



Food and Agriculture
Organization of the
United Nations



FAO/China Intensive Training Course on Tilapia Lake Virus (TiLV)

Sun Yat Sen University, Guangzhou, China

18-24 June 2018

Session 2

Win Surachetpong DVM, PhD, CertAqV, DTBVP

Kathy Tang-Nelson PhD

**TiLV isolation and
Koch's Postulates**

Learning objectives

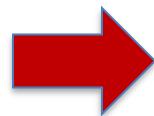
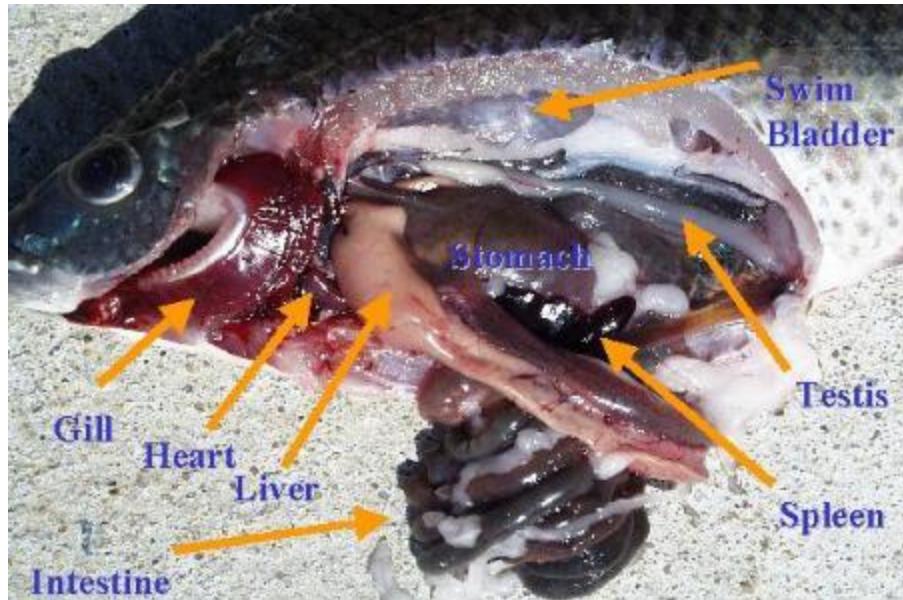
- **Describe how viruses are isolated**
- **Apply the appropriate method to the identification of a virus**
- **Explain Koch's Postulates and experiment to fulfill it**

How can we detect viruses? Identifying the etiology of a new disease

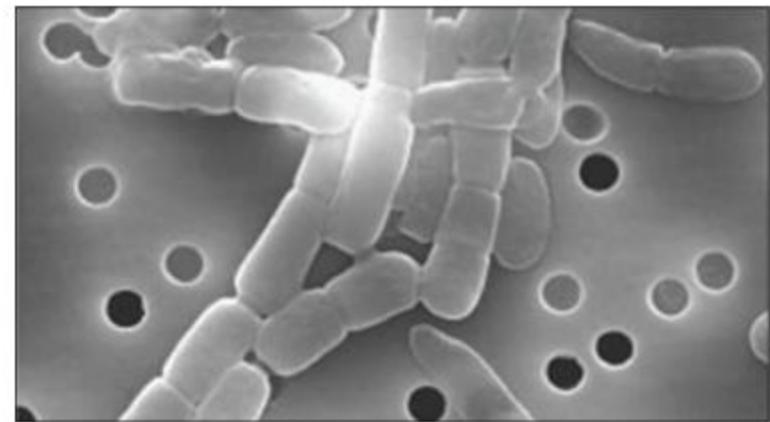
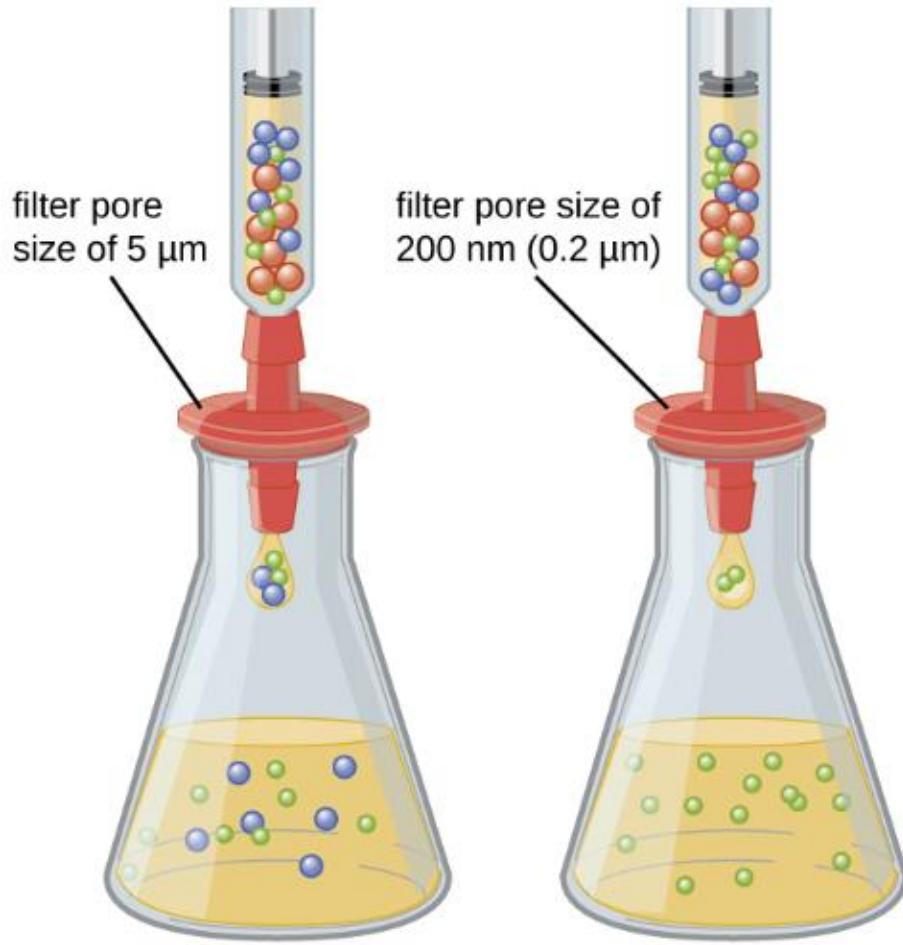
"to open Pandora's box"
means to perform an action
that may seem small or
innocent, but that turns out
to have severely
detrimental and far-
reaching negative
consequences. (Wikipedia)



Virus isolation



Filtration of virus through membrane



Virus isolation: general concept

Viruses are obligate intracellular parasites and can replicate only within living host cells. These include cell cultures, embryonated eggs, and animals.

Cell cultures are the host system most frequently used for virus cultivation.

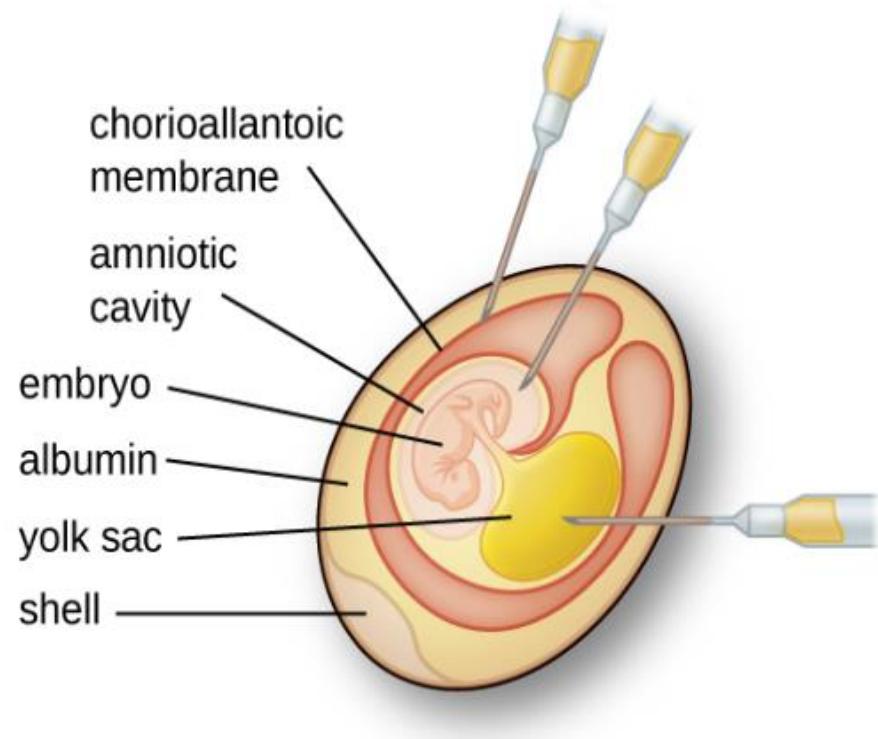


Leland D.S., French M.L.V. 1988. Virus Isolation and Identification. In: Laboratory Diagnosis of Infectious Diseases Principles and Practice. Springer, NY

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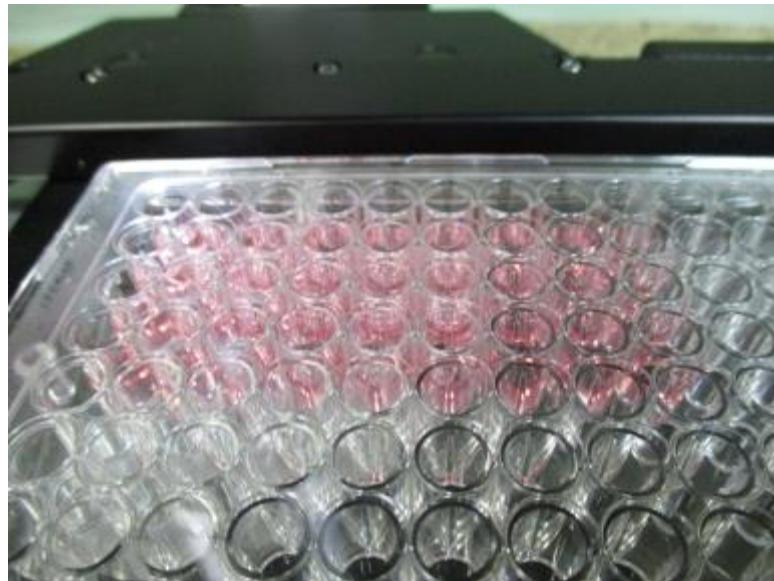
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Cultivation of virus in embryonic eggs

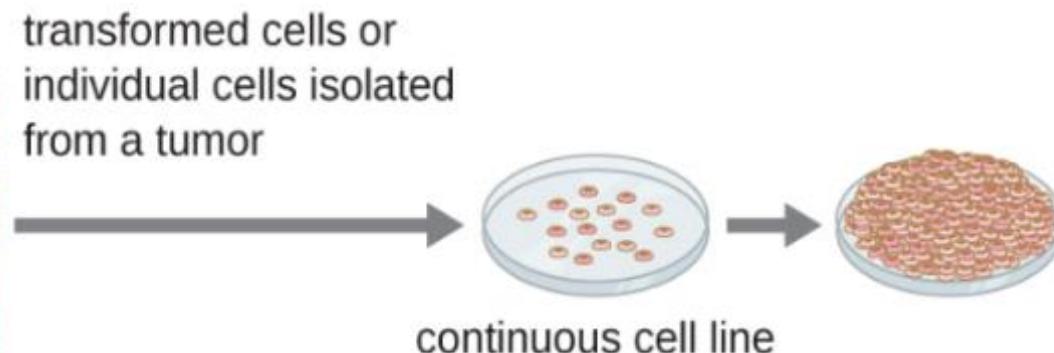
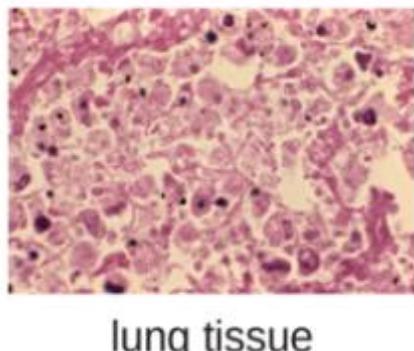
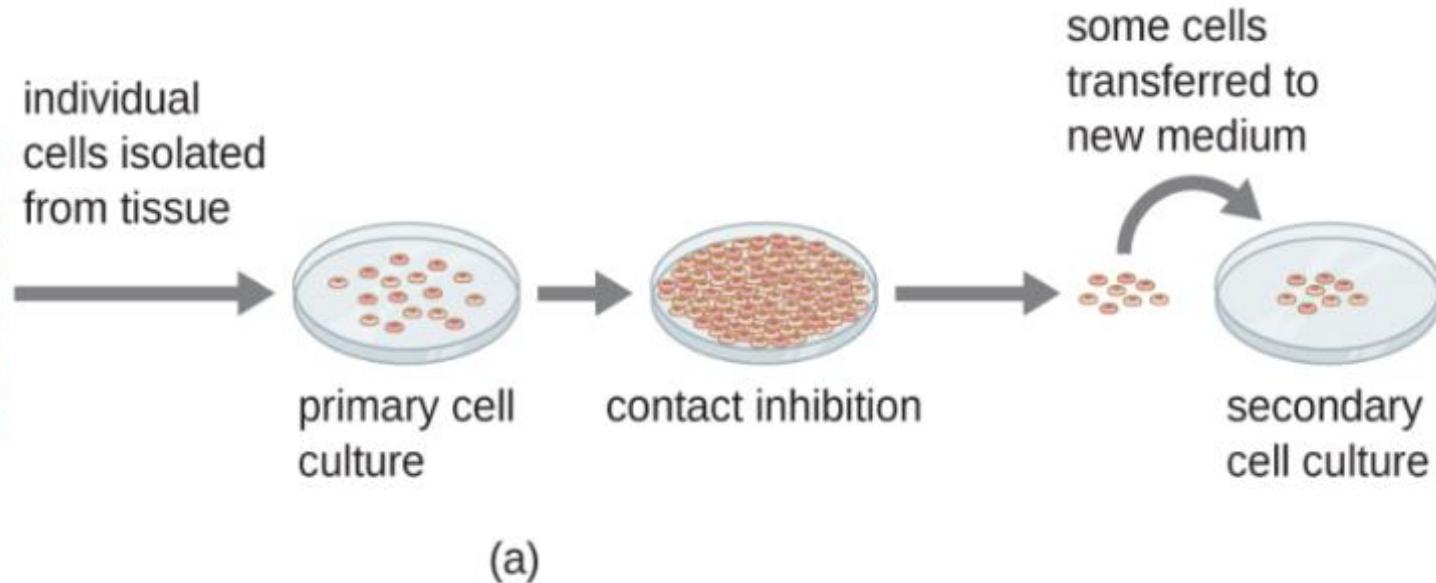
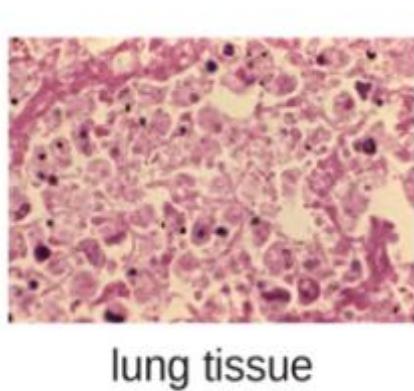


Cell culture

Cell culture techniques are an important for detection and surveillance of fish viruses. Cell culture system require optimal environment, adequate nutrition and regular checkups.

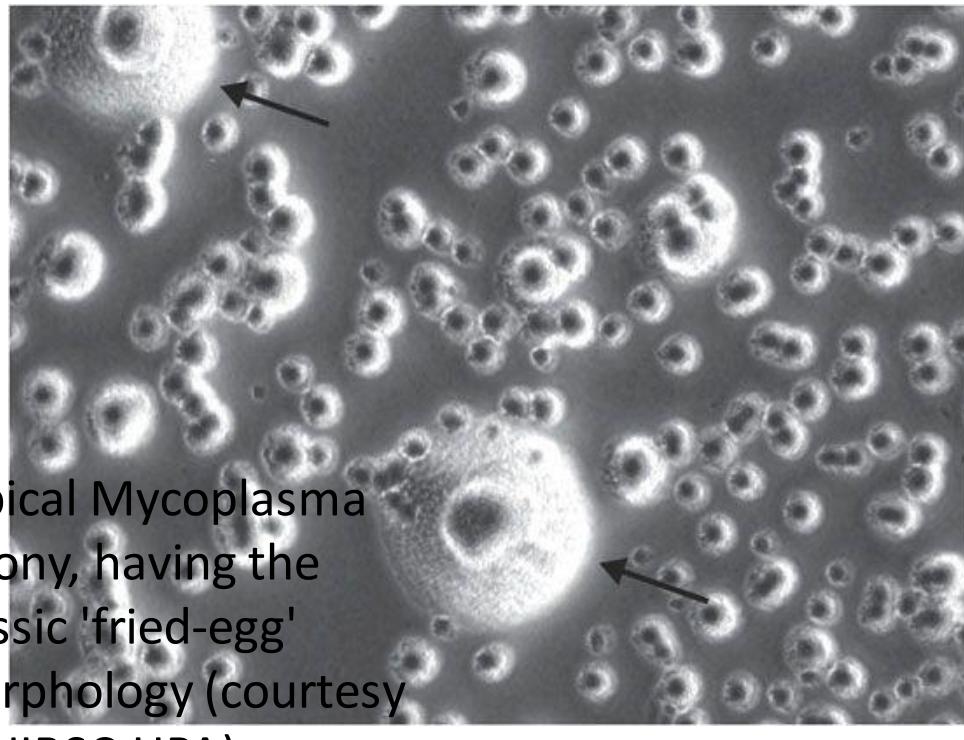


Preparation of cell line for viral propagation

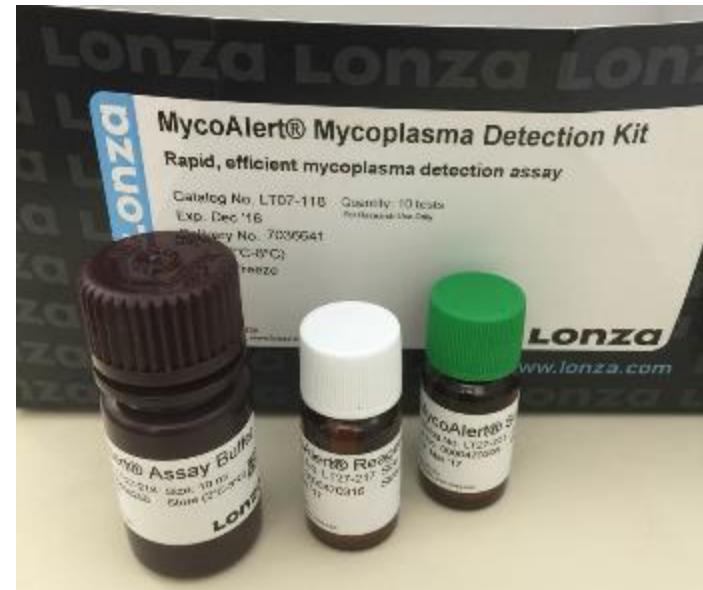


The cell line must be mycoplasma free.

The mycoplasma infection can alter cell culture morphology by deplete nutrition, induce chromosomal breaking and interfere with viral expression and viral isolation.



Typical Mycoplasma colony, having the classic 'fried-egg' morphology (courtesy of NIBSC-HPA).



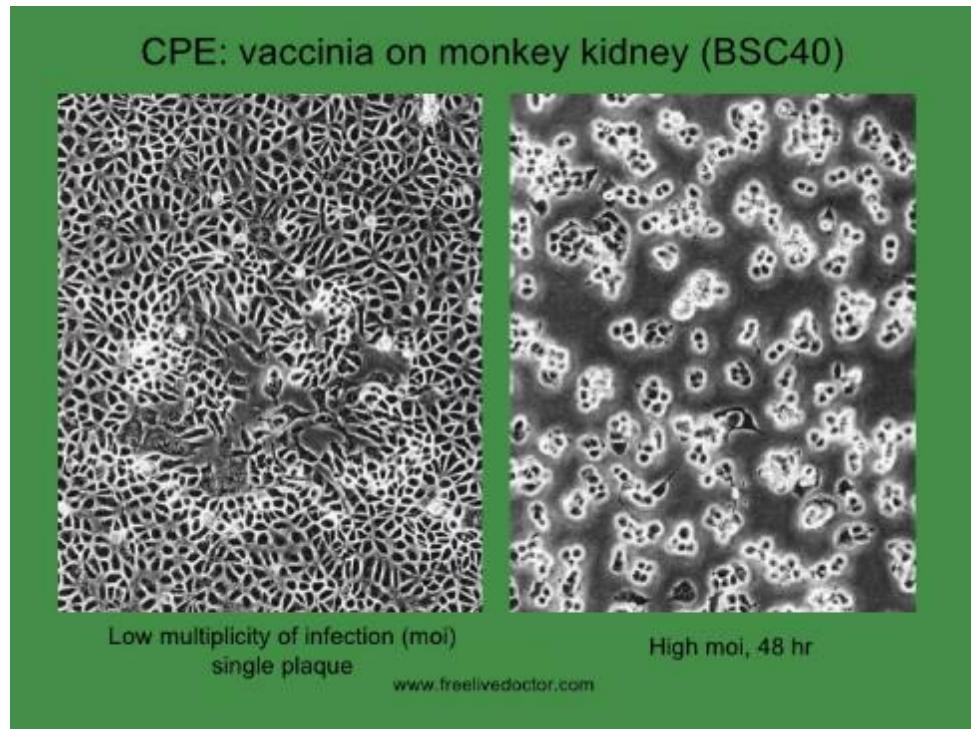
Young, L., J. Sung, G. Stacey and J. R. Masters. 2010. Detection of Mycoplasma in cell cultures. Nature Protocols 5: 929.

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Identifying viruses in cell culture

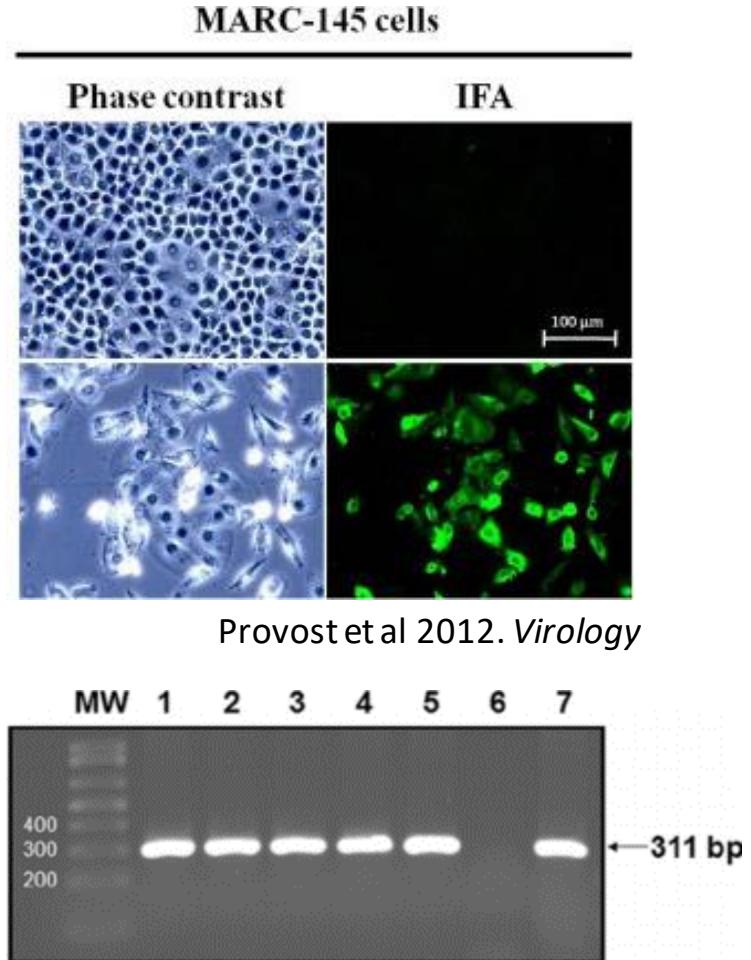
Morphological changes during viral infection

1. Rounding
2. Detachment
3. Syncytia or fusion
4. Shrinkage
5. Increased refractivity
6. Aggregation
7. Loss of adherence
8. Cell lysis or death



Identifying viruses in cell culture

- Immunofluorescent
- In situ hybridization
- RT-PCR, RT-qPCR



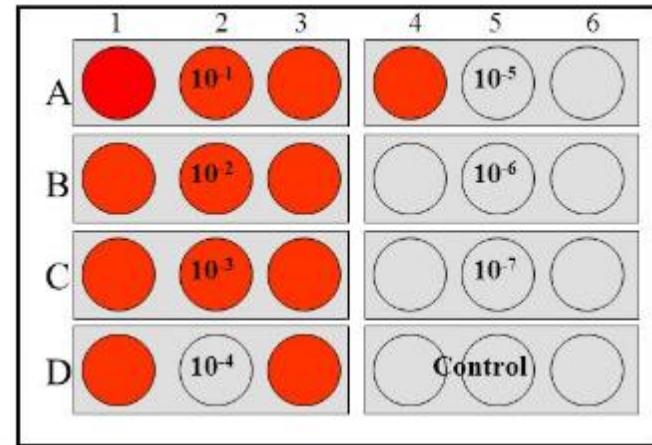
Quantification of virus

- Plaque assay

TCID₅₀

- TCID50/end point dilution assay

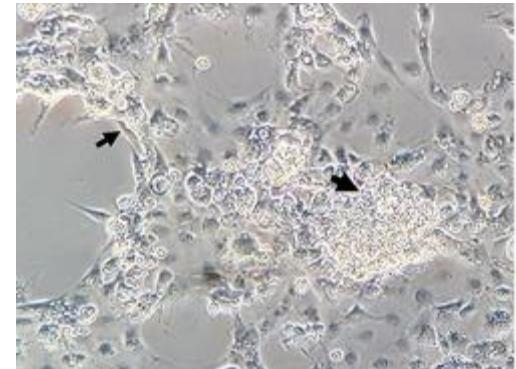
Dilution	Infected	% Infected
10-1	3/3	100
10-2	3/3	100
10-3	3/3	100
10-4	2/3	66
10-5	1/3	33
10-6	0/3	0
10-7	0/3	0



$$\text{Log PD} = \frac{66-50}{66-33} \times (\text{Log} 10)$$

66 Log PD = 0.48
33 Log Dilution above 50 %
0 Infection $10^{-4.48}$

TiLV viral isolation



Clinical specimens

- Organ and tissue
- Mucus

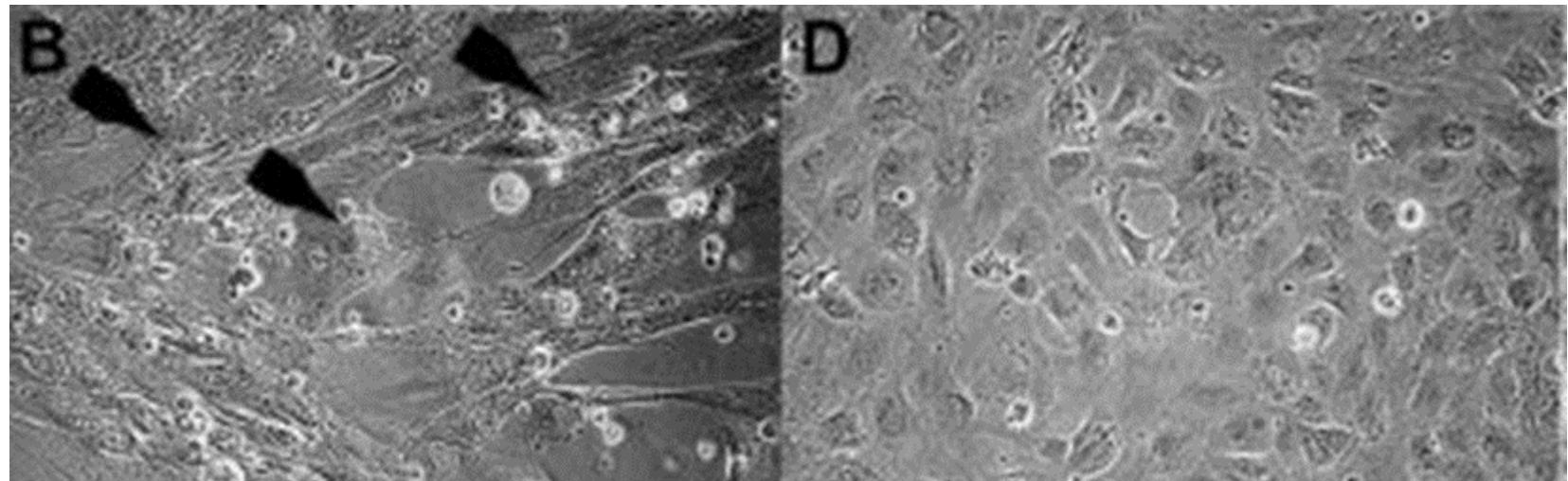
Virion isolation and cultivation in cell culture

**CPE were
observed
in viral
replicated cells**

Cell line for TiLV isolation

Primary tilapia brain cells

The typical elongated cells is normal characters of primary tilapia brain cell culture. CPE show swollen, rounded, and granulated cells (marked with arrows)

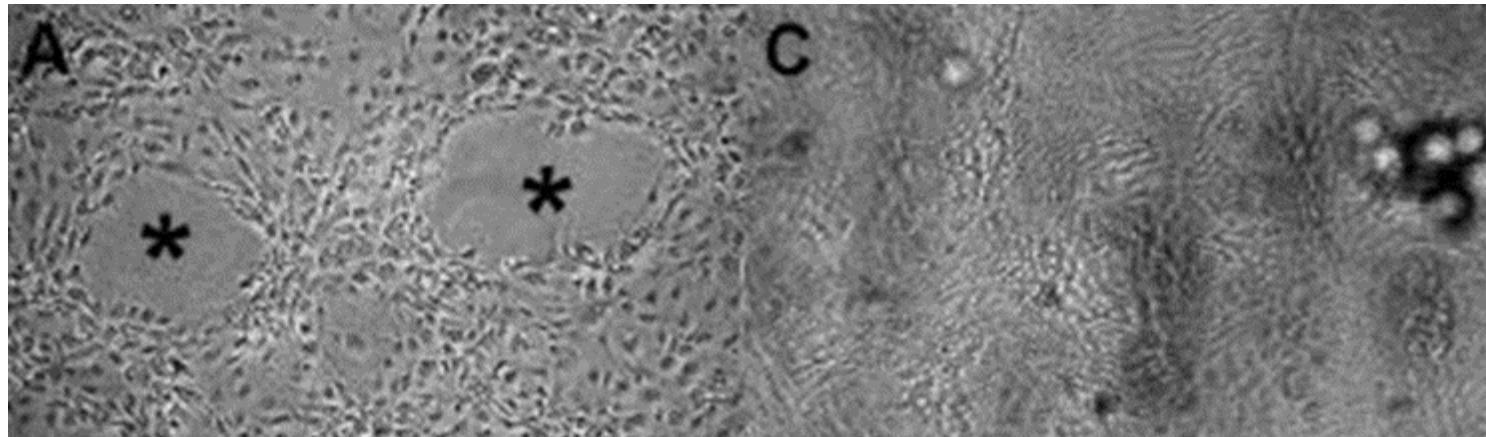


Eyngor et.al., 2014 JCM: 52(12): p4137-4146.

Cell line for TiLV isolation

E-11 cell line

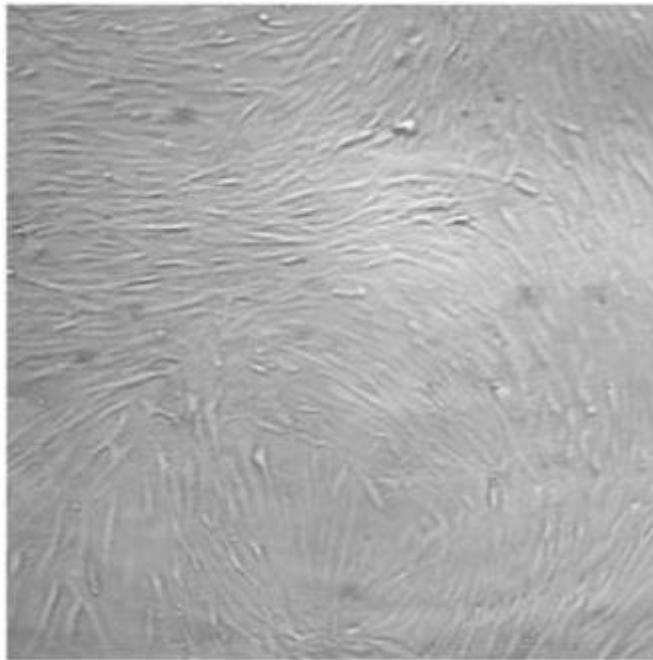
E-11 from the striped snakehead (*Ophicephalus striatus*). The commercial fibroblast cell from whole fry tissue. Because the adherent type, the clear CPE show the cytoplasmic vacuole formation followed by intensive disintegration.



Plaque formation and vacuolated cells at the rims of the plaques. The centers of two plaques are marked with asterisks. Eyangor et.al., 2014 JCM: 52(12): p4137-4146.

Cell line for TiLV isolation

Mozambique tilapia (*Oreochromis mossambicus*) cell lines from brain (OmB)



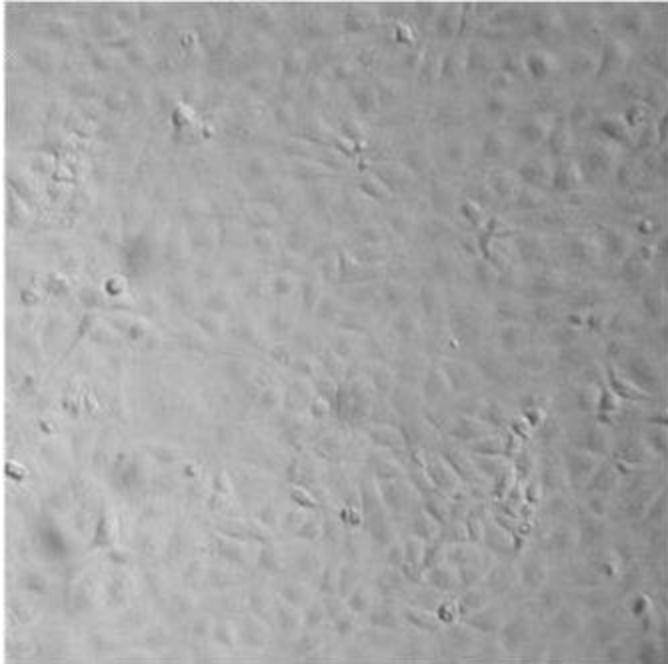
OmB cells are *fibroblast-like cell*.
CPE were characterized by
almost complete detachment from
the plate.

Gardell, A. M., et al. 2014. Derivation and Osmotolerance Characterization of Three Immortalized Tilapia (*Oreochromis mossambicus*) Cell Lines. PLoS ONE, 9(5), e95919.

Kembou Tsofack JE, et al. 2017. Detection of Tilapia lake virus in clinical samples by culturing and nested reverse transcription-PCR. J Clin Microbiol 55:759–767

Cell line for TiLV isolation

Mozambique tilapia (*Oreochromis mossambicus*) cell lines from bulbus arteriosus (TmB)



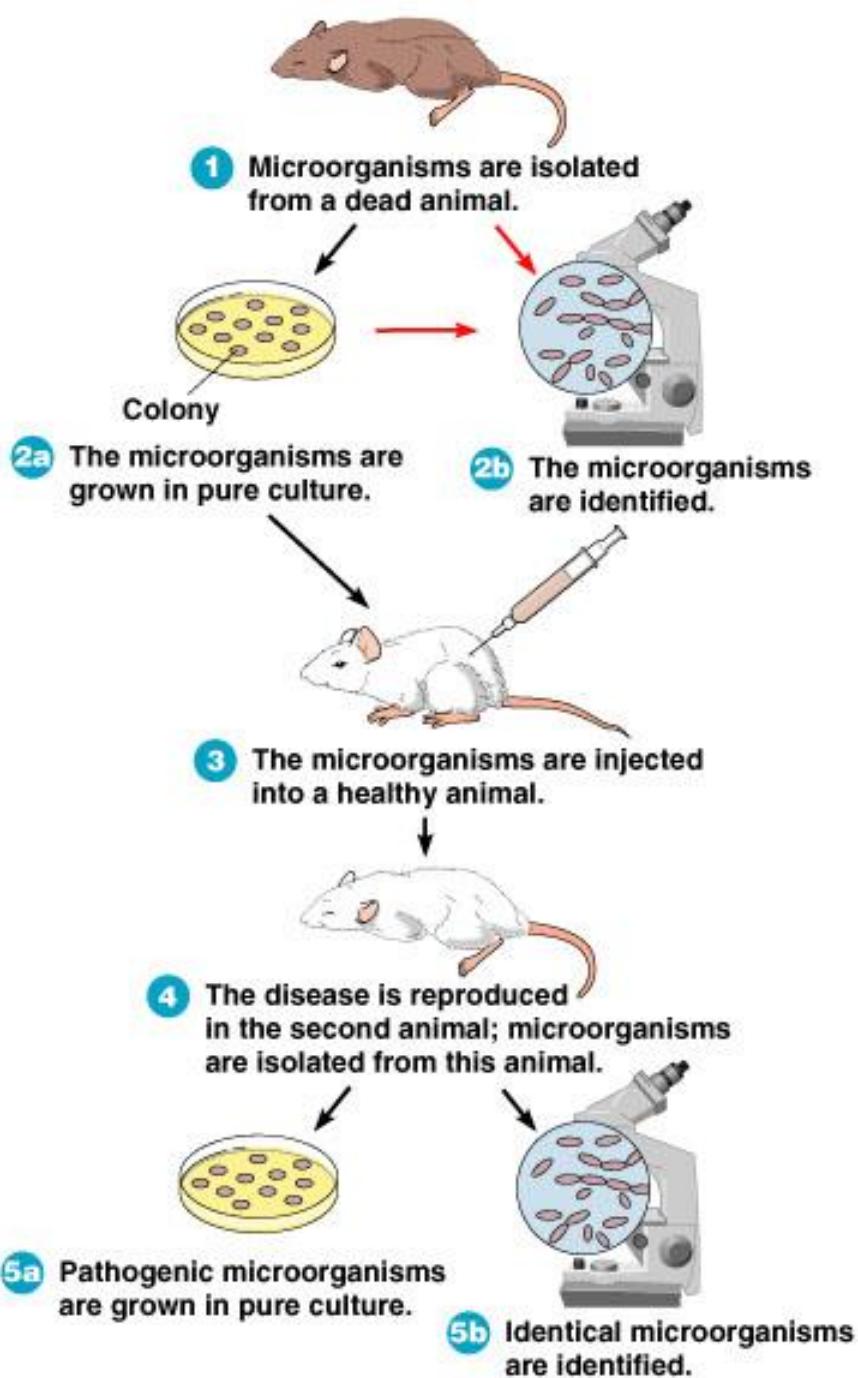
TmB cells are *endothelial cell*.
CPE detecting was more difficult
cause TmB cells did not support the
formation of clear plaques

Gardell, A. M., et al. 2014. Derivation and Osmotolerance Characterization of Three Immortalized Tilapia (*Oreochromis mossambicus*) Cell Lines. PLoS ONE, 9(5), e95919.

Kembou Tsofack JE, et al. 2017. Detection of Tilapia lake virus in clinical samples by culturing and nested reverse transcription-PCR. J Clin Microbiol 55:759–767

Koch's Postulates

Concept of Koch's Postulates



River's Postulates

- 1. Isolate virus from diseased hosts**
- 2. Cultivation of virus in host cells**
- 3. Proof of filterability**
- 4. Production of a comparable disease when the cultivated virus is used to infect experimental animals**
- 5. Reisolation of the same virus from infected challenge animal**
- 6. Detection of a specific immune response to the virus**

TiLV can cause disease in susceptible/normal tilapia

- Koch's Postulates



Veterinary Microbiology

Volume 207, August 2017, Pages 170–177



Experimental infection of Tilapia Lake Virus (TiLV) in Nile tilapia (*Oreochromis niloticus*) and red tilapia (*Oreochromis* spp.)

Puntanat Tattiayapong^{a, b}, Worawan Dachavichitlead^{a, b}, Win Surachetpong^{a, b},  

1. The pathogen is isolated from naturally exposed fish

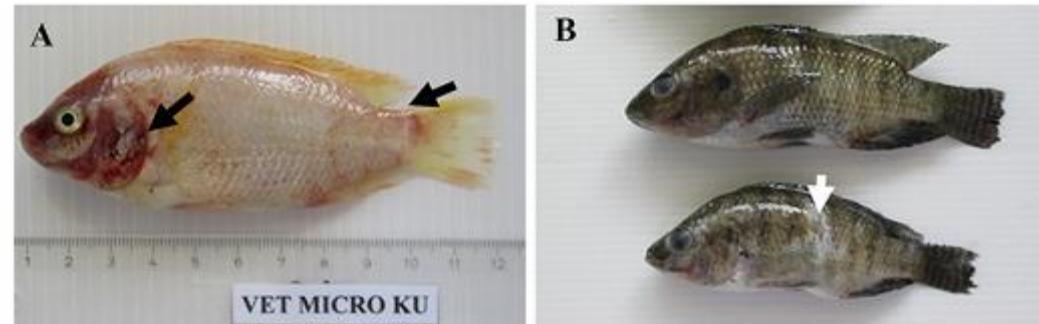


Table 1
Details of clinical isolates.

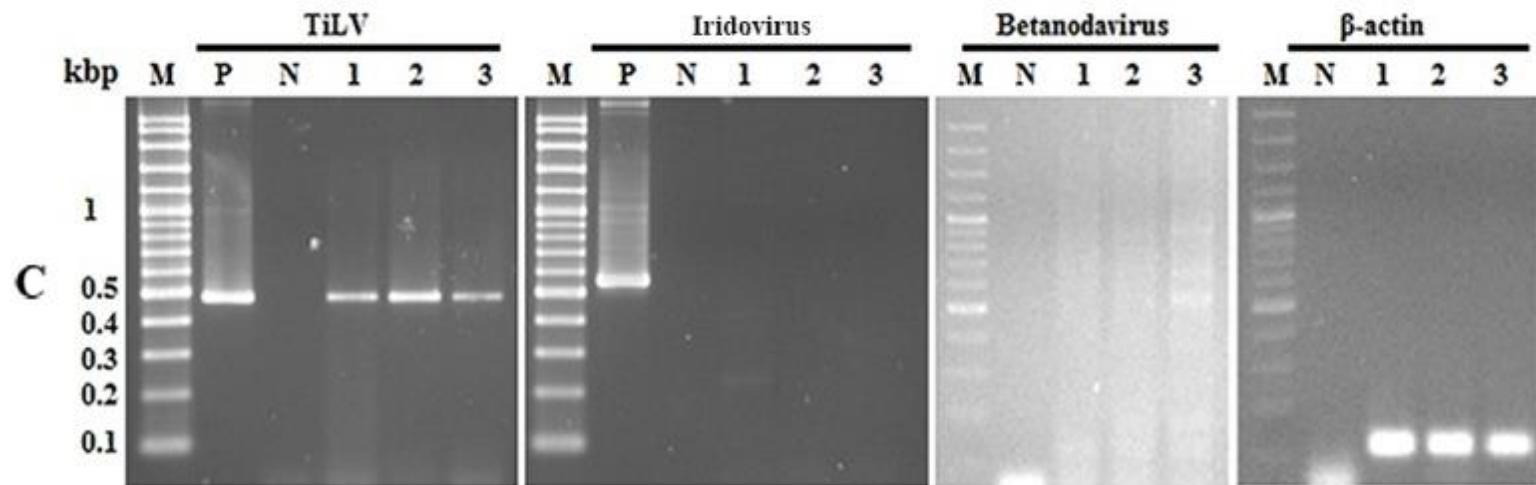
Sample no.	Location	Collection date	Clinical signs	PCR test
1	Ang Thong	02/06/16	+	+
2	Pathum Thani	22/06/16	+	+
3	Pathum Thani	28/06/16	+	+
4	Ang Thong	01/07/16	+	+
5	Pathum Thani	02/08/16	+	+
6	Pathum Thani	05/08/16	+	+
7	Ang Thong	16/08/16	+	+
8	Ang Thong	22/08/16	+	+
9	Kanchanaburi	24/08/16	+	+
10	Pathum Thani	16/09/16	+	+
11	Pathum Thani	02/10/16	+	+
12	Pathum Thani	05/10/16	+	+
13	Pathum Thani	16/10/16	+	+
14	Ang Thong	25/11/16	+	+
15	Pathum Thani	26/11/16	+	+

1. The pathogen is isolated from naturally exposed fish

- Skin redness, skin erosion, corneal opacity

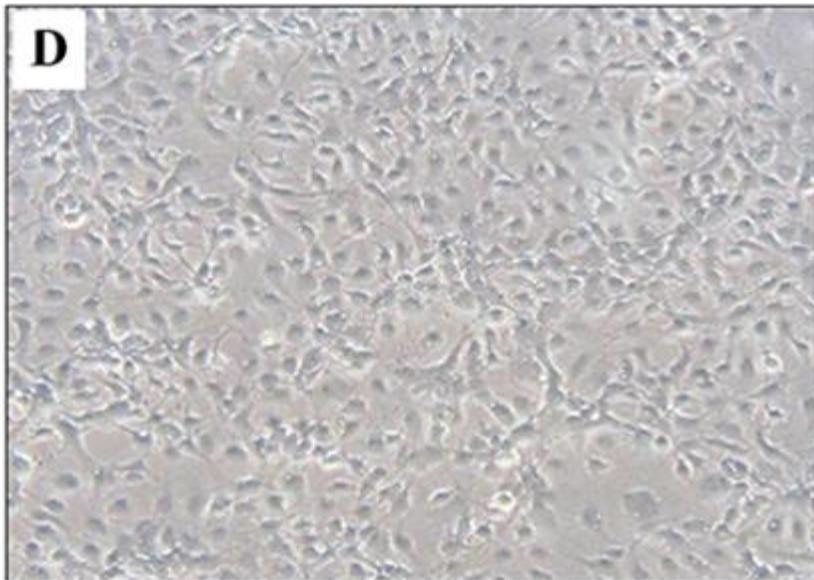


- Detection only TiLV, not other viruses

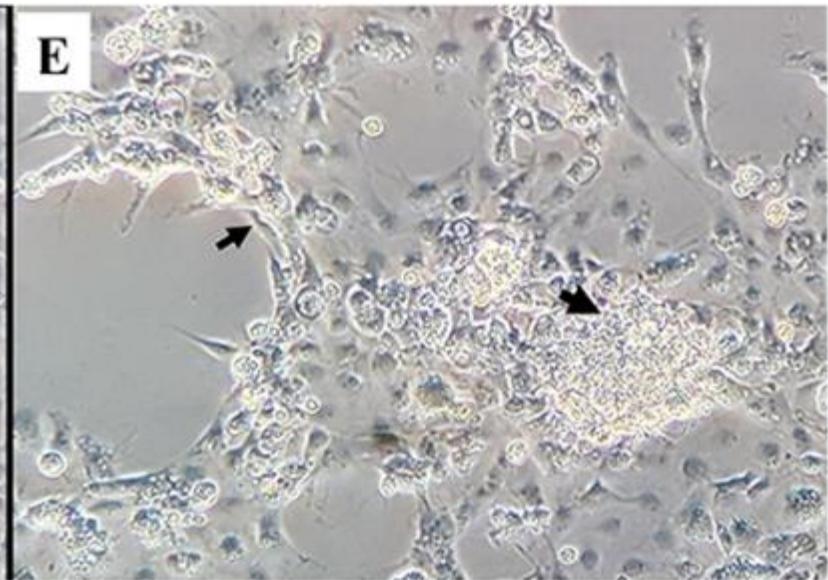


2. The pathogen was isolated in E-11 cells

Normal brain



Infected brain



- 3-5 dpi, CPE were observed in E-11 cells inoculated with infected brain

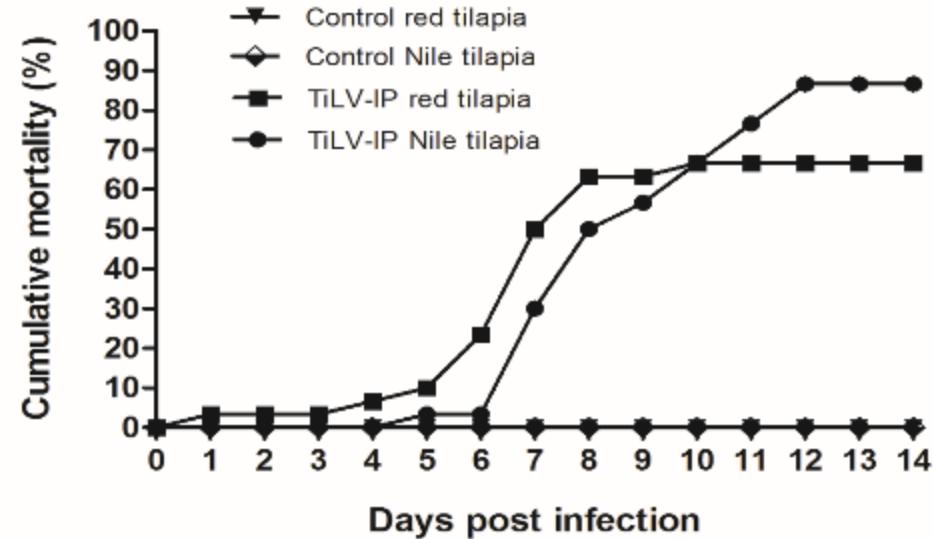
3. The pathogen caused disease in challenged fish

- Challenged Nile and red tilapia developed clinical signs of TiLV infection with high mortality

Clinical signs of challenged fish



Mortality rate



4. The pathogen was isolated in E-11 cells

- CPE formation in E-11 cells inoculated with brain from experimentally challenged tilapia

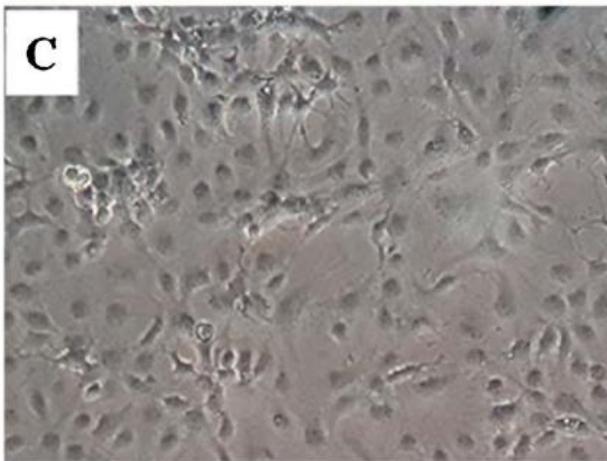
TiLV challenge Nile Tilapia



TiLV challenge Red Tilapia

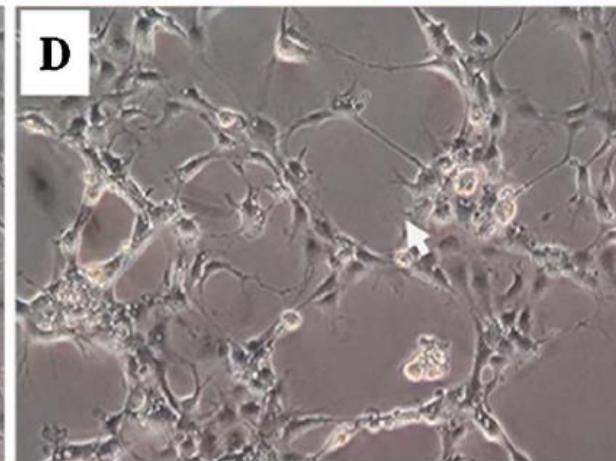


C



Control brain

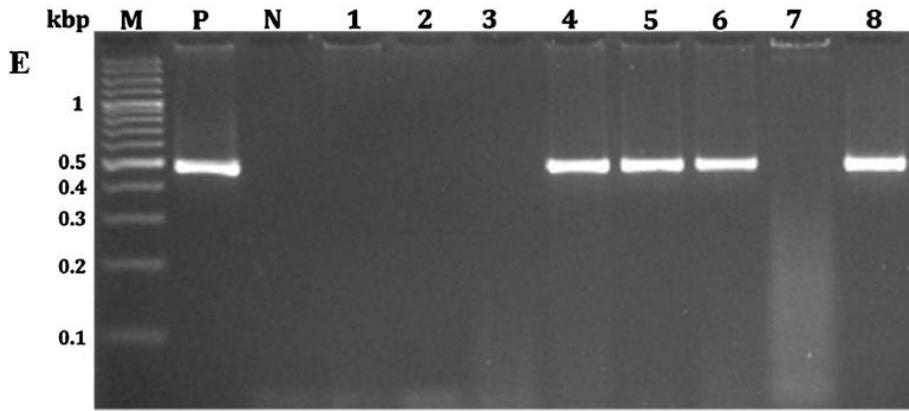
D



TiLV challenge brain

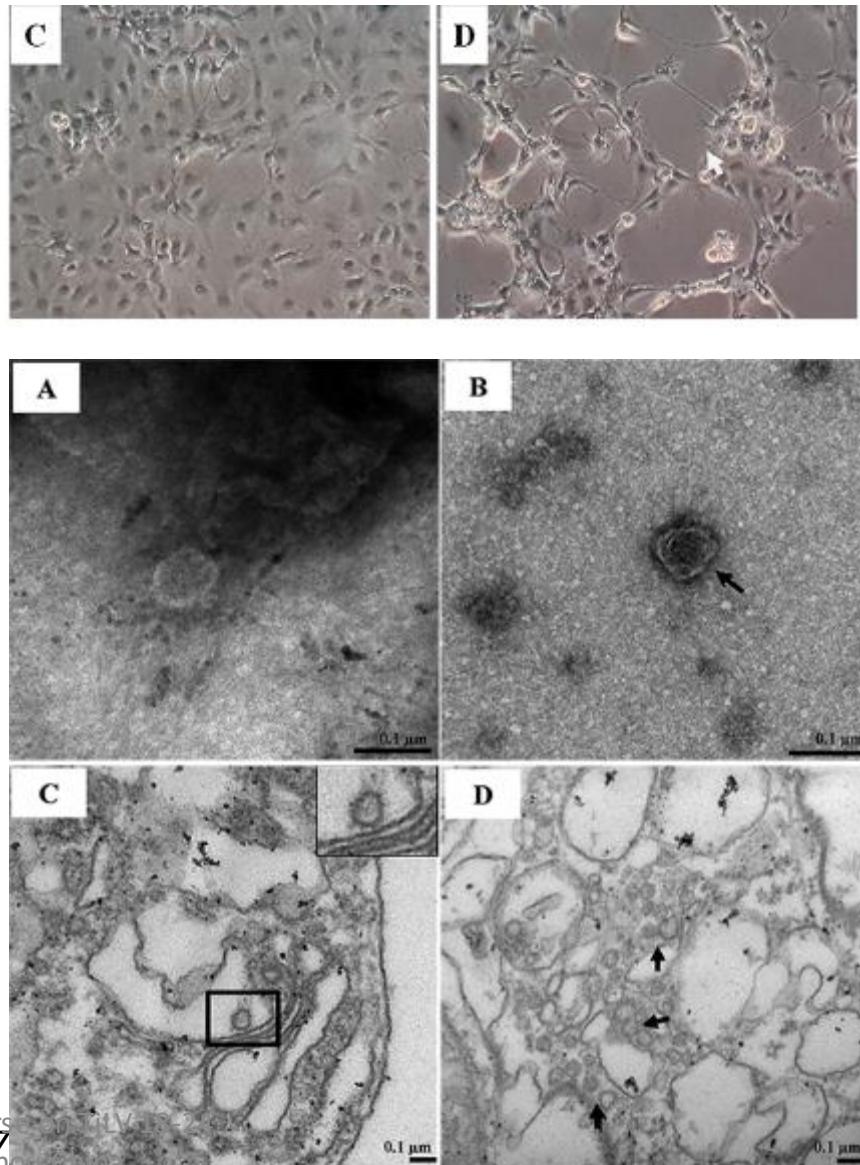
4. The pathogen was isolated from challenged fish

- Confirmation of TiLV using RT-PCR and TEM (infected E-11, and brain tissues of challenged fish)

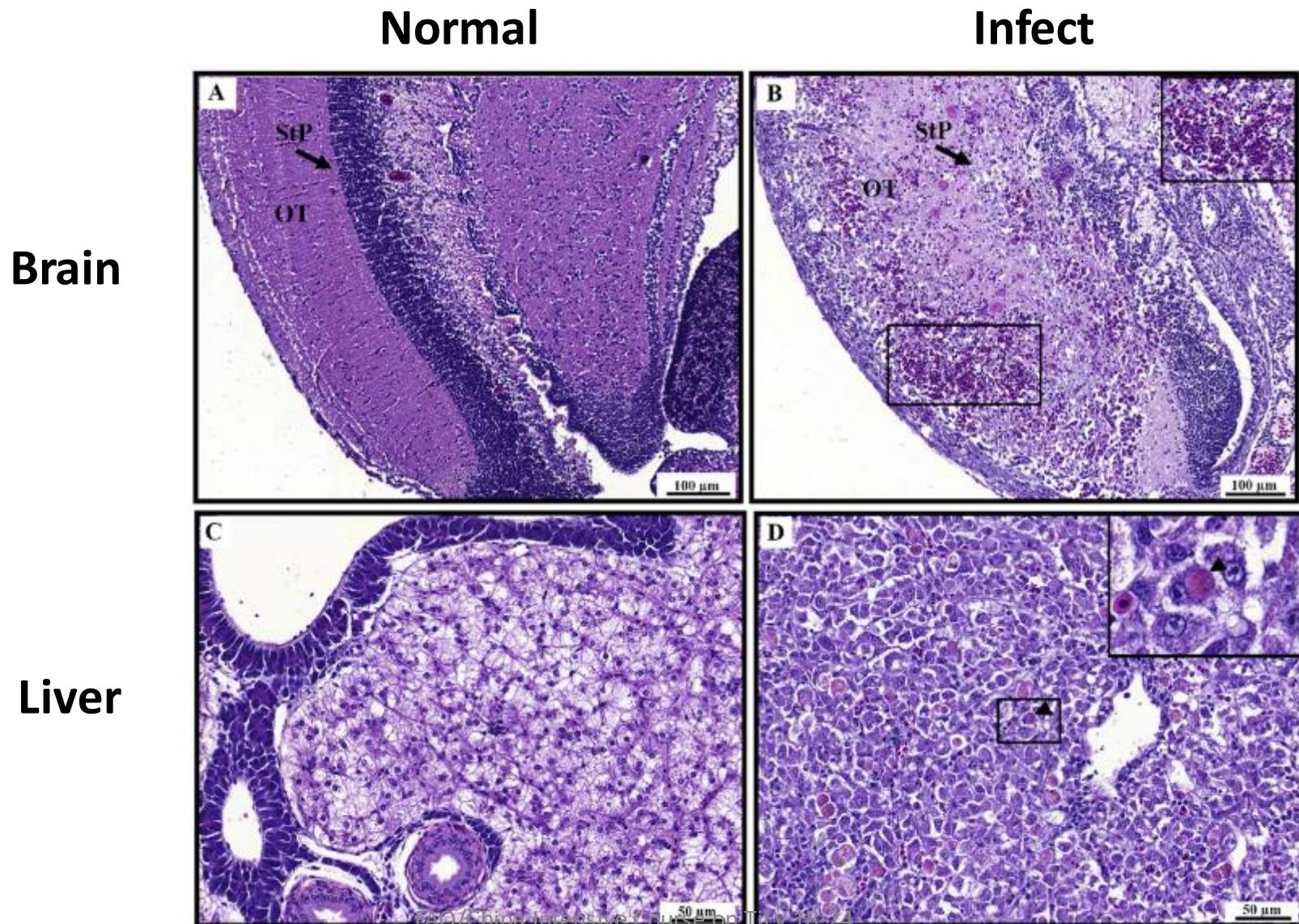


1-3: brain from PBS injected fish

4-7: brain from TiLV injected fish



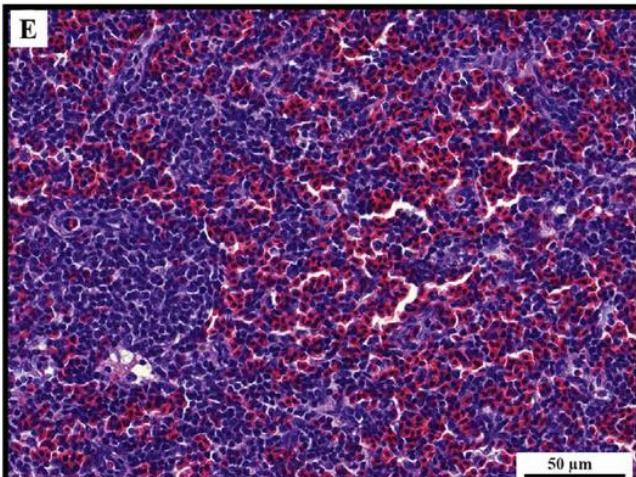
Histopathology of TiLV-challenged fish



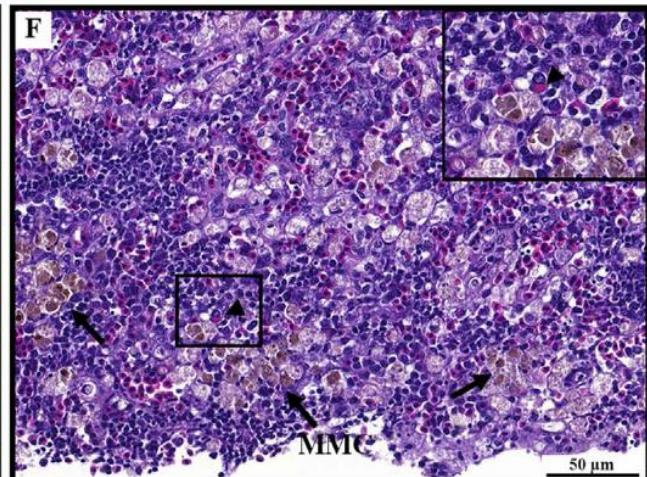
Histopathology of TiLV-challenged fish

Spleen

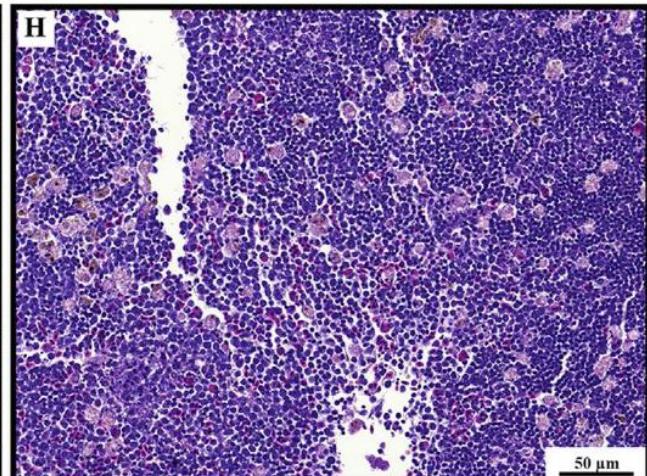
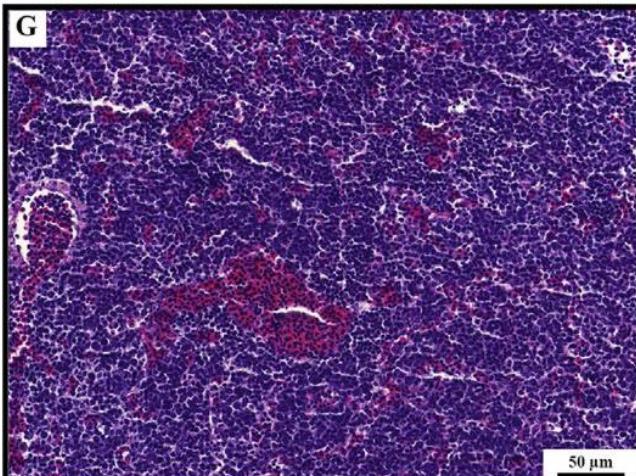
Normal



Infect



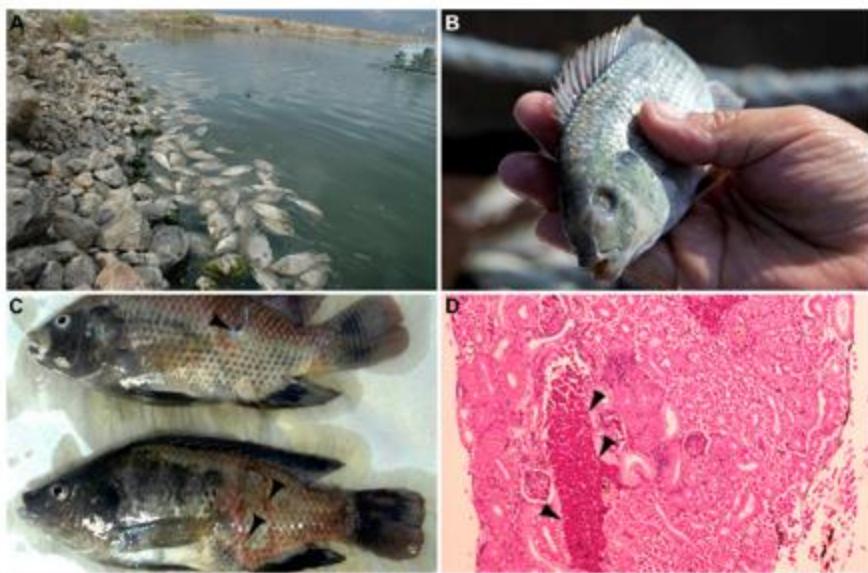
Anterior
kidney



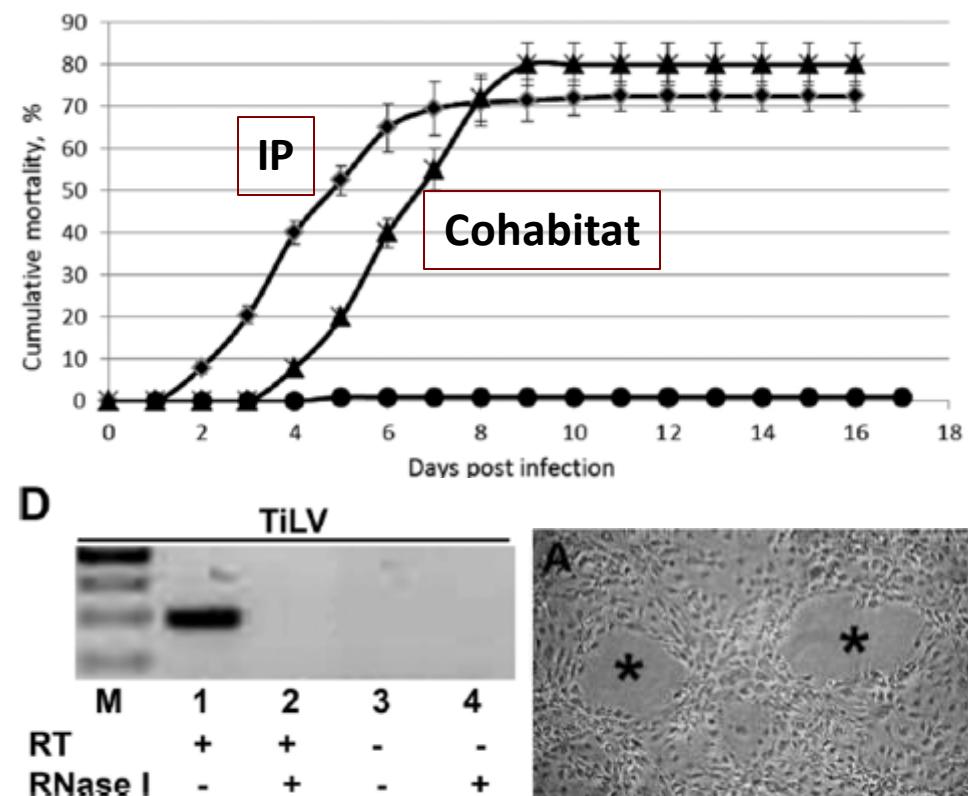
Identification of a Novel RNA Virus Lethal to Tilapia

Marina Eyangor,^a Rachel Zamostiano,^b Japhette Esther Kembou Tsofack,^b Asaf Berkowitz,^a Hillel Bercovier,^c Simon Tinman,^d Menachem Lev,^e Avshalom Hurvitz,^f Marco Galeotti,^g Eran Bacharach,^b Avi Eldar^a

Department of Poultry and Fish Diseases, The Kimron Veterinary Institute, Bet Dagan, Israel^a; Department of Cell Research and Immunology, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel^b; The Hebrew University-Hadassah Medical School, Jerusalem, Israel^c; Department of Animal Facility, Faculty of Life Sciences, Bar Ilan University, Ramat Gan, Israel^d; Ein Gev Fisheries, Kibbutz Ein Gev, Israel^e; Dan Fish Farms, Kibbutz Dan, Upper Galilee, Israel^f; Department of Food Science, Section of Veterinary Pathology, University of Udine, Udine, Italy^g

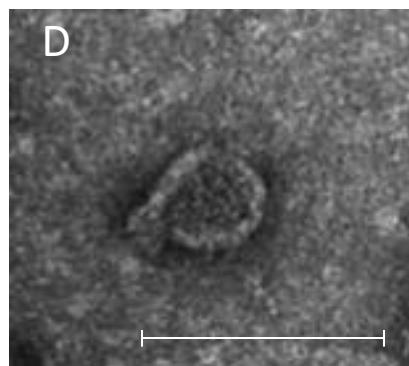
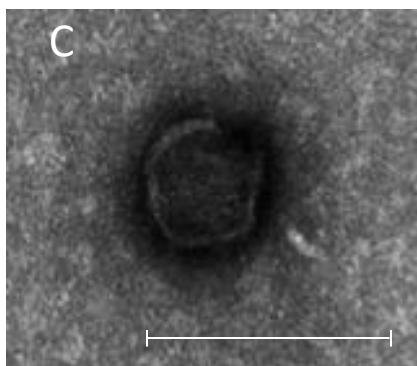
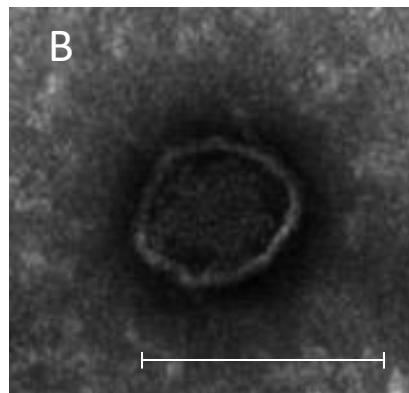
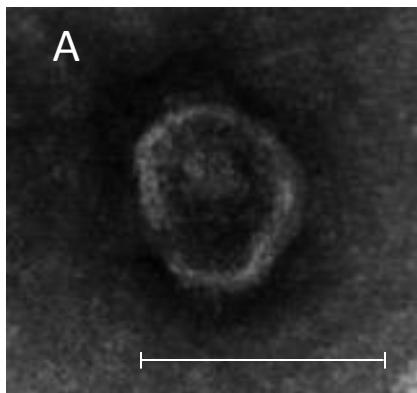


Tilapia Lake Virus: TiLV

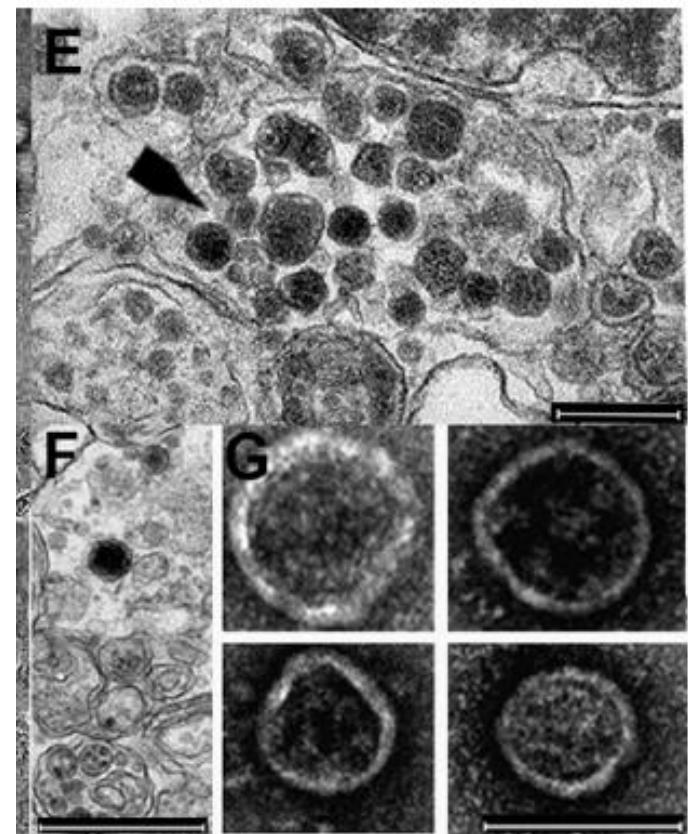


Comparison of Thai and Israel virus

Thai



Israel

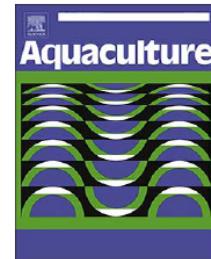


Transmission electron microscope (TEM) of negatively staining of Thai TiLV virions. The virions size are 50-70 nm. Note: The virus has envelope structure (A-F). Scale bar, 100 nm.

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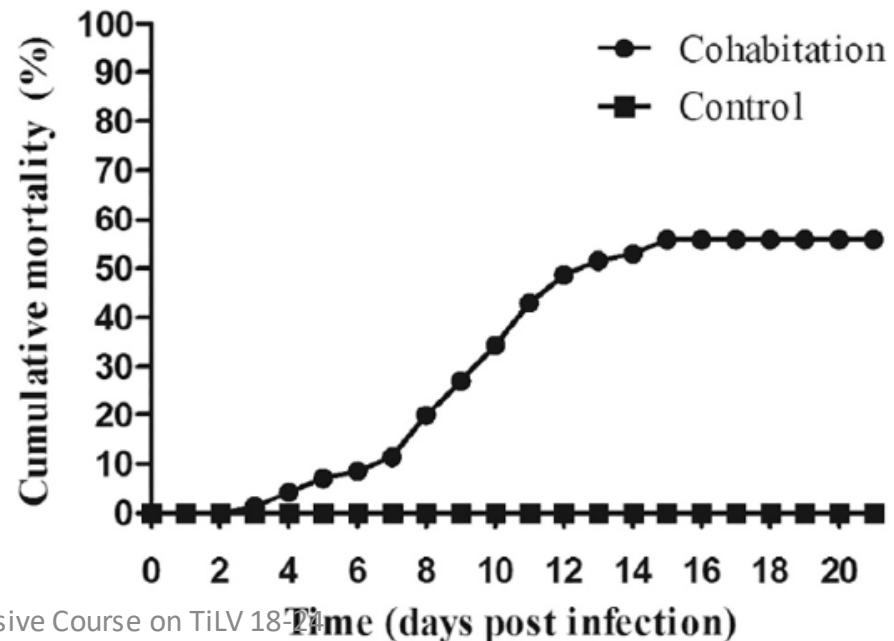
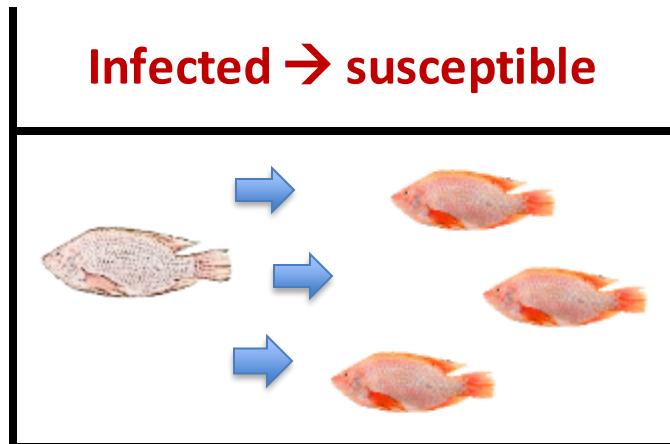
Eyngor et.al., 2014 JCM: 52(12):
p4137-4146.



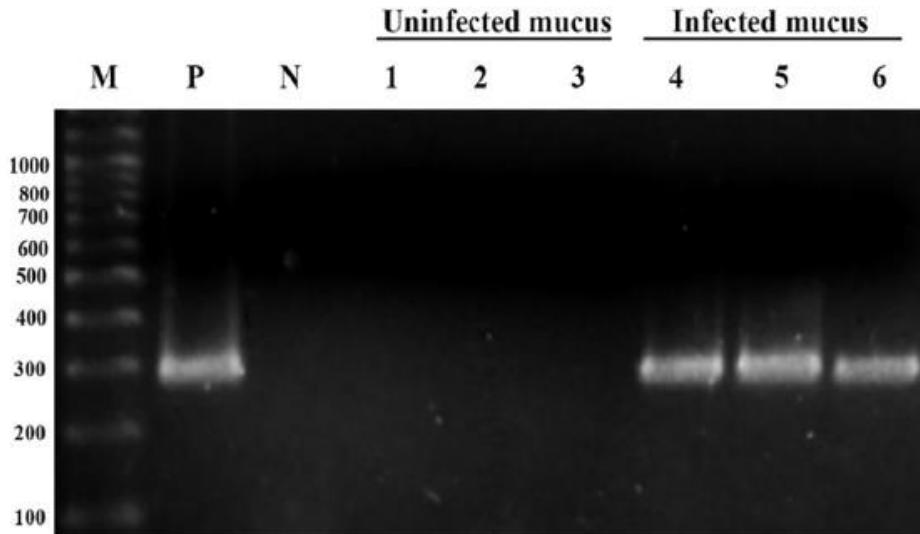
Non-lethal sampling for Tilapia Lake Virus detection by RT-qPCR and cell culture



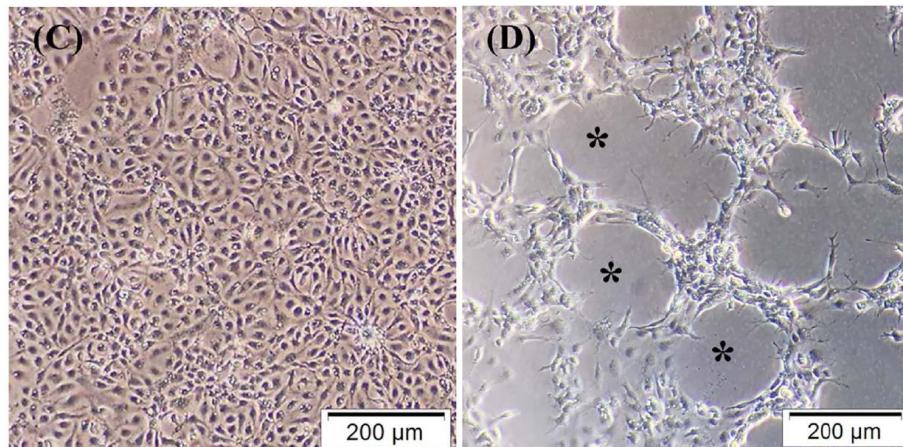
Pavarit Liamnimitr^a, Worrayanee Thammatorn^a, Sonicha U-thoomporn^a, Puntanat Tattiyapong^b, Win Surachetpong^{a,b,*}



Horizontal transmission of TiLV via fish mucus



- **Detection of TiLV in infected tilapia mucus**



- **Virus in mucus is still infective!**

Summary

- **Several cell lines are susceptible to TiLV infection**
- **TiLV isolated in Thailand and Israel can cause disease in challenged tilapia**
- **Fulfilled Koch's postulates of TiLV infection in tilapia**

Acknowledgements



Our team



สำนักงานพัฒนาการวิจัยการเกษตร (องค์การมหาชน)
AGRICULTURAL RESEARCH DEVELOPMENT AGENCY (PUBLIC ORGANIZATION)



Agri&Food
Innovation hub



Prof. Alongkorn Amonsin
Chulalongkorn University



Taveesak Janetanakit
(DVM)