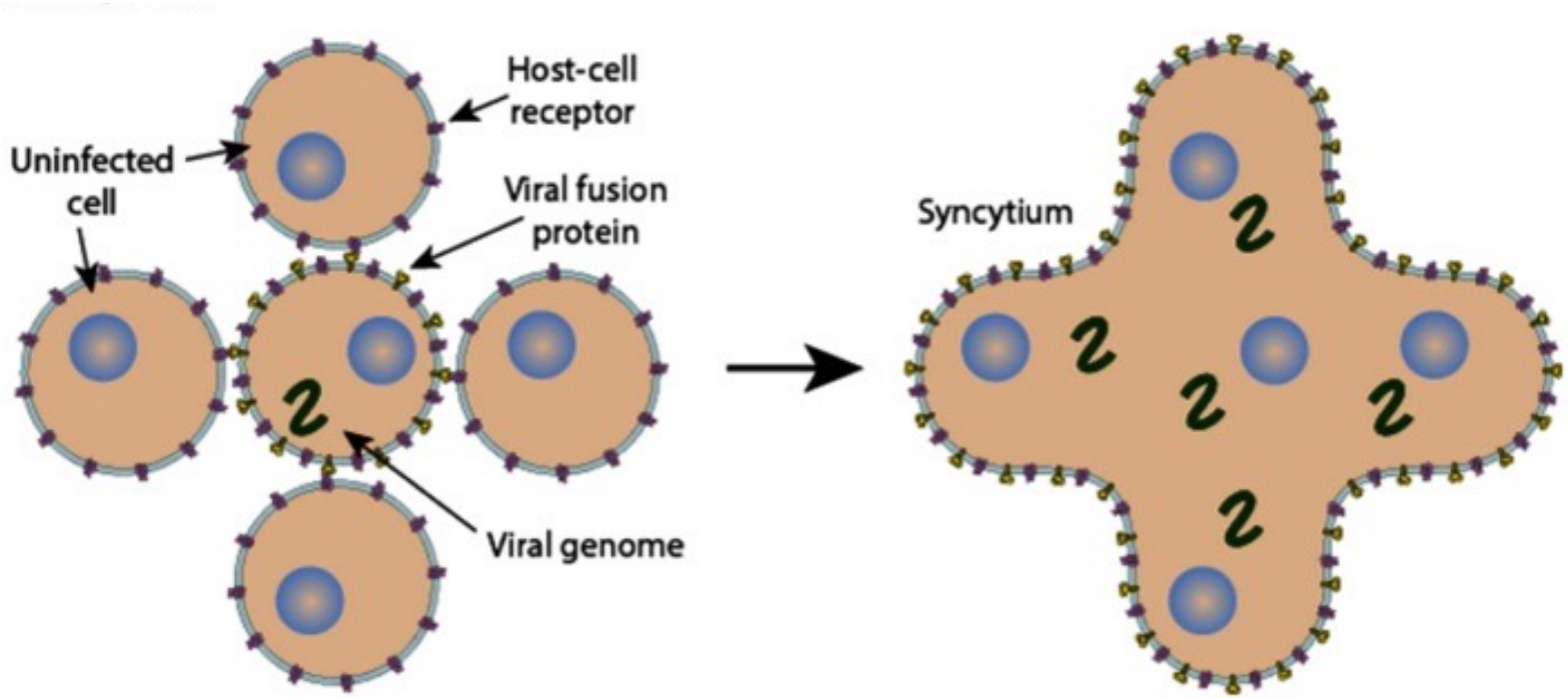


Malaysia (Amal et al. 2018)



Peru (Pulido et al. 2019)

Possible explanation for SHT



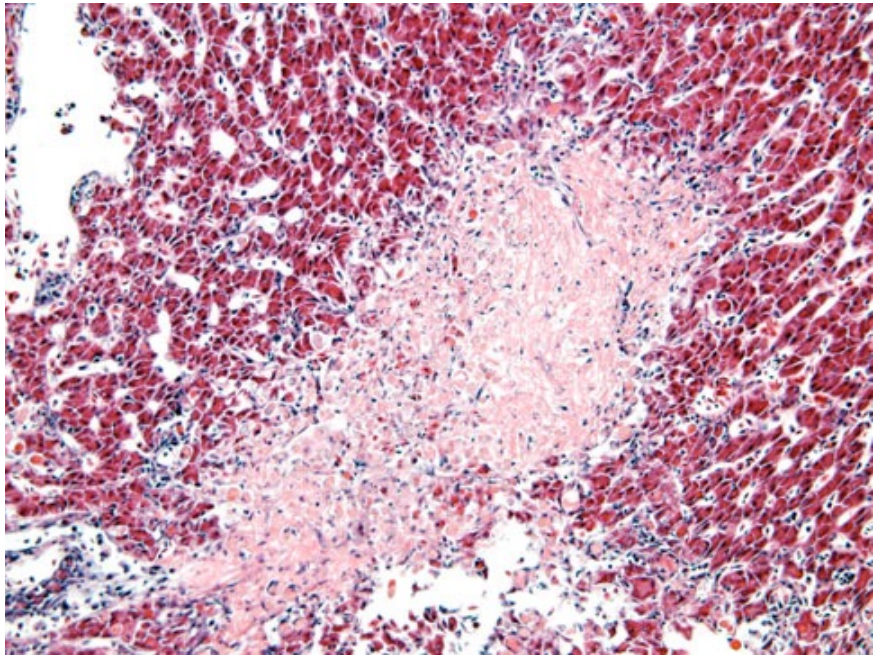
Source: www.expasy.org/viralzone

Other histopathological changes in the liver

Atypical lesions (individual or combination of following lesions):

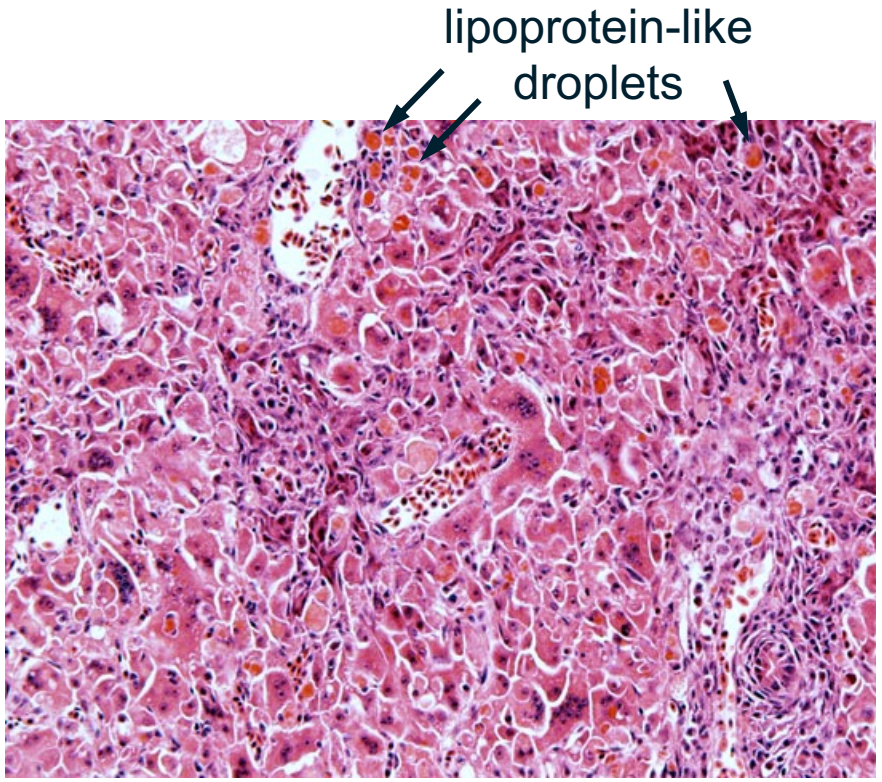
- ❑ multifocal chronic hepatitis
- ❑ presence of intracytoplasmic inclusion bodies (eosinophilic inclusion or lipoprotein droplets)
- ❑ reduction of fat-storage cells
- ❑ foamy cytoplasm
- ❑ hepatocyte disassociation
- ❑ necrotic pancreases and infiltration of lymphocytes
- ❑ hemorrhage
- ❑ cellular necrosis
- ❑ pyknosis and karyorrhexis

Other histopathological changes in the liver



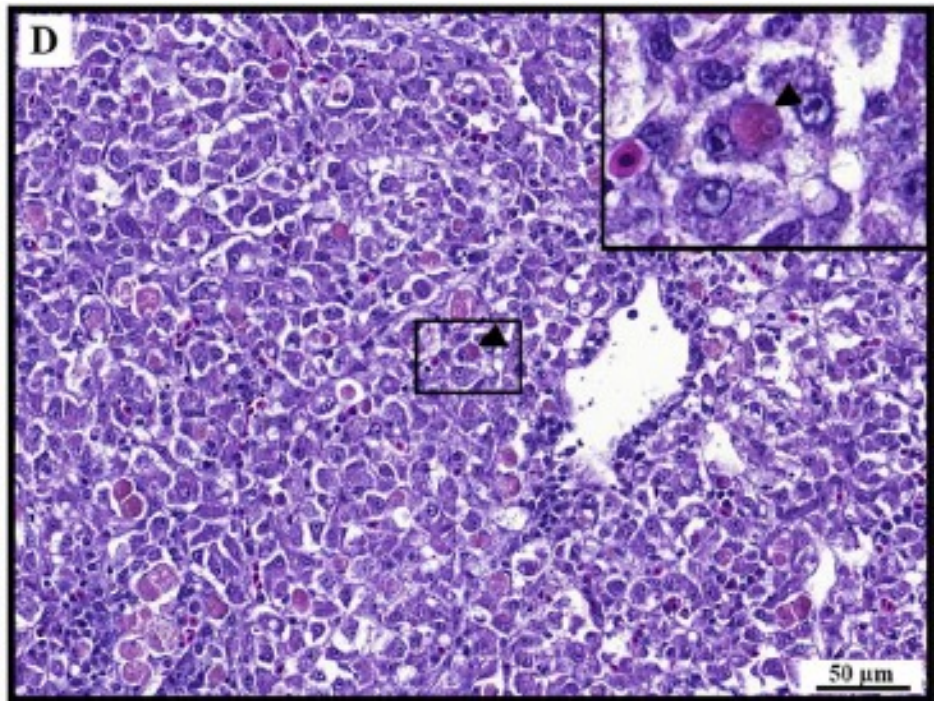
multifocal chronic hepatitis

Ferguson et al. 2014

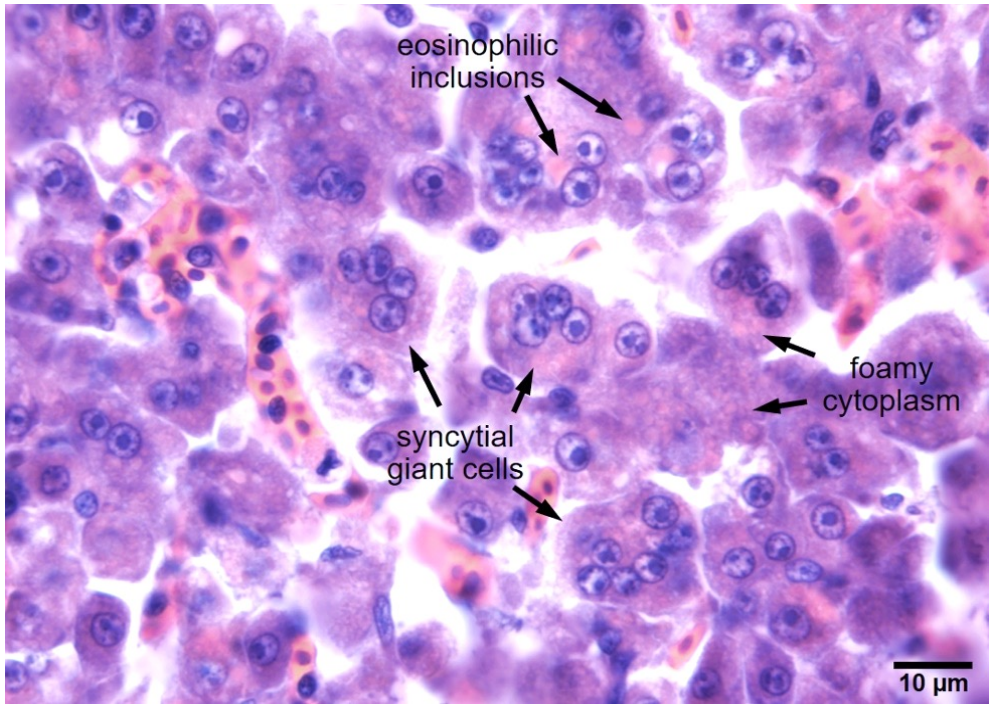


hepatocytes often containing
lipoprotein-like droplets

Other histopathological changes in the liver

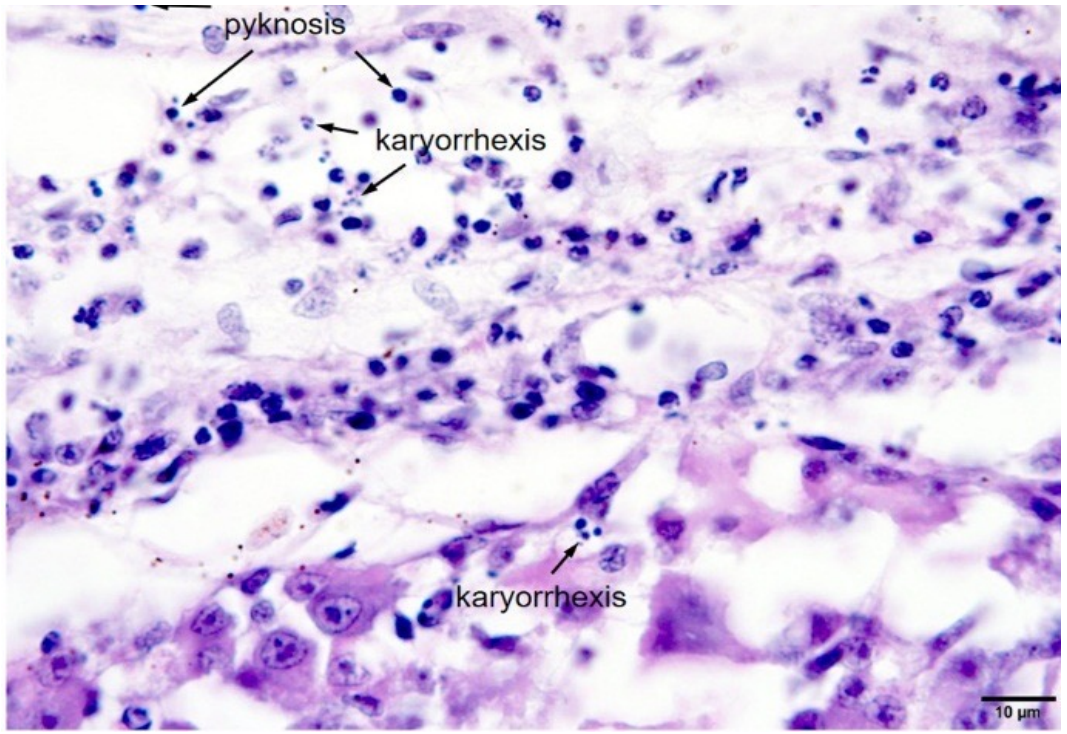


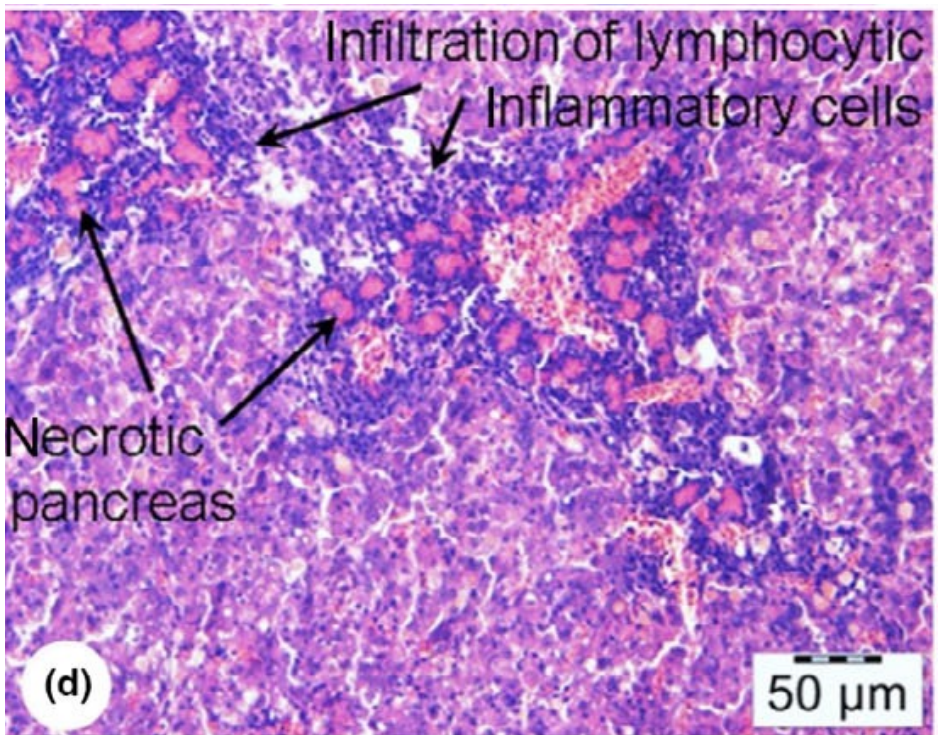
Intracytoplasmic inclusion bodies
Tattiyapong et al. 2017



Syncytial giant cells, intracytoplasmic inclusion bodies, foamy cytoplasm (HT Dong)

Other histopathological changes in the liver

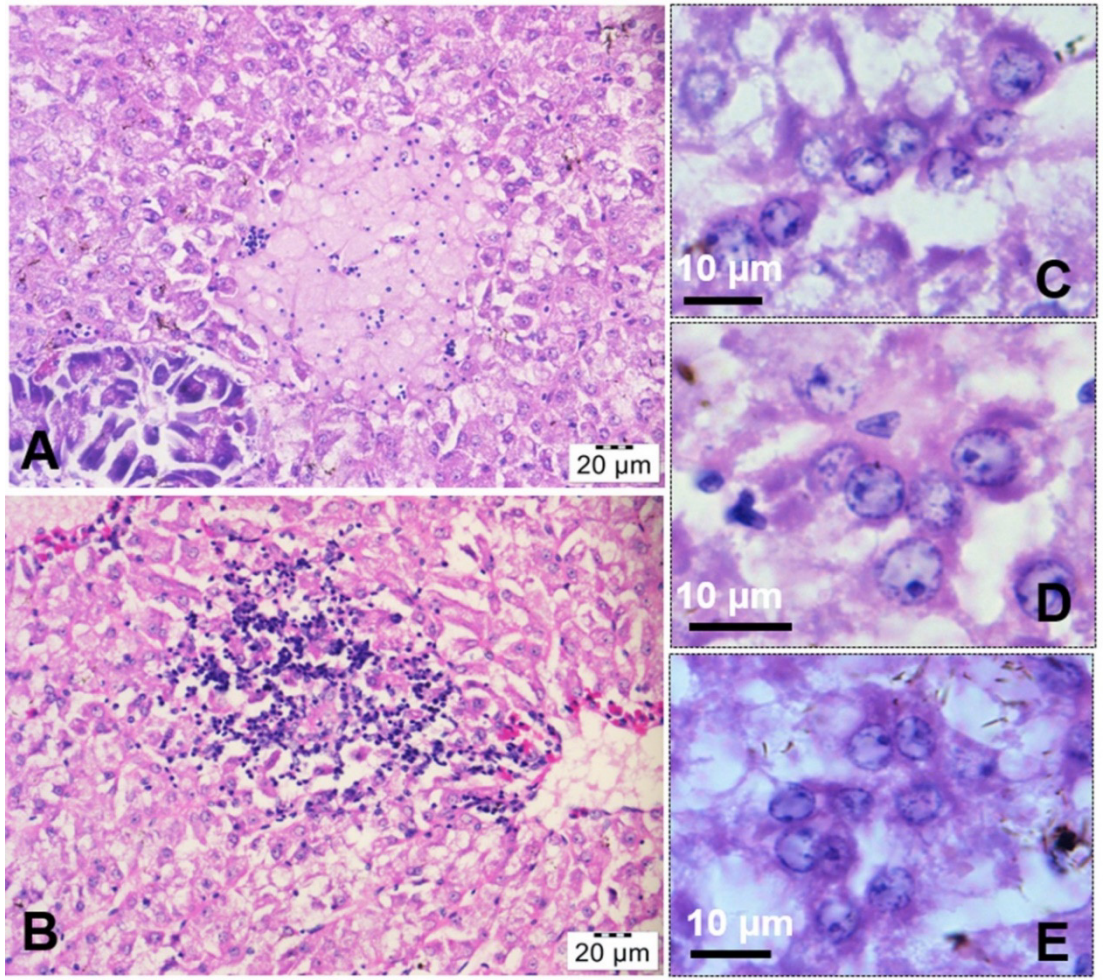




Other histopathological changes in the liver

focal necrosis of hepatocytes

infiltration of lymphocytic inflammatory cells

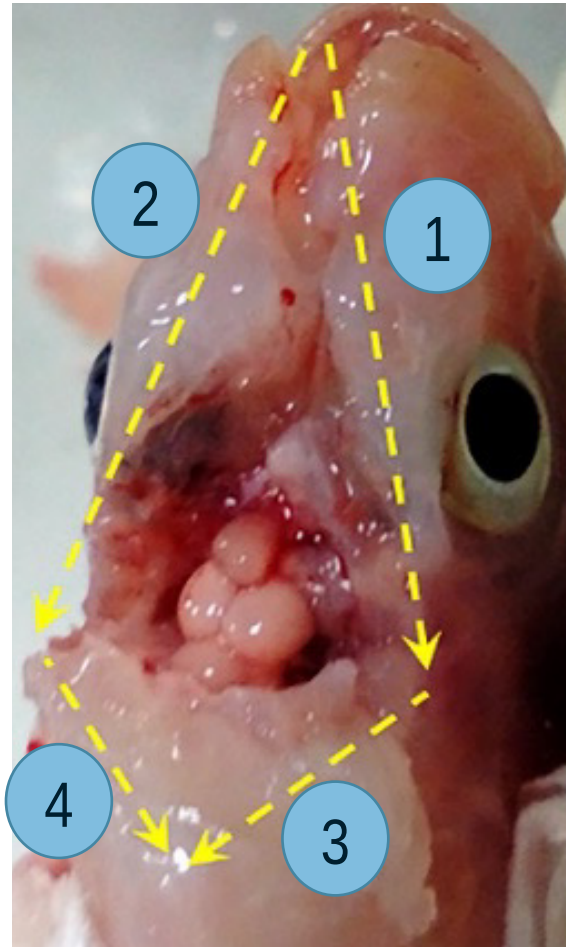


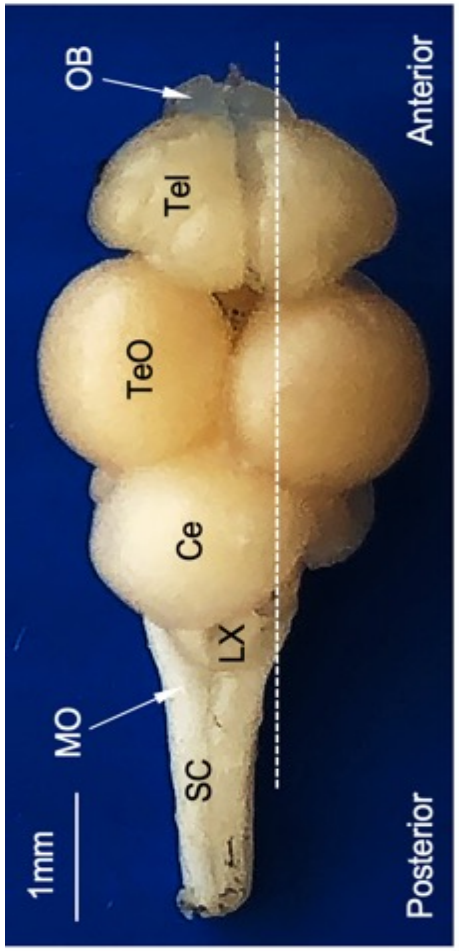
hepatocytes resembling giant cells which contained multiple nuclei

Senapin et al. 2018 Aquaculture

Histopathological alterations in the TiLV infected brain

Normal brain





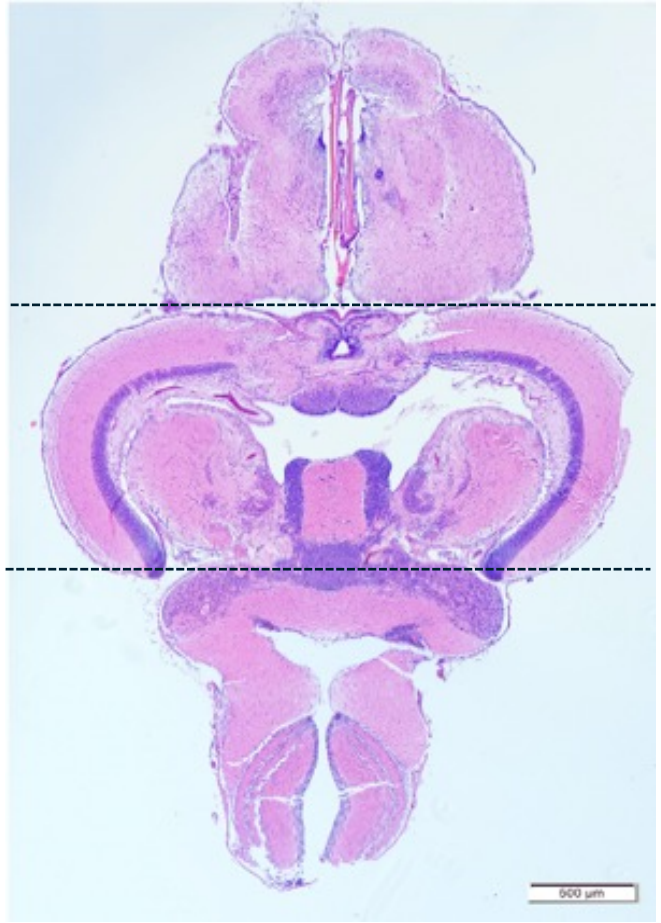
Necropsy

OB: Olfactory bulb
Tel: Telencephalon
TeO: Optic tectum
Ce: Cerebellum
LX: Vagal lobe
SC: Spinal cord

Fore brain

Midbrain

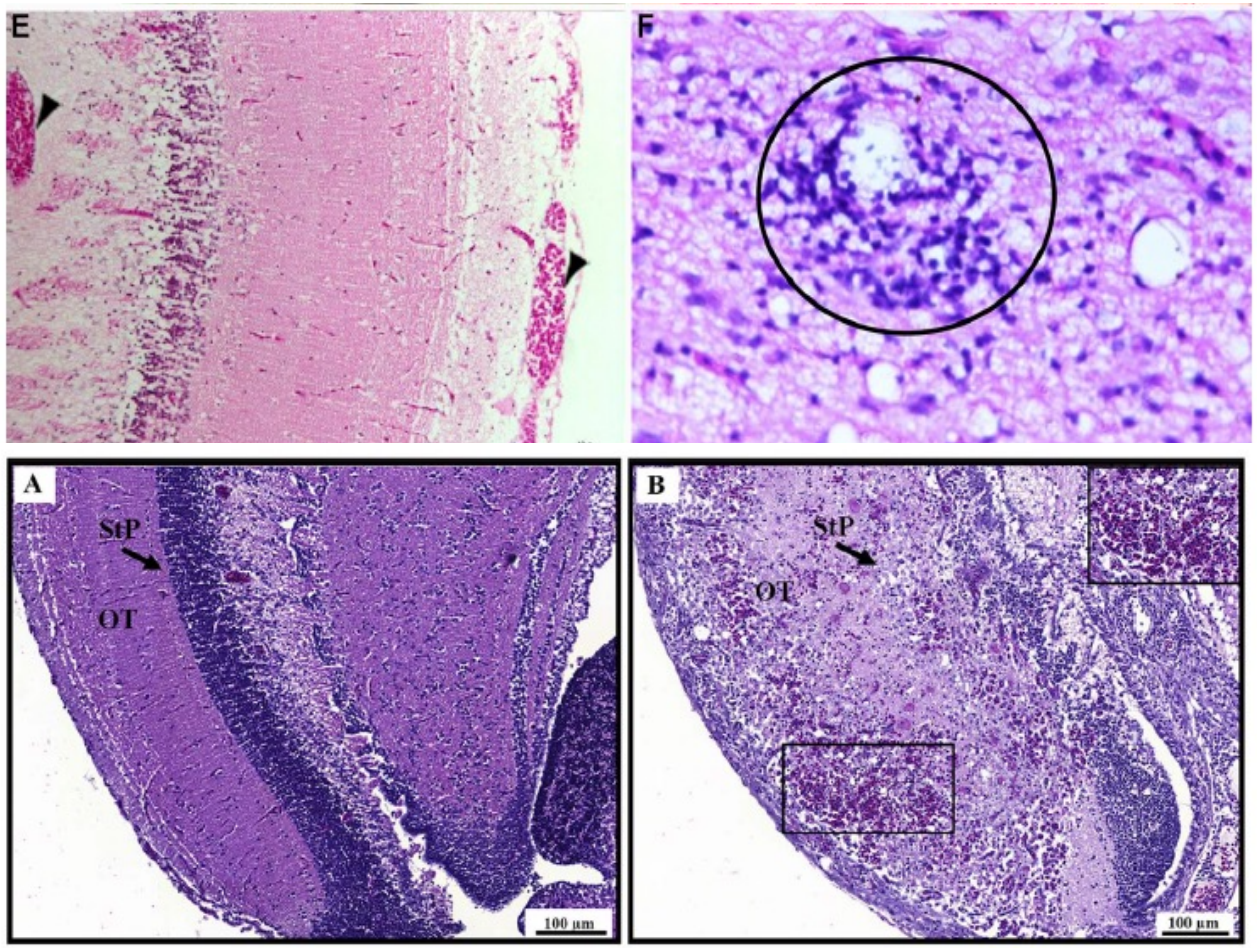
Hindbrain



H&E stained (horizontal) section of the normal brain of tilapia

Dinh-Hung et al. 2021 J Fish Dis

Histopathological alterations in the TiLV infected brain



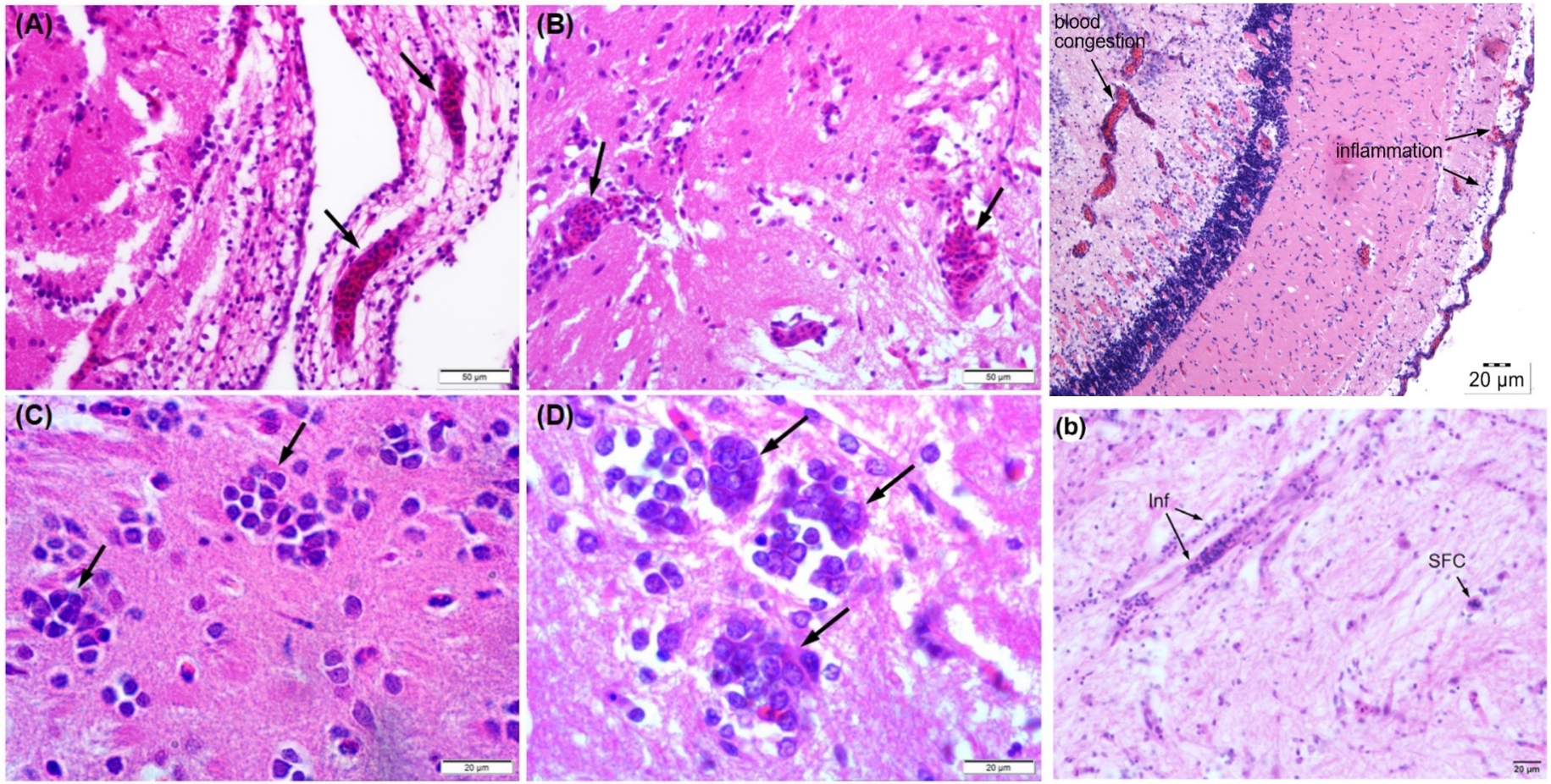
- ✓ congestion
- ✓ perivascular cuffing of lymphocytes in the brain cortex
- ✓ multifocal hemorrhage & blood congestion

Eyngor et al. 2014 J Fish Dis

Tattiyapong et al. 2017 Vet Microbiol

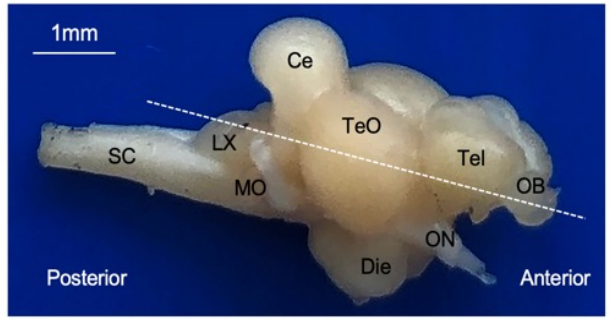
Histopathological alterations in the TiLV infected brain

- ✓ Congestion
- ✓ Inflammation
- ✓ Aggregation of cells
- ✓ Syncytia-like



Dinh-Hung et al. 2021 J Fish Dis
Debnath et al., 2020 J Fish Dis

Histopathological alterations in the TiLV infected brain



Received: 20 January 2021 | Revised: 3 March 2021 | Accepted: 4 March 2021
DOI: 10.1111/jfd.13367

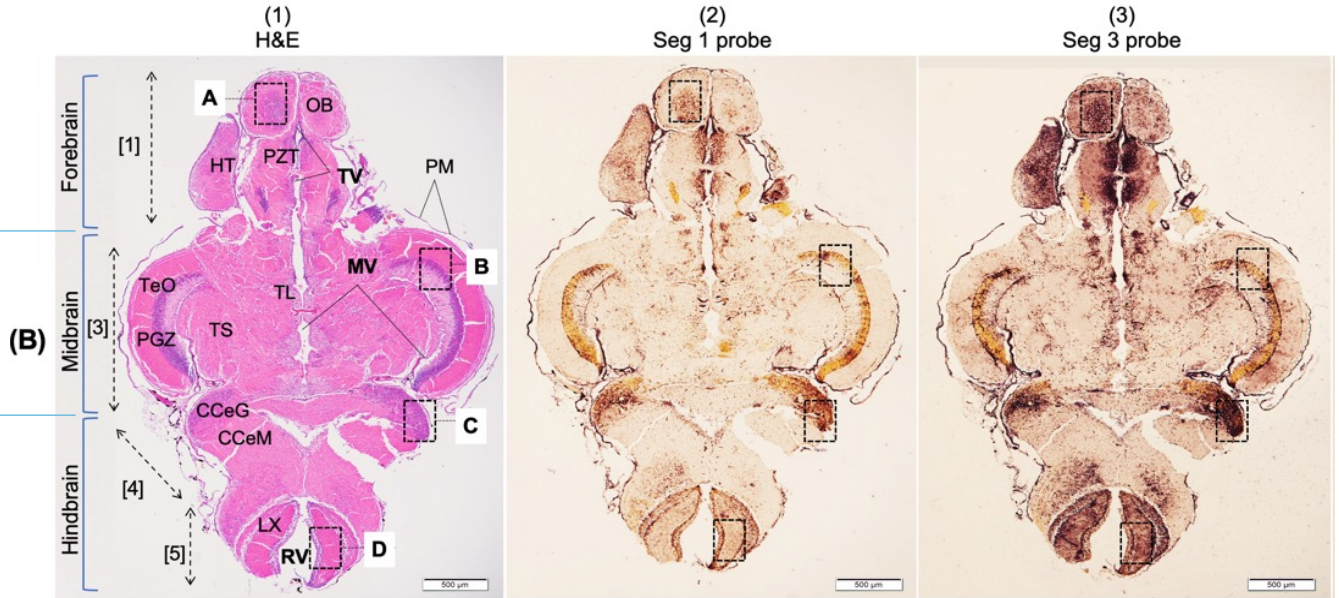
RESEARCH ARTICLE

Journal of Fish Diseases WILEY

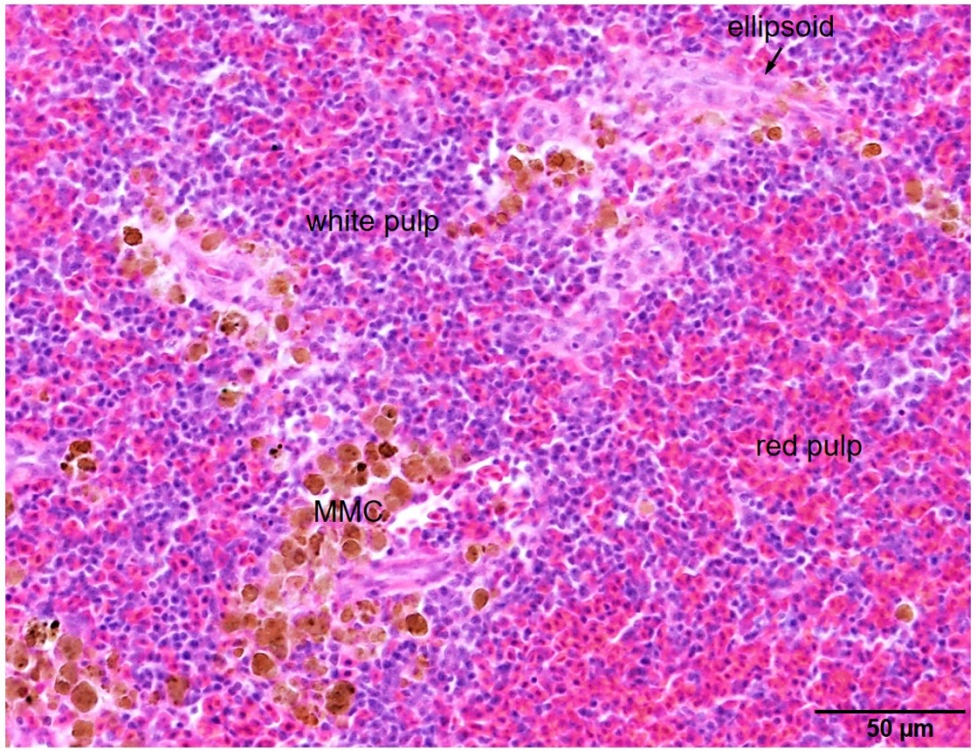
Dissecting the localization of *Tilapia tilapinevirus* in the brain of the experimentally infected Nile tilapia, *Oreochromis niloticus* (L.)

Nguyen Dinh-Hung[1,2] | Pattiya Sangpo[3,4] | Thanapong Kruangkum[4,5] | Pattanapon Kayansamruaj[6] | Tilladit Rung-ruangkijkrai[7] | Saengchan Senapin[4,8] | Channarong Rodkhum[1,2] | Ha Thanh Dong[3]

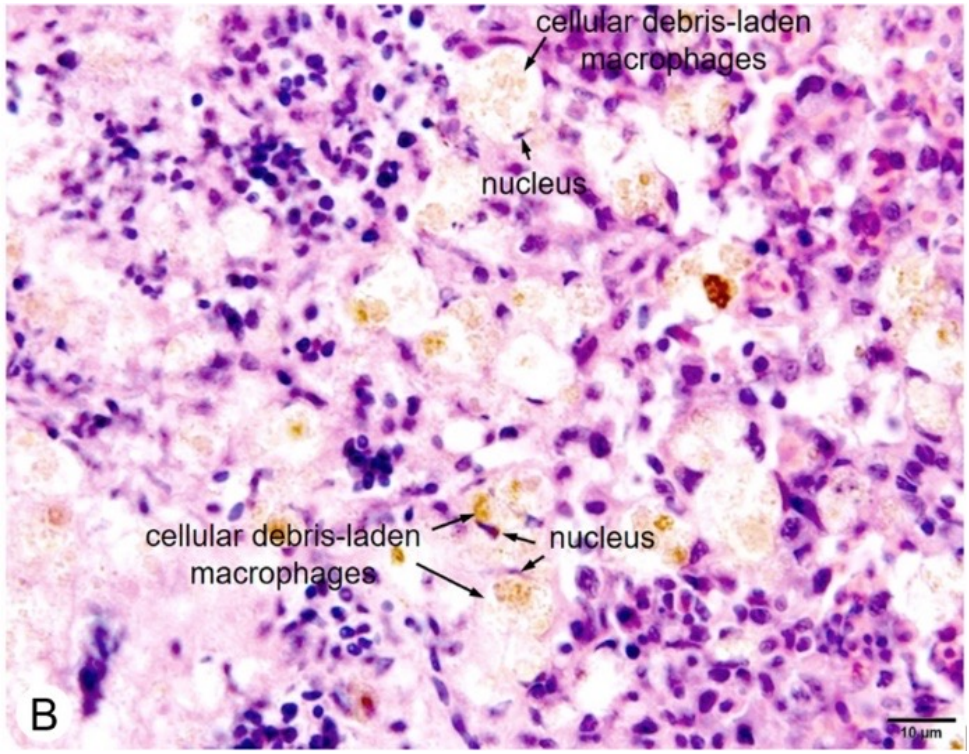
- Loss of appetite, lethargy, stop schooling, stop eating
- Disorders of navigating, food and prey seeking
- Erratic swimming or loss of balance
- Failure of the respiratory and cardiovascular systems



Histopathological changes in the spleen

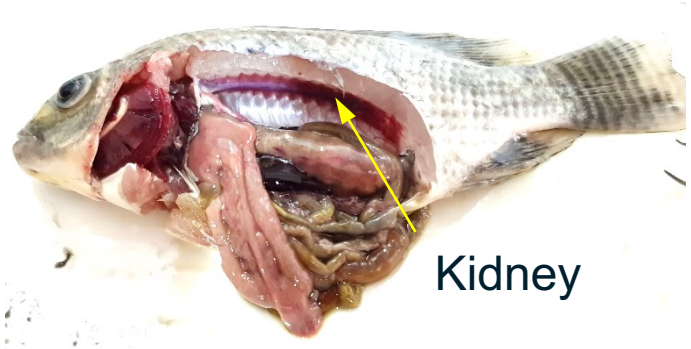


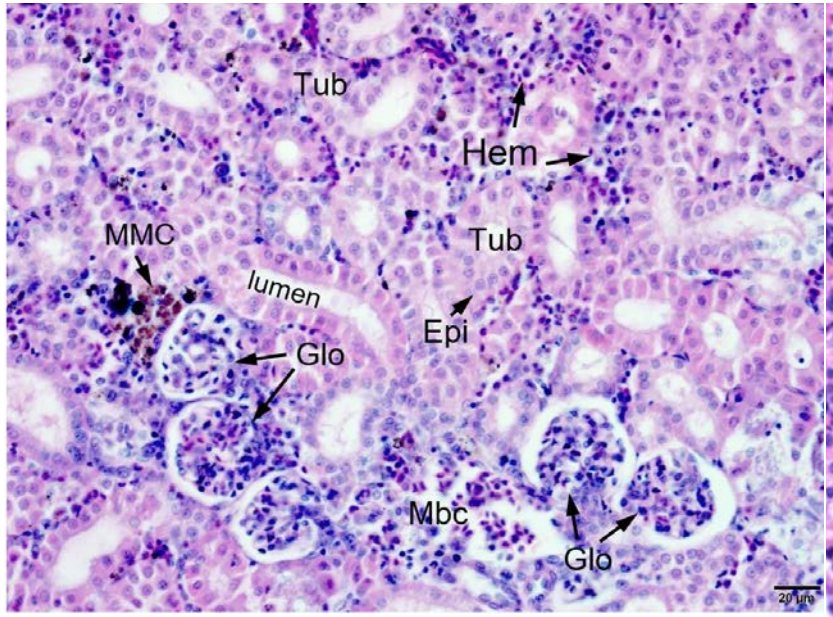
Normal spleen



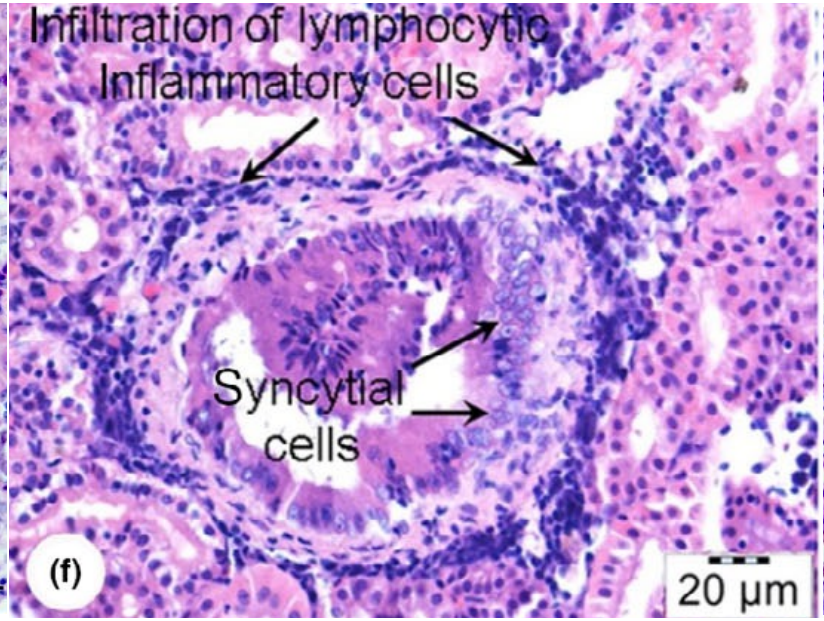
TiLV-infected spleen

Histopathological changes in the kidney

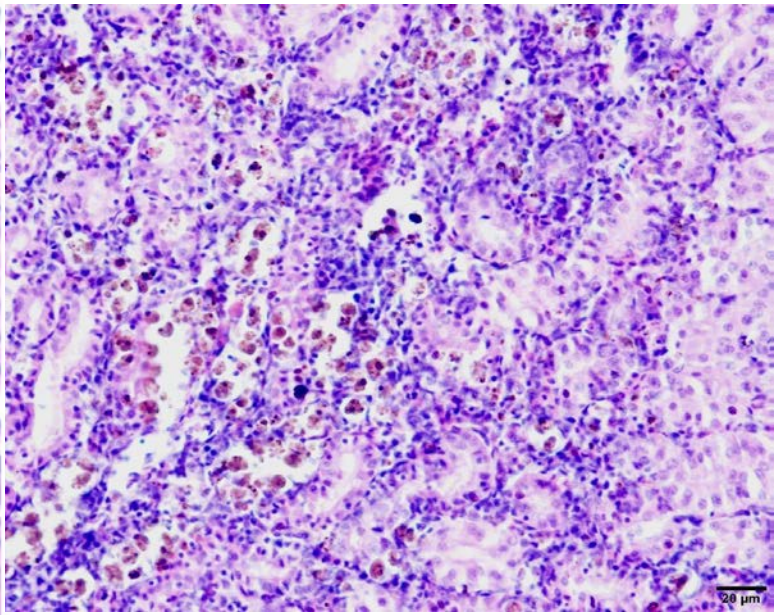




Normal kidney. Epi, epithelial cell; Glo, glomerulus; Hem, hematopoietic tissue; Mbc, mature blood cells; MMC, melano-macrophage center; Tub, tubules



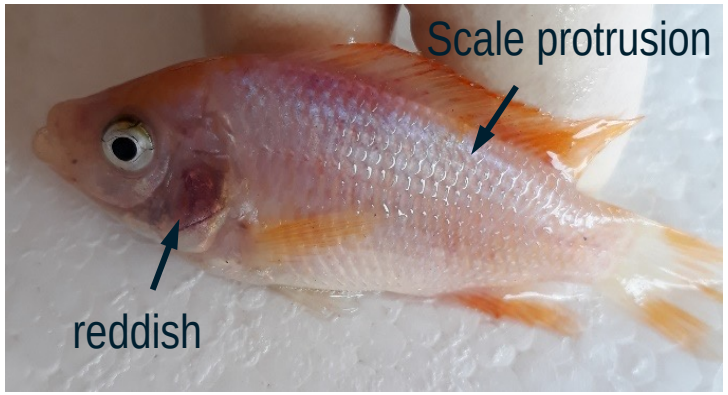
Infiltration of lymphocytic inflammatory cells

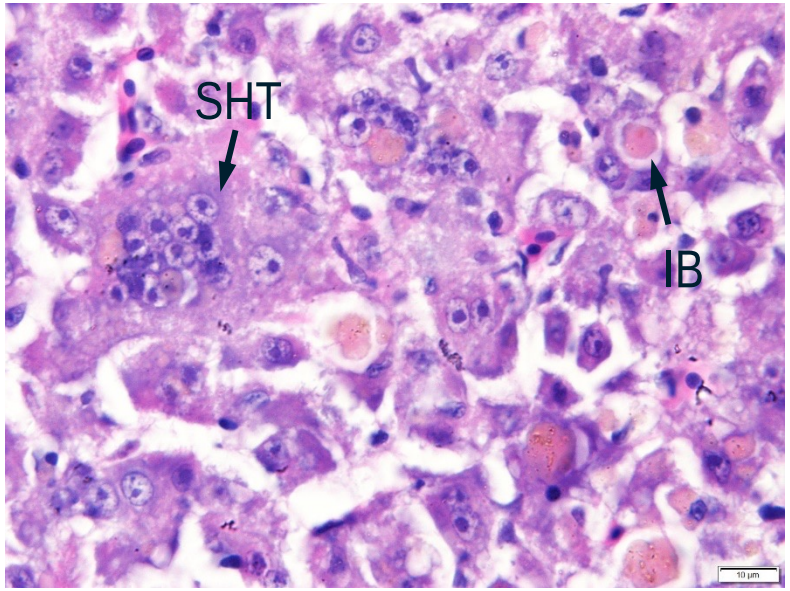


increasing number of MMCs

Organ	Histopathology description
Liver	Typical lesion: presence of syncytial giant cell(s) or multinucleated giant cells. Atypical lesions (individual or combination of following lesions): presence of intracytoplasmic inclusion bodies (eosinophilic inclusion or lipoprotein droplets), reduction of fat-storage cells, hepatocyte disassociation, necrotic pancreases and infiltration of lymphocytes, hemorrhage, cellular necrosis, pyknosis and karyorrhexis, foamy cytoplasm, multifocal chronic hepatitis.
Kidney	Typical lesions: none Atypical lesions: aggregation of lymphocytes, pyknosis and karyorrhexis, increasing number of melano-macrophages centers. Syncytia-like was occasionally seen.
Spleen	Typical lesions: none Atypical lesions: splenic cell degeneration, presence of debris-laden macrophages within splenic ellipsoids, pyknosis and karyorrhexis, increasing number of melano-macrophage centers.
Brain	Typical lesions: none Atypical lesions: severe inflammation with infiltration of massive lymphocytes, encephalitis, perivascular cuffing, blood congestion or sometime hemorrhage, syncytia-like was occasionally seen.

Summary of TiLV Diagnostics (Level I-II)





Level I

1. Observation of animals and environment
2. Gross signs (external & internal)
3. Preserve representative specimens for Level II & III
   - ❑ Histology (Level II)
   - ❑ PCRs (Level III)
   - ❑ TEM (Level III)
   - ❑ Virology (Level III)

Level II: Histopathology

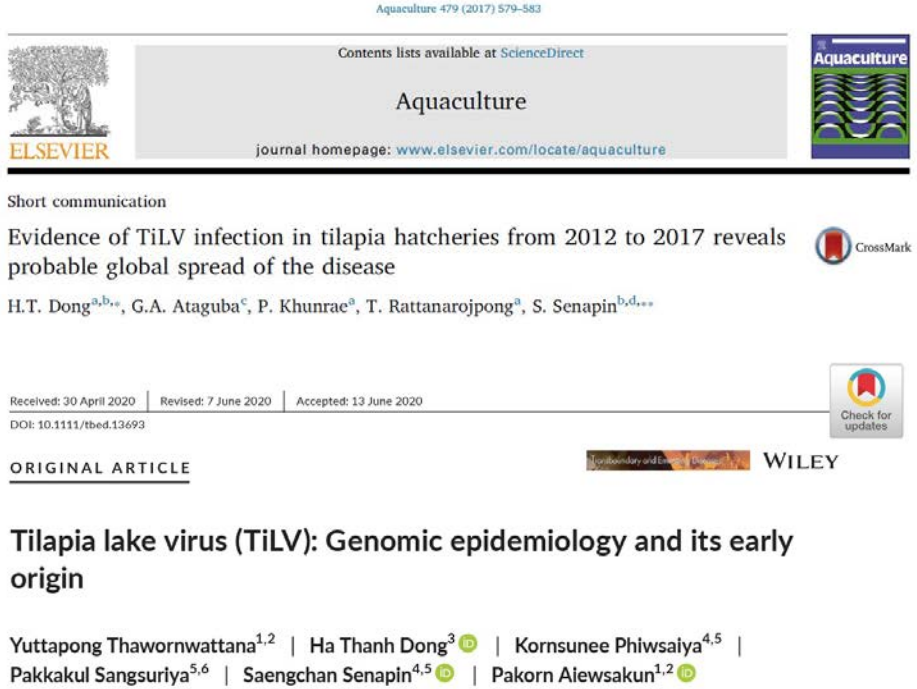
- Syncytial hepatitis (pathognomonic lesion of TiLV)
- Other changes

Linked to

- ☑ case definition in checklist 5
- ☑ study design and sampling in checklist 7

Importance of archived samples (BioBank)

- Tell you something about newly emerging diseases
  - ✓ How long the virus presence
  - ✓ Track the possible spreading network
  - ✓ Genomic epidemiology and evolution
  - ✓ Dating early origin of pathogen



Aquaculture 479 (2017) 579–583

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Short communication

Evidence of TiLV infection in tilapia hatcheries from 2012 to 2017 reveals probable global spread of the disease

H.T. Dong[a,b,*], G.A. Ataguba[c], P. Khunrae[a], T. Rattanarojpong[a], S. Senapin[b,d,**]

Received: 30 April 2020 | Revised: 7 June 2020 | Accepted: 13 June 2020

DOI: 10.1111/tbed.13693

ORIGINAL ARTICLE

WILEY

Tilapia lake virus (TiLV): Genomic epidemiology and its early origin

Yuttapong Thawornwattana[1,2] | Ha Thanh Dong[3] | Kornsunee Phiwsaiya[4,5] | Pakkakul Sangsuriya[5,6] | Saengchan Senapin[4,5] | Pakorn Aiewsakun[1,2]

Based on 17 TiLV whole-genome sequences

"We estimated the origin of TiLV to be between 2003 and 2009, 5–10 years before the first report of the virus in Israel in 2014. Our analyses consistently showed that TiLV started to spread in 2000s, and reached its peak in 2014–2016, matching well with the timing of its first report. From 2016 onwards, the global TiLV population declined steadily."

Food and Agriculture Organization of the United Nations

SUSTAINABLE DEVELOPMENT GOALS

Thank you for your attention!

Ha Thanh Dong
hadongntu@gmail.com

TCP/INT/3707:
Strengthening biosecurity (policy and farm level) governance to deal with Tilapia lake virus

This was also made possible with the support of the Norwegian Agency for Development Cooperation under the project GCP/GLO/979/NOR Improving Biosecurity Governance and Legal Framework for Efficient and Sustainable Aquaculture Production.

Norad