LOCALIZATION OF *TILAPIA TILAPINEVIRUS* BY *IN SITU* HYBRIDIZATION IN THE BRAIN OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

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การระบุตำแหน่งของเชื้อไวรัสทิลาเปีย ทิลาปิน โดยวิธีอินซิทูไฮบริไดซ์เซชั่น ในเนื้อเยื่อสมองของปลานิล (โอรีโอโครมิส นิโลติคัส)

นายดินห์ ฮุง เหวียน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	LOCALIZATION OF *TILAPIA TILAPINEVIRUS* BY *IN SITU* HYBRIDIZATION IN THE BRAIN OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*)
By	Mr. Hung Dinh Nguyen
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ดินห์ ฮุง เหวียน : การระบุตำแหน่งของเชื้อไวรัสทิลาเปีย ทิลาปิน โดยวิธีอินซิทูไฮบริไดซ์เซชั่น ในเนื้อเยื่อสมองของปลานิล (โอรีโอโครมิส นิโลติคัส). (LOCALIZATION OF *TILAPIA TILAPINEVIRUS* BY *IN SITU* HYBRIDIZATION IN THE BRAIN OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*)) อ.ที่ปรึกษาหลัก : ชาญณรงค์ รอดคำ, อ.ที่ปรึกษาร่วม : ฮา ทานห์ ดง

ทิลาเปีย ทิลาปินไวรัส หรือ ทิลาเปีย เลค ไวรัส (*Tilapia tilapinevirus* or tilapia lake virus; TiLV) เป็นเชอื้ ไวรัสอุบัติใหม่ที่ส่งผลกระทบที่เสียหายแก่ฟาร์มปลานิลทั่วโลก ในระหว่างที่เกิดโรค ปลานิลจะมีอาการผิดปรกติ เช่น ผอมโซ ไม่กินอาหาร ว่ายน้ำหมุนวน และไม่ว่ายน้ำรวมฝูง ซึ่งมีความเป็นไปได้ที่อาจเกิดความเสียหายที่สมอง มีการศึกษาก่อนหน้านที้ ี่ได้มีการตรวจหาเชื้อไวรัสจากอวัยวะต่างๆ ของปลาที่ติด เชื้อ อย่างไรก็ตาม ความรู้ในเชิงลึกเกี่ยวกับการปรากฏของเชื้อไวรัสที่ระบบประสาทส่วนกลางหรือสมอง ยังมีอยู่น้อยมาก จากเหตุผลที่กล่าวมาจึงเป็นมูลเหตุจูงใจให้ผู้วิจัยศึกษาถึงการกระจายตัวของเชื้อไวรัส และพยาธิวิทยาเนื้อเยื่อในสมองของปลานิลที่ถกู ทำให้ติดเชื้อ TiLV ในหอ้ งทดลอง การศึกษาการกระจายตัวของไวรัสด้วยวิธีอินซิตู ไฮบริไดซ์เซชั่น เมื่อใช้โพรบที่มีความจำเพาะ พบว่า TiLV มีการกระจายตัวอยู่ทั่วทั้งสมอง และเป็นที่น่าสนใจว่าพบสัญญาณบวกของ TiLV เปน็ จำนวนมากบริเวณสมองส่วนหน้า ซงึ่ สมองส่วนนี้มีหน้าที่เกี่ยวกับพฤติกรรมการเรียนรู้ ความอยากอาหาร และความตั้งใจต่างๆ และยังพบสัญญาณบวกเป็นจำนวนมากที่สมองส่วนหลัง ซึ่งสมองส่วนนี้มีหน้าที่เกี่ยวกับการควบคุมการทรงตัว และสรีรวิทยาทั่วไป จากการพบสัญญาณบวกจำนวนมากที่สมองทั้งสองส่วนดังกล่าว แสดงให้ให้เหน็ ถึงความเกี่ยวข้องระหว่างความผิดปรกติของหน้าที่ของสมอง และอาการป่วยที่เกิดขึ้นของปลานิลที่ติดเชื้อ นอกจากนี้ยังพบว่า กลุ่มเซลล์ที่เชื้อไวรัสมักเข้าไปอาศัย ได้แก่ เซลล์ของหลอดเลอื ด และช่องสมอง (ventricles) ซึ่งบ่งชี้ว่า ไวรัสอาจจะมีช่องทางการเดินทางไปที่สมอง โดยผ่านไปทางระบบไหลเวียนโลหิต จากนั้นอาจจะมีความเป็นไปได้ที่จะแพร่ไปยังน้ำไขสันหลังในช่องสมอง และไปทำให้ติดเชื้อและเกิดความผิดปรกติของสมองขึ้น ความเข้าใจเกี่ยวกับรูปแบบการปรากฏของเชื้อไวรัส TiLV ที่สมอง จะช่วยในการวิเคราะห์แยกแยะอาการทางประสาทของปลาที่ติดเชื้อได้ งานวิจัยนี้เป็นงานวิจัยแรกที่ได้มีการอธิบายเกี่ยวกับการปรากฏ และการกระจายตัวของเชื้อไวรัส TiLV ในทั้งหมดของสมองของปลานิลที่ติดเชื้อ ซึ่งถือเป็นองค์ความรู้ใหม่เกี่ยวกับปฏิสัมพันธ์ของเชอื้ ไวรัส TiLV และปลานิลที่ติดเชื้อไวรัสนี้ นอกจากนี้ยังอาจเป็นแนวทางให้แก่งานวิจัยที่เกี่ยวข้องอื่นๆ ในการเติมเต็มองค์ความรู้ในด้านพยาธิกรรมเนิดของการติด เชื้อ TiLV

สาขาวิชา	วิทยาศาสตร์ทางการสัตวแพทย์และเทคโนโลยี	ลายมือชื่อนิสิต .....
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Hung Dinh Nguyen : LOCALIZATION OF *TILAPIA TILAPINEVIRUS* BY *IN SITU* HYBRIDIZATION IN THE BRAIN OF NILE TILAPIA (*OREOCHROMIS NILOTICUS*). Advisor: Asst. Prof. CHANNARONG RODKHUM, Ph.D. Co-advisor: Ha Thanh Dong, Ph.D.

Tilapia tilapinevirus or tilapia lake virus (TiLV) is an emerging virus that poses a significant negative impact on farmed tilapia globally. Abnormal behaviours such as lethargy, loss of appetite, erratic swimming, stop schooling have been recorded during disease occurrence, which is possibly linked to brain activities. Previous studies described detection of the virus in multiple organs of infected fish; however, little is known about the in-depth localization of the virus in the central nervous system. Herein, we determined the distribution of TiLV and histopathological alterations in the entire brain of Nile tilapia experimentally infected fish. By *in situ* hybridization (ISH) using TiLV-specific probes, the results indicated that the virus was broadly spread throughout the brain. These findings have supported the association between brain dysfunctions and the neurological manifestation of infected fish. Interestingly, the strongest positive TiLV signal was dominantly detected in the forebrain (function as learning, appetitive behavior, and attention) and the hindbrain (function as controlling locomotion and basal physiology). In addition, the permissive cell zone for viral infection mostly was observed to be along the blood vessels and the ventricles, indicating that the virus may effectively enter the brain through the circulatory system and infect broad regions potentially through the cerebrospinal fluid along the ventricles, and subsequently induce the brain dysfunction. Understanding the pattern of viral localization in the brain may help elucidate the neurological disorders of the diseased fish. Our work revealed the first spatial visualization of viral distribution in the whole infected brain, providing new information on fish-virus interactions and also guide future studies to fulfill the disease pathogenesis.

Field of Study:	Veterinary Science and technology	Student's Signature .....
Academic Year:	2020	Advisor's Signature .....
		Co-advisor's Signature .....

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Hung Dinh Nguyen

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bp	Base pairs
CNS	Central nervous system
CFS	Cerebrospinal fluid
ISH	*In situ* hybridization
mg	Milligram
min	Minute
ml	Milliliter
sec	Second
$TCID_{50}$	Median tissue culture infectious dose
TiLV	Tilapia lake virus
US	United States
µm	Micrometer
µL	Microliter

CHAPTER I

INTRODUCTION

1. Importance and rationale

Tilapia is the world second most important group of farmed fish, especially the genus *Oreochromis* (FAO, 2016). The tilapia industry is important for providing food and employment, as well as domestic and export earnings to large populations worldwide (FAO, 2016; Chengula et al., 2019). However, various pathogens including bacteria, fungi, and viruses have been reported as the major threats to tilapia aquaculture (Dong et al., 2015; Kibenge, 2019).

An unknown viral disease associated with abnormal mortalities (>80%) of farm-bred tilapia Chitralada during 2011-2012 was first reported in Ecuador in May 2013. The disease was initially named syncytial hepatitis of tilapia (SHT) based on the pathognomonic lesion in the liver (Ferguson et al. 2014). At the same time, similar mysterious disease episodes occurred in Israel and a novel virus was subsequently discovered and initially termed tilapia lake virus (TiLV) (Eyngor et al., 2014). The causative virus from these cases were later confirmed to be identical (Bacharach et al., 2016; de-Pozo et al., 2017). Since then, the virus has been recognized as an emerging virus officially causing disease outbreaks in 16 countries across 3 continents (Asia, Africa, South America) (Surachetpong et al., 2020; Thawornwattana et al., 2020) and severely impact the tilapia industry (OIE, 2017; FAO, 2018; Jansen et al., 2019). It was initially described as a novel *Orthomyxo*-like virus of fish, negative single-stranded RNA with 10 segments (Eyngor et al., 2014; Bacharach et al., 2016) and which was later taxonomically classified as *Tilapia tilapinevirus* species, a single species belong to the *Tilapinevirus* genus, under the *Amnoonviridae* family (ICTV, 2019).

Tilapia tilapinevirus is a severe contagious pathogen that causes high mortality (20-90%) in both farmed and wild tilapines (Eyngor et al., 2014; Ferguson et al., 2014; Bacharach et al., 2016; Dong et al., 2017; Surachetpong et al., 2017; Tattiyapong et al., 2017; Jansen et al., 2019). The infected fish exhibited variable pathognomonic signs from skin erosion, scale protrusion, or gill pallor to abdominal distension, anaemia, exophthalmia as well as abnormal behaviors; the abnormal behaviors are described as lethargy, loss of appetite, swimming at the water surface, erratic swimming, stop schooling, swirling or loss of balance (Ferguson et al., 2014; Bacharach et al., 2016; Dong et al., 2017; Fathi et al., 2017; Surachetpong et al., 2017; Tattiyapong et al., 2017; Jansen et al., 2019). These unusual behaviors may have involved in injuries within the central nervous system. The previous studies have been mentioned various histopathological changes in the brain of infected fish notably as congestion of blood vessels and perivascular cuffing of lymphocytes (Eyngor et al., 2014; Ferguson et al., 2014; Fathi et al., 2017; Behera et al., 2018; Jansen et al., 2019). Furthermore, the presence of the TiLV genomic RNA in the infected brain also has been determined by using an *in situ* hybridization technique (Bacharach et al., 2016; Dong et al., 2017). However, these findings only stopped at suggesting the brain may be one of the target tissue for transcription and replication of the virus. Particularly, the literature review shows that the fish brain divides into different regions and each part carries out certain functions (Roberts, 2012; Baldisserotto et al., 2019). On the other hand, the possible routes of viral entry into the brain are still unclear. Some other hypotheses have suggested that the virus is disseminated through the blood circulation and then spreaded systematically throughout the fish body (Dong et al., 2020). Unfortunately, currently available data is unclear which part of the brain is affected and the possible injuries linked to the brain function. Moreover, elucidating the localization of the virus in the brain is important for understanding the neuropathogenesis caused by TiLV infection.

Thus, the present study investigated the spatial localization of TiLV in the entire infected brain detected by *in situ* hybridization using the different specific probes. Additionally, a histopathological study of TiLV was also conducted. Lastly, the potential route of TiLV entry into the brain after the intraperitoneal infection was suggested. To our best knowledge, this is the first in-depth study of the distribution of TiLV and its possible effects in infected fish brains, cooperation with the histopathological study, providing the initial step to further understanding the effects of the host-pathogen interaction.

2. Hypothesis

The virus is presented in particular areas of the brain which may be related to neurological signs.

3. Objective

To investigate the spatial localization of TiLV and permissive cell zone in the intact brain of the infected fish.

4. Conceptual framework

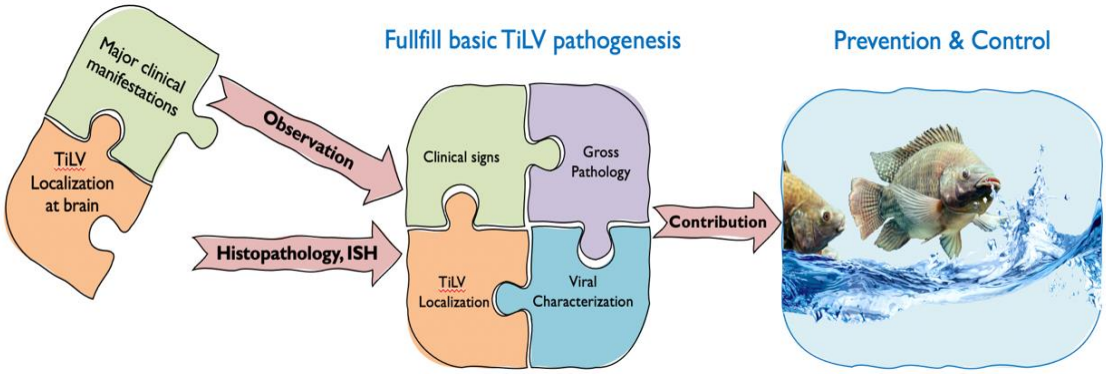

Figure 1. Conceptual framework of the study

CHAPTER II

LITERATURE REVIEW

1. Discovery of Tilapia lake virus and its characterization

Tilapia lake virus (TiLV) is a globally emerging virus affecting cultured Nile tilapia *(Oreochromis niloticus),* red tilapia *(Oreochromis* sp.) and hybrid tilapia *(O. niloticus × O. aureus)* (Jansen et al., 2019). The disease caused by TiLV was first reported in Israel in 2009, while the virus was first characterized in 2014 (Eyngor et al., 2014). To date, the disease has been officially reported in 16 countries located in different parts of the world from Israel (Eyngor et al., 2014; Bacharach et al., 2016), Ecuador (Ferguson et al., 2014), Egypt (Fathi et al., 2017; Nicholson et al., 2017), Colombia (Tsofack et al., 2017), Thailand (Dong et al., 2017; Surachetpong et al., 2017), Chinese Taipei (OIE, 2017b), the Philippines (OIE, 2017c), Malaysia (Amal et al., 2018), India (Behera et al., 2018), Tanzania and Uganda (Mugimba et al., 2018), Mexico (OIE, 2018), Indonesia (Koesharyani et al., 2018), Peru (Pulido et al., 2019), the United States (Ahasan et al., 2020) and Bangladesh (Chaput et al., 2020). The virus has been described as a novel enveloped, negative-sense, single-stranded RNA virus with 10 segments encoding 10 proteins (Eyngor et al., 2014; Bacharach et al., 2016; Surachetpong et al., 2017) and a diameter between 55 and 100 nm (Eyngor et al., 2014; Ferguson et al., 2014; Del-Pozo et al., 2017; Surachetpong et al., 2017). All 10 segments contain an open reading frame (ORF), with the largest segment, segment 1, containing an open reading frame with weak sequence homology to the *influenza* C virus PB1 subunit (Bacharach et al., 2016). The remaining segments show no homology to other known viruses (Eyngor et al. 2014; Bacharach et al. 2016a); however, the conserved complementary sequences at the 50 and 30 termini are similar to the genome organization found in *Orthomyxoviruses* (Bacharach et al.,

2016). The virus has been assigned to the genus *Tilapinevirus* that include a single new species *Tilapia tilapinevirus* (ICTV, 2019).

Current literature showed that TiLV seems to be most important in tilapia fry, fingerlings, and juveniles although viral infections were also evidenced in sub-adult and adult fish (Senapin et al., 2018; Jansen et al., 2019). Additionally, subclinical infections with minor mortality impact were also reported (Senapin et al., 2018). Horizontal transmission of the virus has been confirmed through cohabitation of infected fish with clinically healthy fish, which resulted in 80% mortality, confirming waterborne transmission (Eyngor et al., 2014). Experimental challenge of nine non-tilapia fish species with TiLV revealed that giant gourami is an additional susceptible species and cohabitation between tilapia and giant gourami caused cross-species transmission (Jaemwimol et al., 2018). The virus can cause disease in multiple organs such as spleen, heart and brain, and even in the reproductive organs, and can be transmitted vertically (Yamkasem et al., 2019; Dong et al., 2020). The clinical signs may vary depending on the geographical origin, and include skin erosion and darkening, gill pallor, anemia and swollen abdomen (Ferguson et al., 2014; Dong et al., 2017), with subclinical infections also being reported (Senapin et al., 2018). TiLV can cause high levels of mortalities, but these can vary substantially (ranging from 5 to 90%), natural outbreaks of TiLV reportedly resulted in 20–90% mortality (Eyngor et al., 2014; Ferguson et al., 2014; Bacharach et al., 2016; Del-Pozo et al., 2017; Dong et al., 2017; Fathi et al., 2017; Surachetpong et al., 2017). On the other hand, experimental infection using virus culture caused 66–90% mortality in tilapia juveniles (Eyngor et al., 2014; Tattiyapong et al., 2017; Behera et al., 2018).

2. Disease pathogenesis and diagnostics

The pathogenesis of TiLV disease and the causes of fish death remain mostly unknown. However, a few publications have reported a potential route of TiLV entry; initially, TiLV may enter into naïve fish through direct contact of skin and mucus of infected fish or via the oral route. This has been reproduced in a cohabitation challenge study (Eyngor et al., 2014; Tattiyapong et al., 2017; Liamnimitr et al., 2018) and intragastric challenge study (Pierezan et al., 2019). After the virus crosses the epithelial barrier in the intestine, it might distribute to internal organs and starts its replication cycle and then spreads systematically (Keller et al., 2003; Dong et al., 2020). Previous studies reported detection of viral genomic RNAs in gills, liver, brain, heart, anterior kidney and spleen of infected fish (Bacharach et al., 2016; Del-Pozo et al., 2017; Dong et al., 2017; Fathi et al., 2017; Tattiyapong et al., 2017; Mugimba et al., 2018). Moreover, the viral genomic RNA has been found in the liver and brain of infected fish using ISH assay, suggesting that these organs may be the target tissues for viral transcription and replication (Bacharach et al., 2016; Dong et al., 2017). In addition to the liver and brain, positive hybridization signals were also detected in the anterior kidneys, gills, gastrointestinal tract and spleen, as well as in the connective tissues of infected fish (Bacharach et al., 2016; Dong et al., 2017; Jaemwimol et al., 2018; Jansen et al., 2019). A recent study demonstrated that a lysosomotropic agent had no effect on TiLV replication, suggesting that TiLV can replicate without acidic pH in contrast to other orthomyxoviruses (Chengula et al., 2019).

Currently, diagnosis of TiLV infection has mainly focused on the detection of viral genomic RNA using reverse transcription polymerase chain reaction (RT-PCR) (Eyngor et al., 2014; Dong et al., 2017; Tsofack et al., 2017); RT-quantitative PCR (RT-qPCR) (Nicholson et al., 2017; Tattiyapong et al., 2017; Tsofack et al., 2017;

Waiyamitra et al., 2018), virus isolation in the cell culture (Eyngor et al., 2014; Tsofack et al., 2017); the reverse transcription loop-mediated isothermal amplification (RT-LAMP) (Phusantisampan et al., 2019; Yin et al., 2019) and the recombinant protein-based indirect ELISA (Hu et al., 2020). Moreover, at histological level, syncytial hepatitis is being reported the most common histopathological feature for TiLV presumptive diagnosis (Eyngor et al., 2014; Ferguson et al., 2014; Bacharach et al., 2016; Del-Pozo et al., 2017; Dong et al., 2017; Fathi et al., 2017; Tattiyapong et al., 2017; Senapin et al., 2018; Jansen et al., 2019). Results from *in situ* hybridization (ISH) indicate that TiLV replication and transcription occur at sites of pathology (i.e. the liver in samples with liver lesions and the central nervous system in samples with central nervous system lesions) (Bacharach et al., 2016). In samples collected from Thailand, ISH yielded positive signals in multiple organs (liver, kidney, brain, gills, spleen, and muscle connective tissue), with the strongest signals found in liver, kidney, and gills (Dong et al., 2017). In early 2020, evidence of potential vertical transmission of tilapia lake virus was proved by the presence of the virus inside tested testis, ovary of infected broodstock, and the fertilized eggs (Dong et al.). Although TiLV has been detected in various infected tissues in previous studies, there is no in-depth studies on localization of the virus in different parts of the whole brain. Therefore it necessities the importance of understanding the disease pathogenesis, since little work has been done to investigate this. An intimate relationship between viral localization and the changes of histological lesions was demonstrated which supports an etiological role for the virus in disease activity (Coulton and De Belleroche, 2012).

3. The structure and function of fish brain

As in the teleost fish brain, the brain of tilapia is divided into forebrain (*prosencephalon*), midbrain (*mesencephalon*), and hindbrain (*rhombencephalon*). The forebrain was divided further into telencephalon and diencephalon, while the hindbrain was divided into the metencephalon or cerebellum and medulla oblongata. The telencephalon comprises telencephalic hemispheres and the olfactory bulb. The diencephalon is located rostroventral to the midbrain and is largely covered by the optic tectum. The hindbrain is covered rostrally by the cerebellum and includes caudally the prominent paired vagal lobes. Finally, the medulla oblongata grades into the spinal cord. Overview morphology and histology of the teleost fish brain are defined in Figure 2 and Figure 3. Features of the tilapia brain with similar descriptions have been recently highlighted in Red tilapia (*Oreochromis* sp) (Palang et al., 2020) and Nile tilapia (*Oreochromis niloticus*) (Cham et al., 2017). The main functions of different regions of the fish brain are defined in Table 1 and Figure 4. The most anterior portion of a fish's brain, the telencephalon contains the olfactory lobes and its activity includes, but is not limited to, recognition of odors. The diencephalon connects with the pineal gland at the top of the skull. Through the lower section of the diencephalon known as the hypothalamus the diencephalon connects with the pituitary gland, as well. The diencephalon integrates incoming and outgoing neural and hormonal signals. The midbrain of fishes includes the optic lobes, and besides vision, it is integral to learning. The primary function of the cerebellum is muscle coordination and swimming. The medulla oblongata serves as a relaying center between the spinal cord and the higher brain areas. The brain and spinal cord together are termed the central nervous system (CNS).

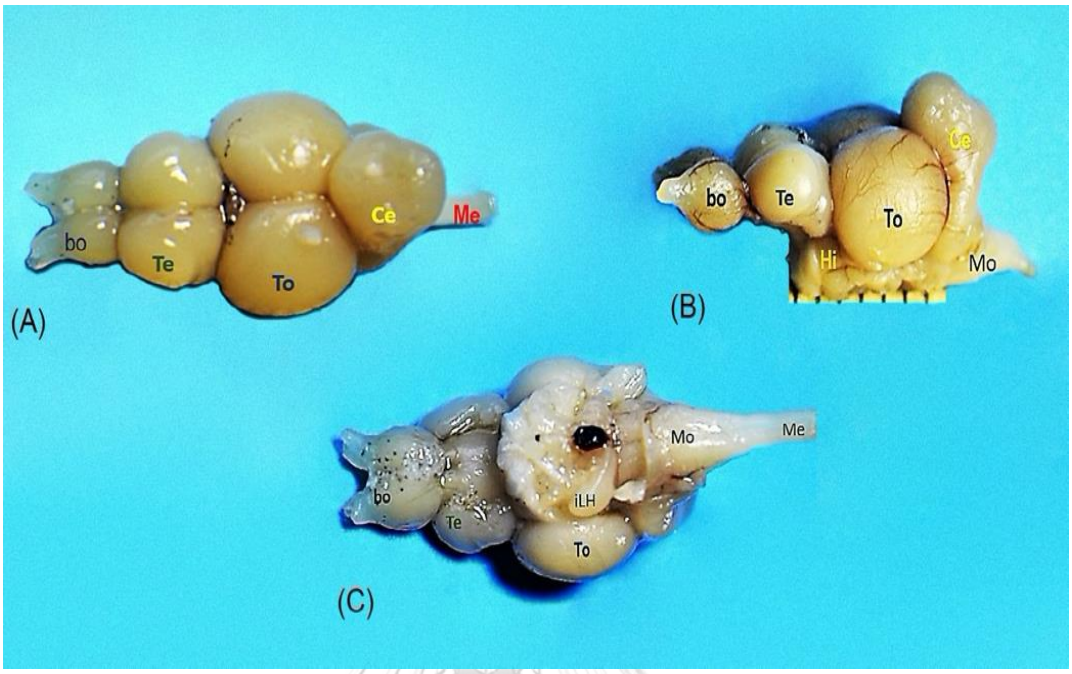

Figure 2. The brain morphology of juvenile P. *brachypomus* (40g). Dorsal (A), lateral (B), and ventral (C) views. bo, bulbus olfactorius (olfactory bulb); Ce, cerebellum; Hi, hypothalamus; iHL, inferior hypothalamic lobe; Mo, medulla oblongata; S, spinal cord; Te, telencephalon; To, Tectum opticum. (adopted from Baldisserotto et al., 2019).

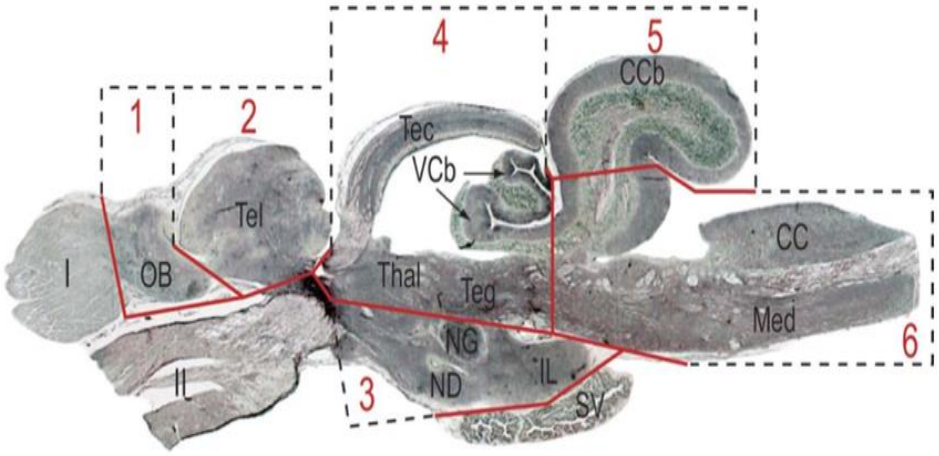

Figure 3. Parasagittal histological section of the brain of *Dissostichus mawsoni* showing cuts made (red) to divide the brain into six regions for the calculation of relative weights. Other major features of the brain are also indicated. Bodian's Protargol. Magnification x5. 1, olfactory bulb; 2, telencephalon; 3, diencephalon; 4, mesencephalon; 5, corpus division of the cerebellum; 6, medulla; CC, crista cerebellaris of the rhombencephalon; CCb, corpus division of the cerebellum; IL, inferior lobe of the diencephalon; Med, medulla of the rhombencephalon; OB, olfactory bulb; ND, nucleus diffusus of the inferior lobe; NG, nucleus glomerulosus of the diencephalon; SV, saccus vasculosus; Tec, tectum of the mesencephalon; Teg, tegmentum of the mesencephalon; Tel, telencephalon; Thal, thalamus of the diencephalon; VCb, valvula division of the cerebellum; I, olfactory nerve (proximal expansion); II, optic nerve (adopted from Eastman and Lannoo, 2011).

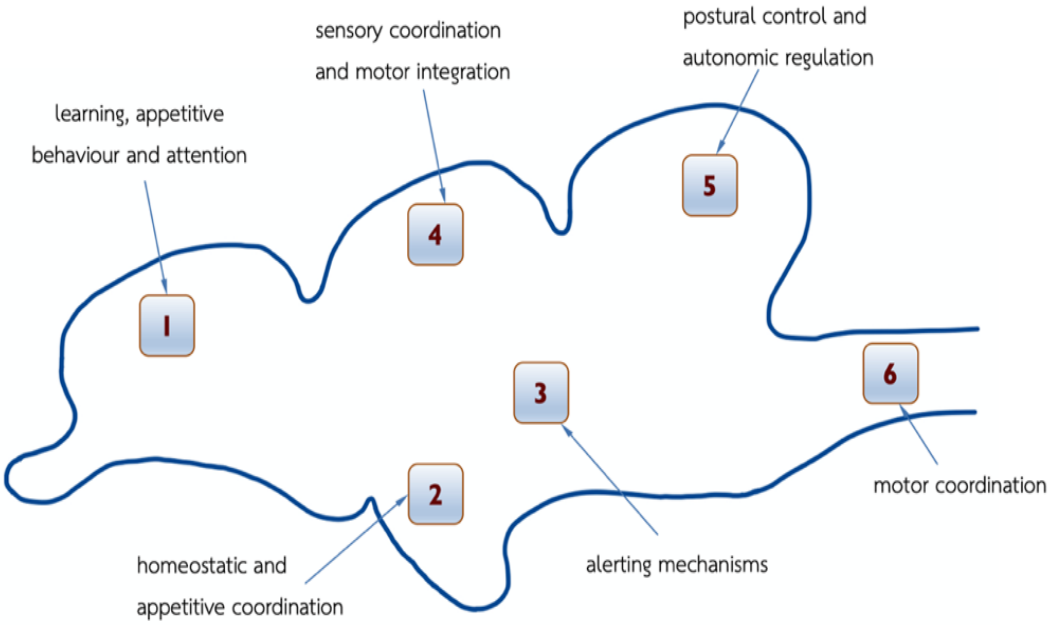

Figure 4. Generalised fish brain showing functions of different regions. (modified from Roberts, 2012).

Table 1. Divisions of the central nervous system and its functions (adopted from Baldisserotto et al., 2019)

Brain region		Main funtion
Forebrain	**Telencephalon (Cerebrum)**	*Olfactory bulb:* Interpretation of olfactory signals
		Hemispheres: learning, memory, and attention
	Diencephalon	*Thalamus:* promotes the filtration of sensory information
		Hypothalamus: control of thermoregulation, participates in the osmoregulatory control, food intake, emotional state, endocrine system
Midbrain		*Tegmentum:* participates in motor control
		Optic tectum: center for integration of visual information with other sensorial information
		Toris semicircularis: receives auditory (and sometimes electrosensory) input
Hindbrain	**Metencephalon (Cerebellum)**	It is related to precise and fast motion control, and in electric fish is related to the interpretation of electroreceptors
	Medulla oblongata	It determines the basic rhythm and regulation of the respiratory and cardiovascular systems. It contains most of the motor and sensory cranial nerve nuclei. It is a place of passage of the neural pathways, making the connection between the spinal cord and the encephalon

4. *In situ* hybridization principles

In situ hybridization (ISH) is a type of hybridization that uses a labeled complementary DNA, RNA or modified nucleic acids strand to localize a specific DNA or RNA sequence in a portion or section of tissue (*in situ*) or if the tissue is small enough, in the entire tissue (whole mount ISH), in cells, and in circulating tumor cells (CTCs). This differs from immunohistochemistry, which is usually localized proteins in tissues.

In situ hybridization is used to reveal the position of specific nucleic acid sequences on chromosomes or in tissue, an important step in understanding gene organization, regulation, and function. The key techniques currently in use include in situ hybridization to mRNA with oligonucleotide and RNA probes, light and electron microscopy analysis, double detection of RNAs, and RNA plus protein and fluorescence *in situ* hybridization use for chromosome sequence detection. The ISH DNA can be used to determine the structure of a chromosome. RNA *in situ* hybridization (RNA) (ISH) RNA is used to measure and locate RNAs (mRNAs, lncRNAs, and miRNAs) in tissue sections, cells, entire mass binding, and cyclic tumor cells. This technique, which was pioneered by *Joseph Gall*, works by hybridizing a labeled RNA or DNA molecule that is complementary to the RNA or DNA of interest (1969).

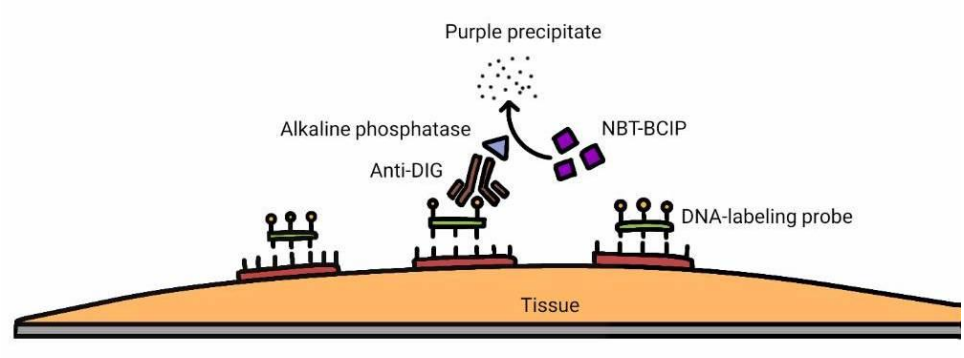

Figure 5. Principles of *in situ* hybridization *(Personal drawing)*

The principle of ISH is based on labeling a complementary RNA probe with a hapten, such as a digoxigenin (DIG), fluorescein, or biotin, which can be visualized after hybridization to a targeted mRNA (Broadbent and Read, 1999). DIG is the most common hapten used for probe labeling because it is naturally produced in Digitalis plants and hence is absent in animal cells. Probe synthesis is carried out by cloning a cDNA of the desired targeted gene transcript into a transcription vector so that the insert is flanked by promoters for different RNA polymerases, typically T3, T7, or SP6 (Harland, 1991). In this way, using one of these RNA polymerases either sense or antisense strand of an insert can be synthesized. An antisense transcript serves as an RNA probe and a sense transcript can be used as a negative control. Probes are synthesized in the presence of uridine-5′-triphosphate nucleotides conjugated to DIG, which is efficiently incorporated by T3, T7, and SP6 RNA polymerases. Upon hybridization of a labeled probe, the embryos are treated with single-strand specific RNAases in order to eliminate nonhybridized single-stranded antisense probe, and then the remaining double-stranded hybrids can be visualized with an anti-DIG antibody conjugated to a fluorochrome, such as alkaline phosphatase (AP) or horseradish peroxidase (HPO), together with appropriate chromogenic substrates. This generates a visible signal that corresponds to the location of the specific mRNA being assessed (Fig 5) (Harland, 1991).

The basis of the specificity of ISH is the interaction of probe to target nucleic acid. Nucleic acid chemistry dictates optimal conditions for the hybridization reaction. Optimal results require attention to tissue fixation, careful selection of probe, and monitoring assay conditions. Appropriate controls are essential. The application of ISH for the detection of infectious diseases has been described for many different organisms. Currently, an *in situ* hybridization (ISH) method has been described and has been used to reveal TiLV tissue tropism. ISH revealed that mRNA of the virus was detected in both the nucleus and the cytoplasm of the infected cells (Bacharach et

al., 2016; Dong et al., 2017; Dong et al., 2020). Another study shows that ISH yielded positive signals in multiple organs (liver, kidney, brain, gills, spleen, and muscle connective tissue), with the strongest signals found in liver, kidney, and gills (Dong et al., 2017). Most recently, evidence of potential vertical transmission of tilapia lake virus was proved by the presence of the virus inside tested testis, ovary of infected broodstock, and the fertilized eggs (Dong et al., 2020).

CHAPTER III

MATERIALS AND METHODS

1. Viral preparation

Tilapia lake virus strain named NV18R, which was isolated from diseased red tilapia (*Oreochromis* sp.) then was propagated in E11 cell line as previously described (Eyngor et al., 2014). The 50% tissue culture infectious dose ($TCID_{50}$) was calculated by the method of Reed and Muench (1938). The purified viral was confirmed by TiLV-specific PCR (Dong et al., 2017) and stored at -80°C until used. Prior to the experiment, the original stock ($10^{7.5}$ $TCID_{50}$) was thawed and diluted with saline solution (0.9% NaCl) to be used as an inoculum dose of $10^{5.5}$ $TCID_{50}$ for injection into fish.

2. Experimental setup

Apparently normal Nile tilapia (*Oreochromis niloticus*) fingerlings (10g ± 1g body weight) were obtained from a tilapia hatchery with no history of TiLV infection. The fish were acclimatized in a laboratory rearing facility for 10 days and water temperature set at 28°C. Prior to challenge test, ten fish were randomly selected for the detection of TiLV by semi-nested RT-PCR (Dong et al., 2017) to warrant TiLV negative status. In order to obtain disease, the fishes were divided into three groups with 20 individuals per one and were held in 50L fiberglass tanks. Each tank was provided with a separate water supply, drainage, and air stones, working as a flow-through system. Commercial tilapia feed pellets (CP) containing 28% crude protein were provided twice daily (9.00 and 16.00 hr) at 3% biomass per day. The 50% water in each tank was replaced every three days. The fish in group 1 and group 2 were injected with TiLV at a dose of $10^{5.5}$ $TCID_{50}$/0.1mL/fish, while the fish in the group 3

were injected with 0.1 mL of 0.9% saline solution without virus as control. The experimental protocols and animals used were performed in strict accordance with recommendations and guidelines of the Animal Ethics Committee, Laboratory Animal Resource Unit, Faculty of Medicine, The National University of Malaysia (Number: **WORLDFISH/2019/JEROME DELAMARE/27-NOV./1071-DEC.-2019-NOV.-2021.**).

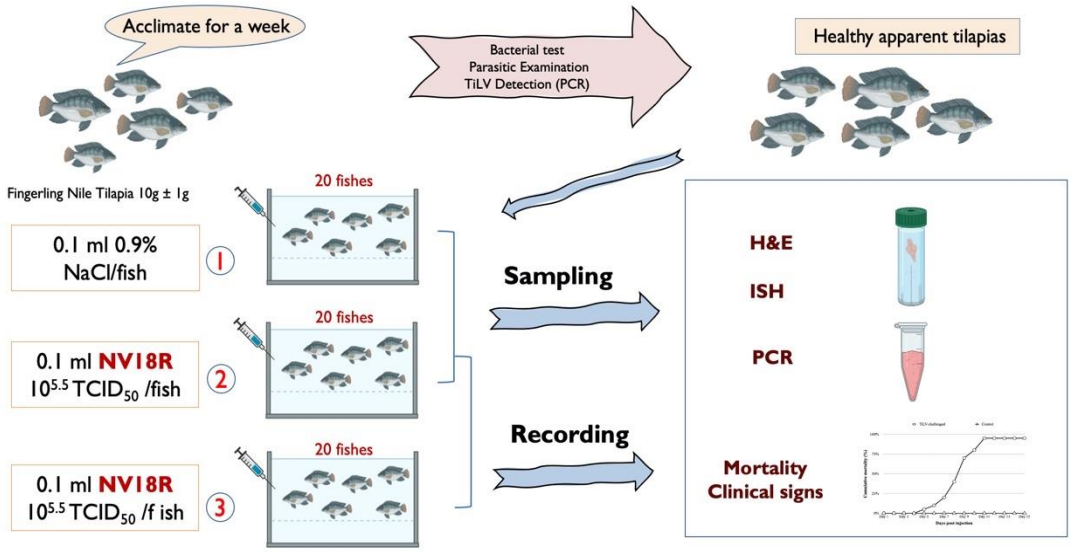

Figure 6. Overview of experimental design in this study

3. Clinical observation and brain collection

Following the infection, infected fishes in group 1 were observed continuously for 16 days to record gross pathology and mortality. While, in the group 2, moribund fish showed clear signs, which prioritized neurological manifestation such as loss of appetite, lethargy, abnormal behavior (e.g. swimming at the surface, stop schooling, erratic swimming, or loss of balance), were euthanized with an overdose of (150ppm/L) of Clove Oil, cleaned by a dried cloth, and opened the skull. The intact brain tissues was carefully isolated and preserved in 10% neutral buffered formalin for 24 hours and then immersed in 70% ethanol before being processed for routine histology and ISH, as described below.

4. Labelling and probes preparation

Two TiLV-specific DIG-labelling probes targeting two different TiLV genomic segments were used in this study. The 274 bp probe derived from TiLV segment 1 was prepared from primers TiLV/nSeg1F; 5′-TCT GAT CTA TAG TGT CTG GGC C-3′ and TiLV/nSeg1RN; 5′-CCA CTT GTG ACT CTG AAA CAG–3′ (Taengphu et al., 2019) while the 250 bp probe derived from TiLV segment 3 was employed primers ME1; 5’-GTT GGG CAC AAG GCA TCC TA-3’ and 7450/150R; 5’-TAT CAC GTG CGT ACT CGT TCA GT-3’ (Dong et al., 2017). An unrelated probe 282 bp acquired from a shrimp virus namely infectious myonecrosis virus (IMNV) (F13N; 5’-TGT TTA TGC TTG GGA TGG AA-3’ and R13N; 5’-TCG AAA GTT GTT GGC TGA TG-3’) (Senapin et al., 2007) and no probe were used as negative controls. The probes were prepared using Digoxigenin (DIG) a commercial PCR DIG-labeling mix (Roche Molecular Biochemicals). Briefly, RNA extracted from internal organs of tilapia infected with TiLV were used as a template for one step of RT-PCR to amplify to 274 bp and 250 bp genomic segment 1 and 3 performed by Dong *et al* (2017), and then the amplicons were cloned into a suitable vector (namely pGEM®-T Easy – Promega, US). The plasmids contain TiLV segment 1 and 3, separately were then used labeling with DIG by PCR. The PCR reaction of 25 µL composed of 200 ng of plasmid template, 1 µM of each primer, 0.5 µL of SuperScript III RT/Platinum Taq Mix (Invitrogen), and 1X of the supplied buffer, dNTP in the reaction was replaced by 0.5µL DIG-labeling mix. The RT reactions were heated inactivation at 94°C for 5min. PCR cycling then was carried out for 30 cycles at 94°C for 30s, 55°C for 30s, 72 °C for 30s, final extension step at 72 °C for 5 min. The DIG-labeled TiLV probes for each segment was obtained after purifying amplified product by NucleoSpin™ Gel PCR Clean-up Kit (Fisher Scientific) according to the manufacturer’s protocol. Pure PCR Purification were measured for concentration by NanoDrop™ One Spectrophotometer (Thermo Fisher Scientific) and stored at -20 °C until use.

5. Tissue processing and histopathology

The preserved tissues were dehydrated by incubating in several increasing concentrations of ethanol (70–100%) and then transferred to xylene automatically by Leica TP1020 Tissue Processor (Leica Biosystems, US). The tissues were infiltrated and embedded in paraffin. Each paraffin-embedded tissue was sequentially cut at 5 µm thickness into 5 consecutive sections and were mounted on HistoGrip (Zymed, US) coated glass slides. The purposed tissue sections including 2 TiLV specific probes (segment 1 and segment 3), 2 negative controls (IMNV and no probe) and one for hematoxylin and eosin (HE) staining. One slide in groups of five as described were then conventionally HE stained and observed histopathology changes under the microscope Olympus BX51 (Olympus, Japan). The remaining set 4 slides were incubated at 42°C 48hours for the ISH technique afterwards.

6. *In situ* hybridization (ISH) assay

The ISH assay was performed from that of a routinely used ISH in our previous study (Senapin et al., 2015; Dong et al., 2017; Dong et al., 2020) with a few steps of optimization, as described below.

6.1. Pre-hybridization and hybridization

Unstained 5 µm sections on HistoGram coated slide was deparaffinized by triplicate in xylene for 5 min followed by a graded series of ethanol (95%, 80%, 75% twice each for 5 min), distilled water, and finally in TNE buffer (100 mM Tris–HCl, 10 mM EDTA, pH 8.1). Tissues were digested with proteinase K (prepared just prior to use) at a final concentration 10 µg ml^{-1} for 15 min at 37°C, then treated by 4% paraformaldehyde for 5 min at 4°C and immersed in distilled water for 5 min. After rapidly treating with acid acetic for 20 seconds and washing in distilled water, each

section were then covered with pre-hybridization buffer (4× SSC containing 50% (v/v) deionized formamide) at 37 °C for at least 10 mins. Each probe was diluted to hybridization buffer (50% deionized formamide, 50% dextran sulfate, 50× Denhardt's solution (Sigma, US), 20 × SSC, 10 mg mL^{-1} salmon sperm DNA (Invitrogen, Breda, the Netherlands), heated at 95°C for 10 min, and then chilled on ice. Each specific probe was added to the tissue sections, then covered by coverslips, and incubated overnight at 42 °C in a humid chamber.

6.2. Post-hybridization

Post-hybridization was motivated sequentially washing twice each, with 2×, 1× and 0.5× SSC at 42°C, 37°C, 37°C for 15 min, respectively, equilibrated by 5 min washing with buffer I (1 M Tris–HCl, 1.5 M NaCl, pH 7.5). Tissue sections were then blocked with blocking solution buffer II (containing 0.1% Triton X-100 and 2% normal sheep serum) at room temperature for 30 min before covering with anti-DIG alkaline phosphatase conjugate anti-digoxigenin antibody (Roche, diluted 1:500 in buffer II) for 1 h at 45°C. After washing twice for 10 min each with Buffer I, the sections were treated for 10 min in buffer III (100 mM Tris–HCl, 1.5 M NaCl, 50 mM MgCl2, pH 9.5). The sections were incubated for 1 to 24 hours in the dark at room temperature with development solution (NBT–BCIP substrate Roche, US).

6.3. Detection and visualization

The antibody-antigen complexes were subsequently revealed by NBT/BCIP substrate. Once the optimal color was observed the reaction was then stopped by washing tissue sections with 1x TE buffer at RT for 15 min and afterward drip in distilled water. The slides were then counterstained with 0.5% Bismarck Brown (Sigma Aldrich, US) for 2 min. The sections were washed in running water for 5 min then dry at room temperature before immersing twice in 100% xylene for 5 min

each. The slides were then mounted, coverslipped and observed and photographed under using a light microscope BX51 (Olympus, Japan). Localization of TiLV were interpreted in parallel with the slides of TiLV-specific probes, negative controls (unrelated probe and no probe), and H&E stained sections. TiLV signal density in the brain was subjectively scored on a four-point scale as follows: + + + (*high signal*), + + (*moderate signal*), + (*low signal*), and – (*absent signal*) following Cham et al (2017). Nomenclature for the brain area (Table 2) was based on those described by Simoes et al (2012), Ogawa et al (2016), and Cham et al (2017).

CHAPTER IV

RESULTS

1. Clinical observations and cumulative mortality in experimental challenge

After infection, the fish showed no abnormalities and natural in appetitive behavior as well as swimming activity during the first 2 days. Starting at 3 dpi, some of the fish had been lost of appetitive but were swimming normally, and others exhibited as swimming alone, separated from the group, accompanied by swimming on the surface water. This followed by the fish became lethargy, with marked reduced eating activity from 4dpi. Mortality started at 5 dpi and lasted until 11 dpi with accumulative mortality at 95% (Figure 7A). Prior to death, three to four fishes displayed erratic swimming, swirling, or loss of balance by 6 to 9 dpi. A majority of fish died within 24 hours after appearing of abdominal swelling, exophthalmia and dark discoloration of the skin (Figure 7B). In addition, some sick fish also displayed scale protrusion and skin erosion (data not shown). Internally, post-mortem changes including necrotic and pale liver (arrows); and enlarged pleen (white arrowhead) were frequently noticed as well as ascitic fluid (black arrowhead) was observed (Figure 7C). In contrast, no clinical signs of infection or mortality were recorded in the control group.

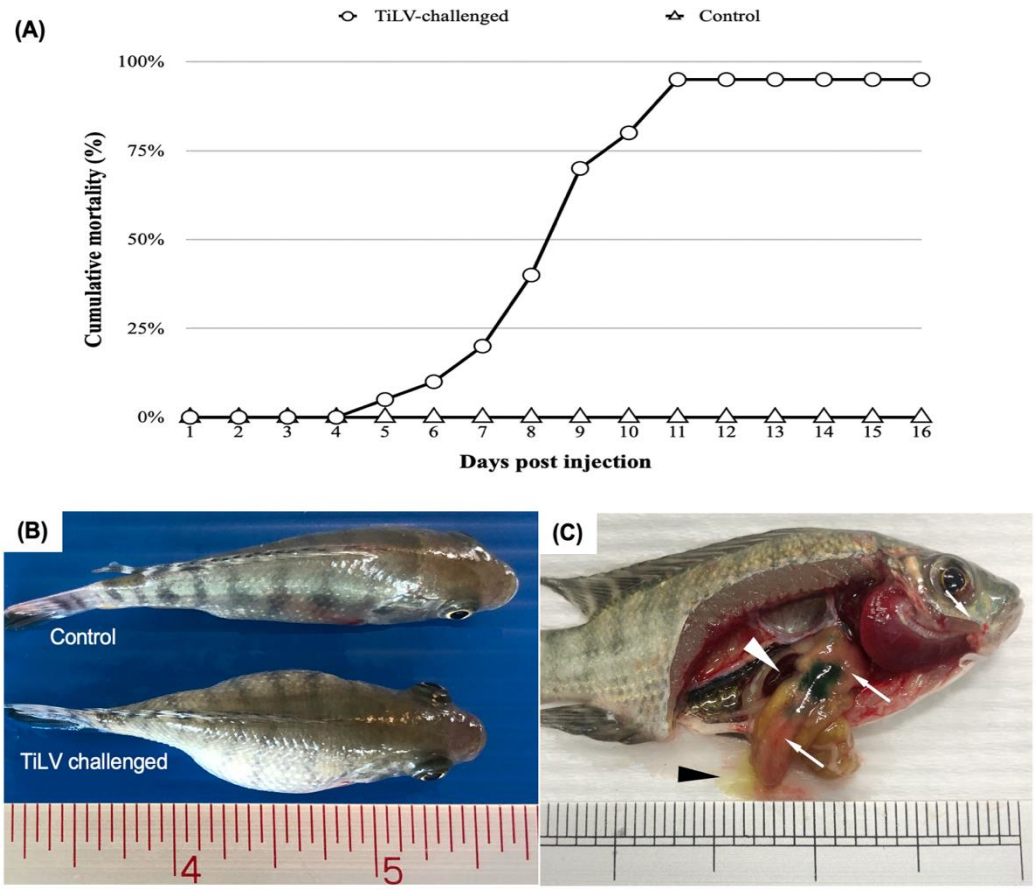

Figure 7. Experimental challenge of TiLV in Nile tilapia. The cumulative mortality rate of Nile Tilapia (n = 20) injected (I/P) with TiLV at 0.1mL × $10^{5.5}$ $TCID_{50}$/fish and the control were injected (I/P) with 0.1 mL of 0.9% saline solution (A). Distinct clinical signs of infected fish include exophthalmia and abdominal swelling (B); necrotic and pale liver (arrows), enlarged spleen (white arrowhead) as well as ascitic fluid (black arrowhead) (C).

2. Preparation of ISH probes

Probes were successfully labelled with DIG for the 274-bp, the 250-bp, and the 282-bp probes, which was evident by the differential molecular weight of unlabelled and labelled probes following electrophoresis (Figure 8).

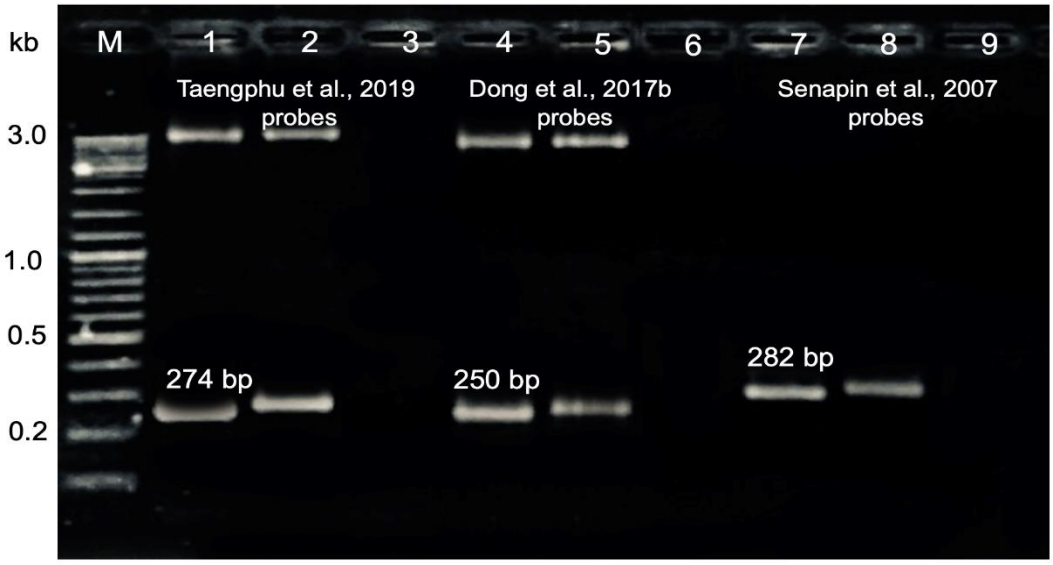

Figure 8. DIG labelling probes were prepared by PCR and confirmed under the agarose gel electrophoresis. Lane 1 (unlabelled), lane 2 (labelled), and lane 3 (no template control) which represented the probes were prepared from the TiLV segment 1 primer set; lane 4 (unlabelled), lane 5 (labelled), and lane 6 (no template control) which represented the probes were prepared from the TiLV segment 3 primer set; lane 7 (unlabelled), lane 8 (labelled), and lane 9 (no template control) which represented the probes were prepared from the IMNV primer set. Lane M was a DNA marker (2-Log DNA Ladder, New England Biolabs); #Marks band derived from the amplified of the pGEM plasmid.

3. TiLV localization in the fish brain

To analyze the distribution of TiLV in the brain of infected fish, *in situ* hybridization (ISH) was performed on horizontal and parasagittal sections of the whole-brain. The results of ISH using two specific probes targeted TiLV genome segment 1 and segment 3 exhibited similar results of positive signal distribution while no signal was detected in the sections assayed with an unrelated probe and no probe. ISH positive signals (dark color) were widely distributed in various parts of the brain. However, the forebrain and hindbrain showed stronger signal intensities compare to that of the midbrain. Details on the distribution of the TiLV positive signals and their densities were shown in Table 2. Representative microphotographs of the four consecutive horizontal and parasagittal whole-brain sections (H&E staining, ISH with segment 1 probe, segment 3 probe, and unrelated probe) of a diseased fish were shown in Figure 9 and Figure 11. The strongly positive areas of the telencephalon, mesencephalon, cerebellum, and medulla oblongata were visualized at higher magnifications (Figure 10). There were no ISH positive signals detected in the brain of non-infected control fish that were assayed in the same manner as shown in Figure 12.

3.1. The forebrain *(prosencephalon)*

The forebrain comprises of two main parts namely telencephalon (or cerebrum) and diencephalon. In the telencephalon, all subdivisions are positively marked; the granular layer of olfactory bulb (OB), hemispheres of telencephalon (HT), and the periventricular zone (PZT) shown more intensely signals (Figure 9B, 10A). In the diencephalon, strong positive staining was found throughout the hypothalamus, specifically along the periventricular zone of hypothlamus (PZH) and diffuse nucleus of inferior lobe (DIL) (Figure 11B, C).

3.2. The midbrain *(mesencephalon)*

The midbrain or mesencephalon divides into some main parts including optic tectum (TeO), torus longitudinalis (TL), torus semicircularis (TS), and tegmentum (Teg). Diffuse staining of positively labelled cells was observed in the optic tectum (TeO). Stronger signals were observed in the in the periventricular grey zone of the optic tectum (PGZ) (Figure 9B, 10B, 11B). In the tegmentum (Teg), genetic material was weaker compared to other areas (Figure 11B).

3.3. The hind brain *(rhombencephalon)*

The hindbrain consists of metencephalon (or cerebellum) and medulla oblongata. There were relative stronger positively labelled cells in the hindbrain compared to the midbrain. Within the cerebellum, staining was observed in the granular layers of the corpus cerebellum (CCeG) (Figure 9B, 10C, 11B). In the medulla oblongata, TiLV RNA were highly staining in the vagal lobe (LX) (Figure 9B, 10D). Fewer TiLV-positive signals were seen in the intermediate reticular formation and inferior reticular formation (IMRF and IRF, respectively) (Figure 11B).

Table 2. Distribution of TiLV signals in the brain of infected fish

Brain region		Abbreviation	Densities of signal
Forebrain	**Telencephalon**		
	Olfactory bulb	OB	+++
	Hemisphere of telencephalon	HT	+++
	Periventricular zone of telencephalon	PZT	+++
	Diencephalon		
	Thalamus	Tha	-/+
	Optic nerve	ON	-/+
	Hypothalamus	Hyp	++
	Diffuse nucleus of inferior lobe	DIL	++
	Periventricular zone of hypothalamus	PZH	+++
Midbrain	**Mesencephalon**		
	Optic tectum	TeO	+
	Periventricular gray zone of optic tectum	PGZ	++
	Tegmentum	Teg	+
	Torus longitudinalis	TL	++
	Torus semicircularis	TS	+
Hindbrain	**Metencephalon**		
	Corpus division of cerebellum	CCe	++
	Valvula division of cerebellum	VCe	+
	Granular zone of cerebellum	CCeG	+++
	Molecular zone of cerebellum	CCeM	+
	Medulla oblongata		
	Intermediate reticular formation	IMRF	-/+
	Inferior reticular formation	IRF	-/+
	Vagal lobe (lobe of nerve X)	LX	+++

(+ + +, high; + +, moderate; +, low; –, absent)

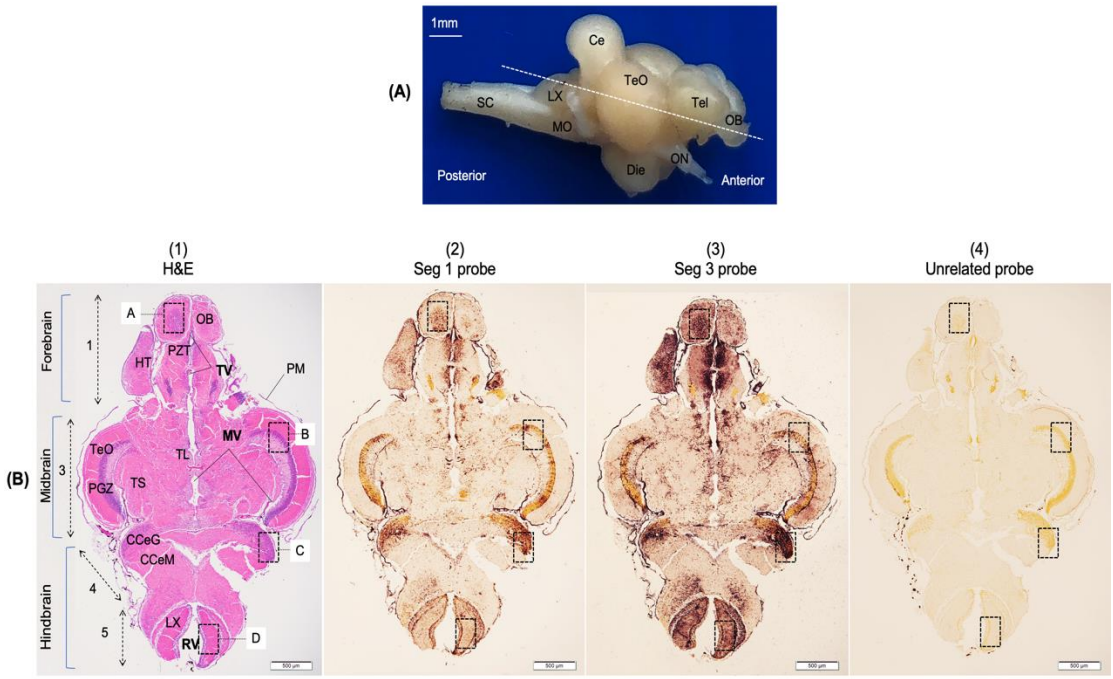

Figure 9. The spatial localization of TiLV in the brain of infected fish *(horizontal sections).* (A): Lateral view of tilapia brain with approximate slice position (dashed white line). (B): Comparison of horizontal consecutive sections from infected fish were stained with H&E (B.1), ISH with TiLV probe based on genome segment 1 (B.2), genome seg 3 (B.3) and ISH with unrelated probe as the control (B.4). The horizontal section was possible to define four major areas: telencephalon (1), mesencephalon (3), mesencephalon (4), medulla oblongata (5). The scale bars are shown in the pictures. The strongest TiLV positive signals (dark brown) were dominantly detected in primitive meninges (PM), the forebrain (telencephalon), and the hindbrain (metencephalon and medulla oblongata). Dashed regions (A, B, C, D) are exhibited the high magnification images in Fig 10. MV: Mesencephalic ventricle, TV: Telencaphalic ventricle, RV: Rhombencephalic ventricle, SC: Spinal cord. Others abbreviation used in captions is listed in Table 2.

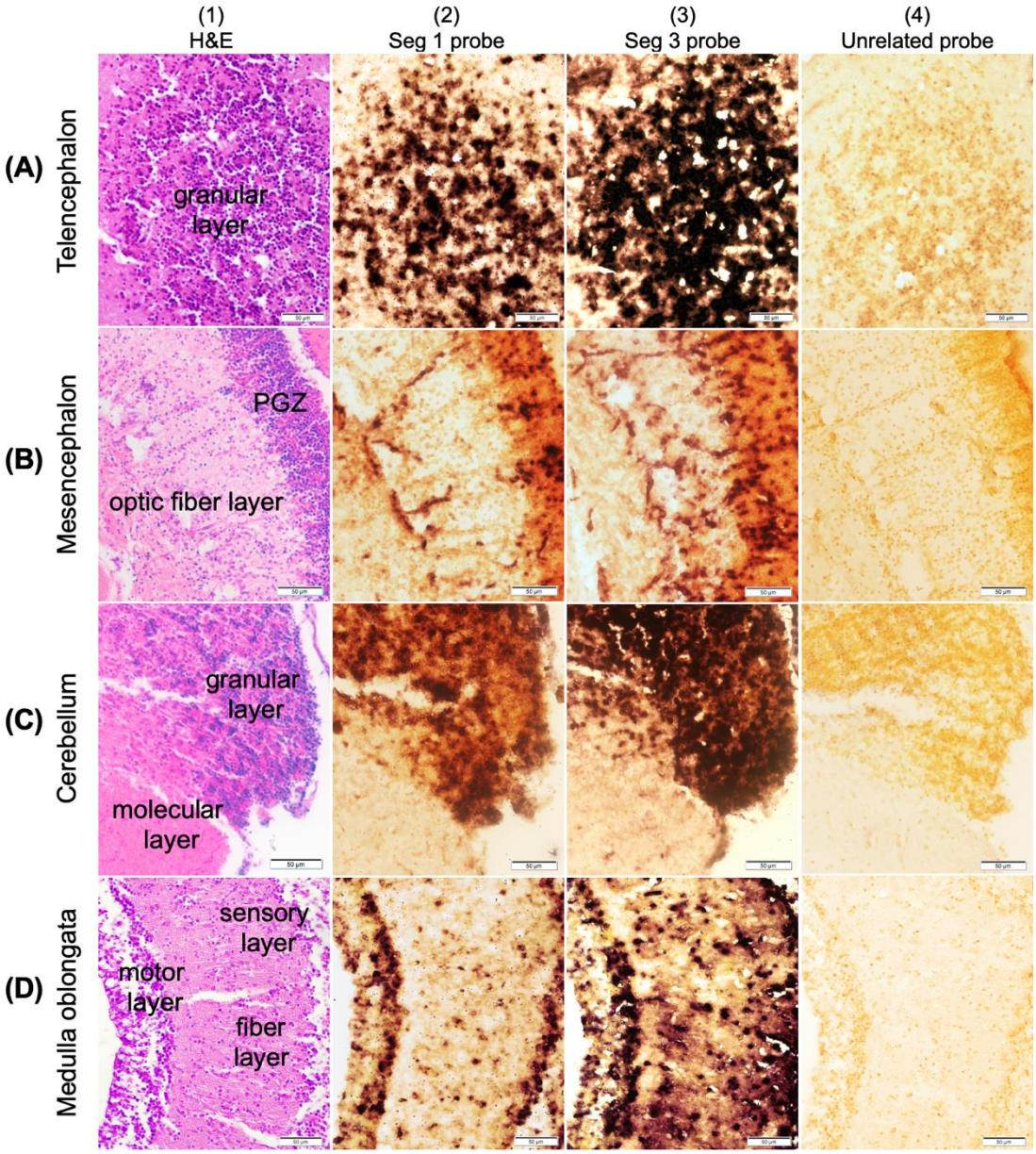

Fig 10. The representative of higher magnification photomicrographs of TiLV distribution in the brain of infected tilapia. The scale bars are shown in the pictures. Strong TiLV positive signals were detected in the cellular layer of the olfactory bulb (BO) in the telencephalon (A); periventricular grey zone (PGZ) of optic tectum in the mesencephalon (B); granular zone of the corpus cerebellum (CCeG) (C); motor layer, sensory layer and fiber layer of the medulla oblongata (D).

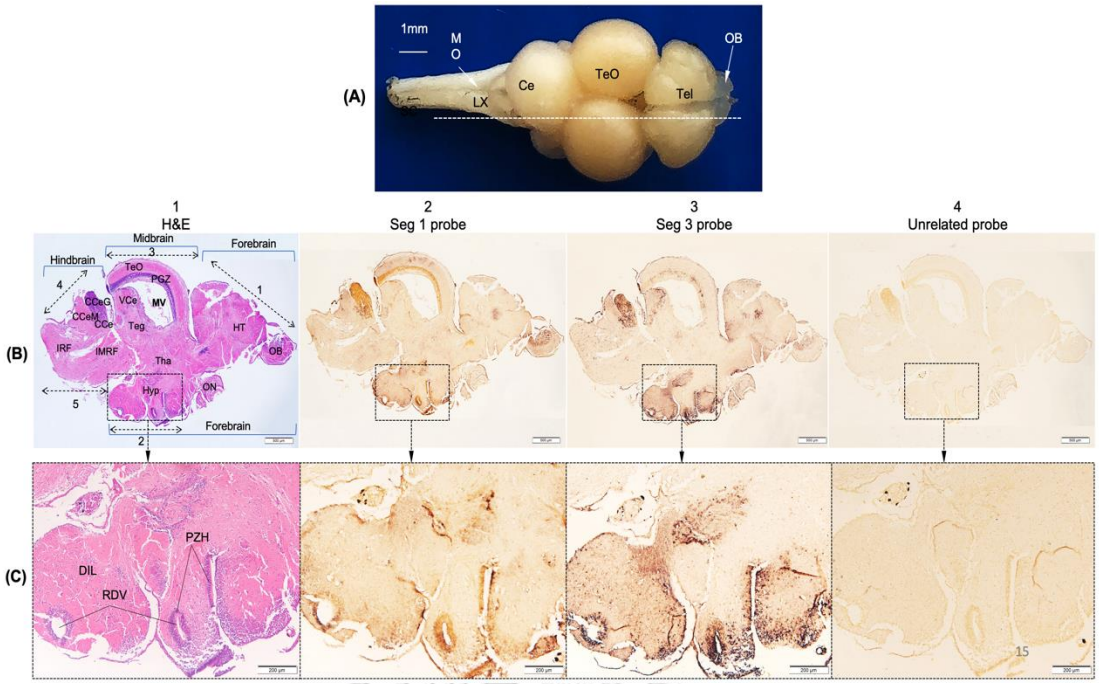

Figure 11. The spatial localization of TiLV in the brain of infected fish *(parasagittal sections).* (A): Dorsal view of tilapia brain with approximate slice position (dashed white line). (B) and (C, higher magnification of hypothalamus): Comparison of parasagittal consecutive sections from infected fish were stained with H&E (B.1, C.1), ISH with TiLV probe based on genome segment 1 (B.2, C.2), genome seg 3 (B.3, C.3), and ISH with unrelated probe as the control (B.4, C.4). The parasagittal section was possible to define five major areas: telencephalon (1), diencephalon (2), mesencephalon (3), mesencephalon (4), medulla oblongata (5). The scale bars are shown in the pictures. The same results that the strongest TiLV positive signals (dark brown) were dominantly detected in primitive meninges (PM), the forebrain (telencephalon and diencephalon), and the hindbrain (metencephalon). DV: Diencephalic ventricle, MV: Mesencephalic ventricle, RDV: Recess of diencephalic ventricle, SC: Spinal cord. Others abbreviation used in captions is listed in Table 2.

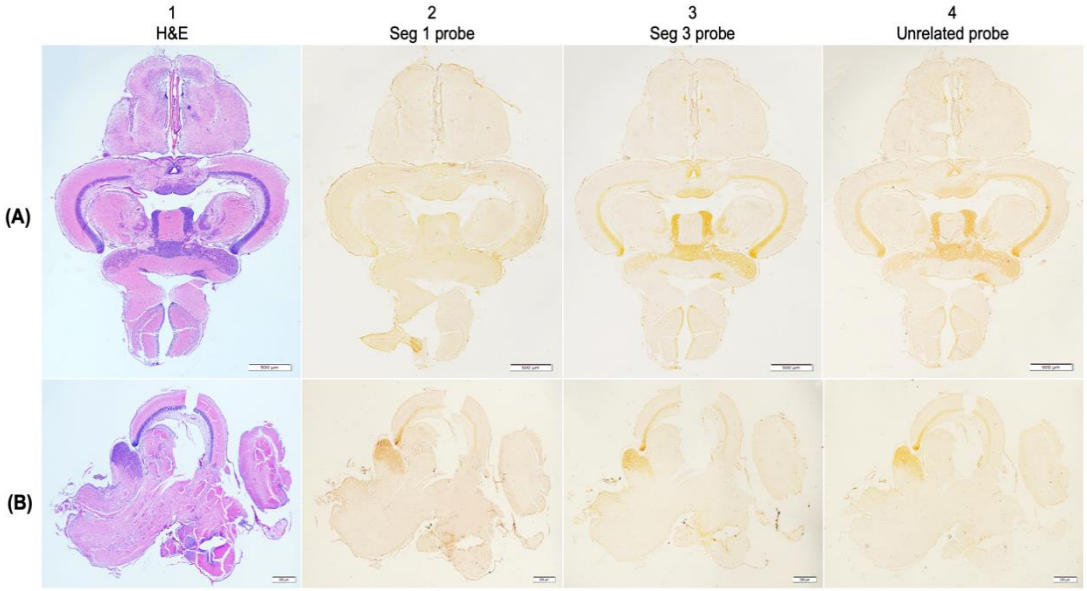

Figure 12. The spatial localization of TiLV in the brain of non-infected fish. (A) and (B): Comparison of horizontal and parasagittal consecutive sections (respectively) were stained with H&E (A.1, B.1), ISH with TiLV probe based on genome segment 1 (A.2, B.2), genome seg 3 (A.3, B.3) and ISH with the unrelated probe as the control (A.4, B.4). No positive signals were found in any brain regions of non-infected fish.

4. The permissive cell zone and pattern of TiLV distribution area

Although the viral signals were presented throughout the brain, ISH revealed strong positive signals of TiLV infected location in the primitive meninges (PM) (Figure 13) and in the periventricular region (both area close to cerebrospinal fluid) (Figure 14). Additional, it should be noted that the heavy ISH signals were also detected in the epithelium of the blood vessels (arrowheads in Figure 13A, B and Figure 14C). In the latter one, the signal intensity exhibited a unique pattern with a gradual increase, more intensive infected cells were detected close to the ventricle, while less infected cells were observed away from the ventricle (see Table 2). In particular, TiLV signals were observed in the ependymal cells lining the ventricles and in the choroid plexus epithelial cells (Figure 14).

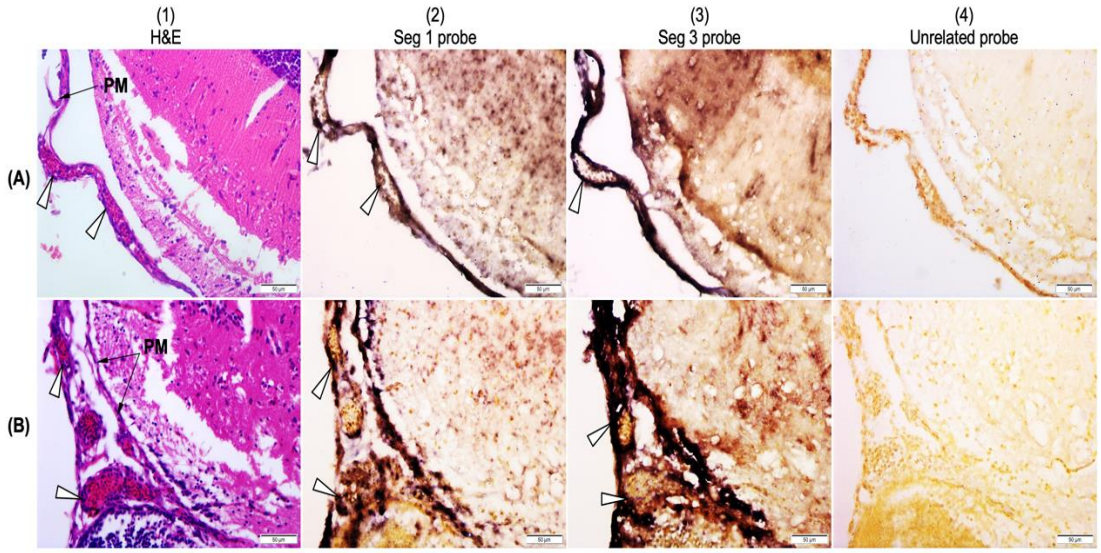

Figure 13. *In situ* hybridization detection of TiLV in the brain *(blood vessels and primitive meninges)*. H&E stained sections (A.1 and B.1), ISH with TiLV-specific probe (A.2, A.3, B.2, and B.3), and ISH sections with unrelated probe (A.4 and B.4). Congestion of blood vessels was found in various areas. Viral RNA was detected in the endothelial cells of the blood vessels (arrowhead) and the primitive meninges (PM) (dark staining).

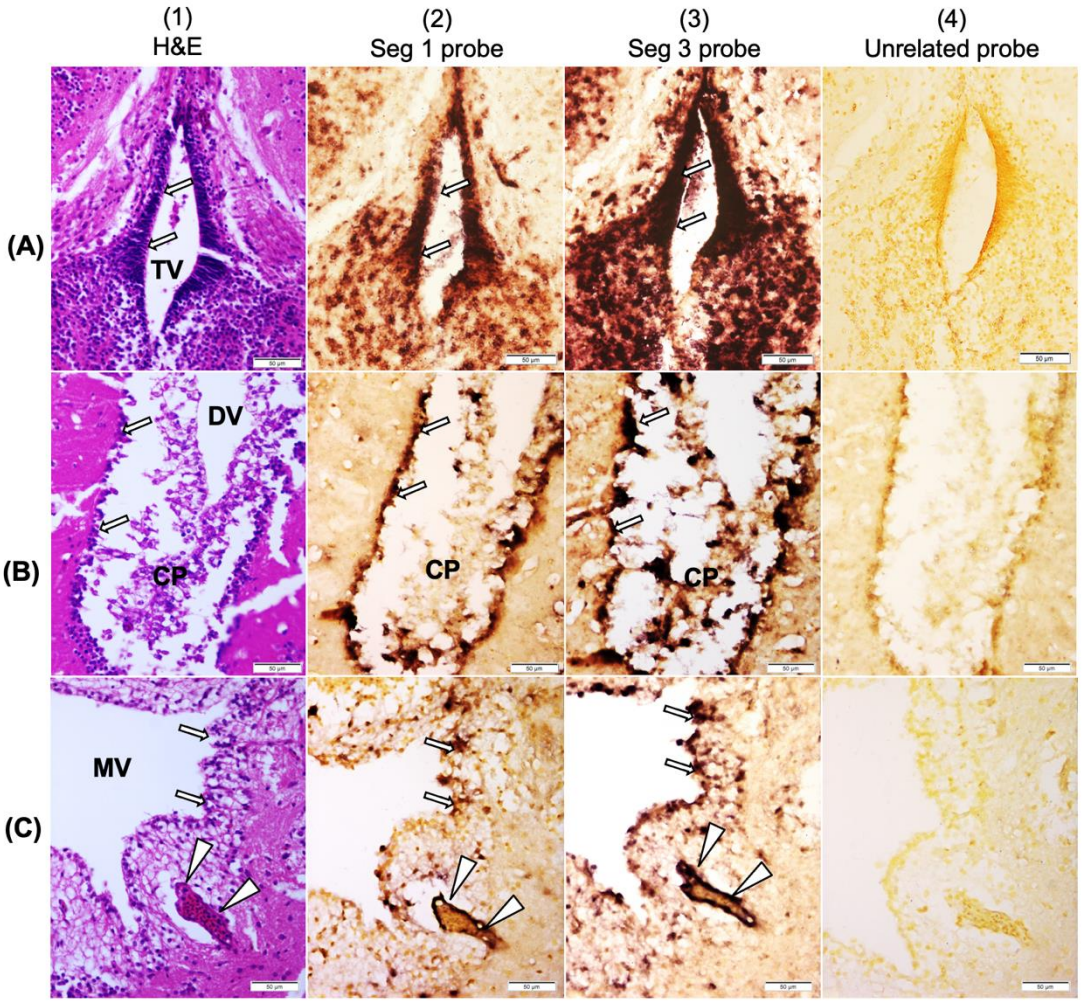

Figure 14. *In situ* hybridization detection of TiLV in the brain *(ventricles and choroid plexus)*. H&E stained sections (A.1, B.1 and C.1), ISH with TiLV-specific probe (A.2, A.3, B.2, B.3, C.2 and C.3), and ISH sections with unrelated probe (A.4, B.4 and C.4). Viral RNA was detected in the periventricular regions including the ventricular ependymal cells ventricular ependymal cells (arrows), choroid plexus (CP) and endothelial cells (arrowheads) of the blood vessels (dark staining). DV: Diencephalic ventricle, MV: Mesencephalic ventricle, TV: Telencaphalic Ventricle.

5. Histopathological assessment of the infected brain

Next, we examined histopathological alterations to correlate signal detection at sites that detect the presence of the virus. Cells degeneration were visible within the location that showing the TiLV positive signals (Figure 15B, D, F, H). Degeneration with loosely cell layer around the ventricles (Figure 15B, H). No apparent histological alterations were visible in the normal brain (Figure 15A, C, E, G). In addition, we found some typical histopathology alterations in various areas of the infected brain such as congestion of blood vessels (Figure 16A, B) and cells aggression (Figure 16C,D).

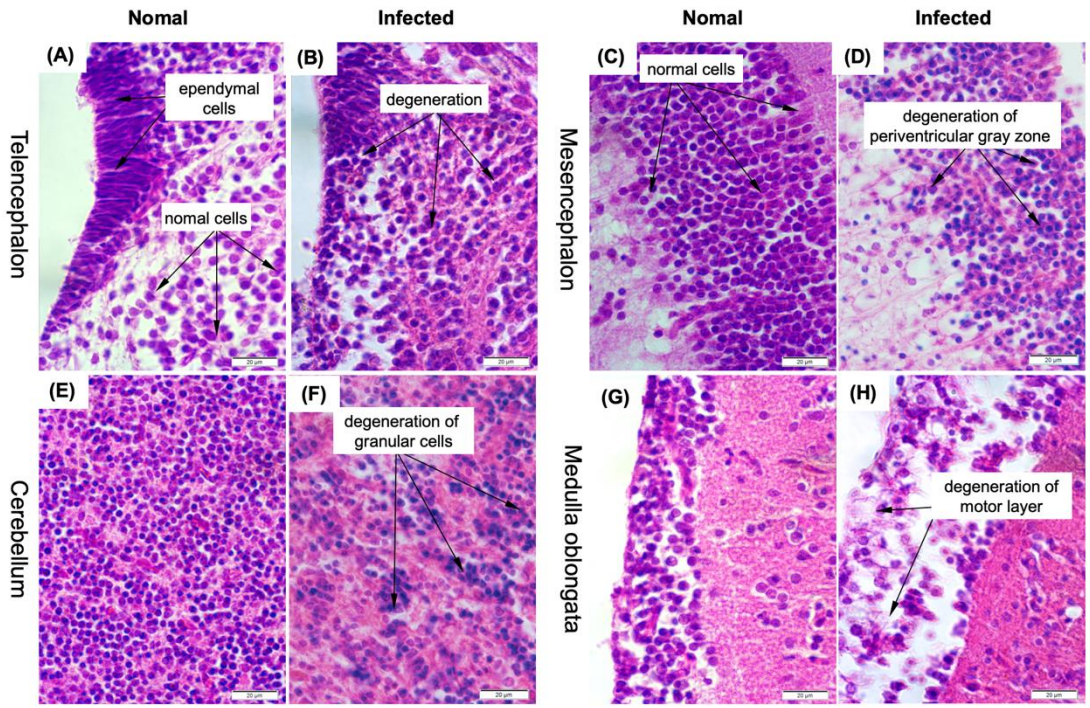

Figure 15. Histopathology of the brain of Nile tilapia from the control and infected fish. (A, C, E, G) Brain histology of the four areas in the normal fish. (B, D, F, H) Brain histology of the four areas in the infected fish. Cells degeneration were observed at sites that detect the presence of the virus.

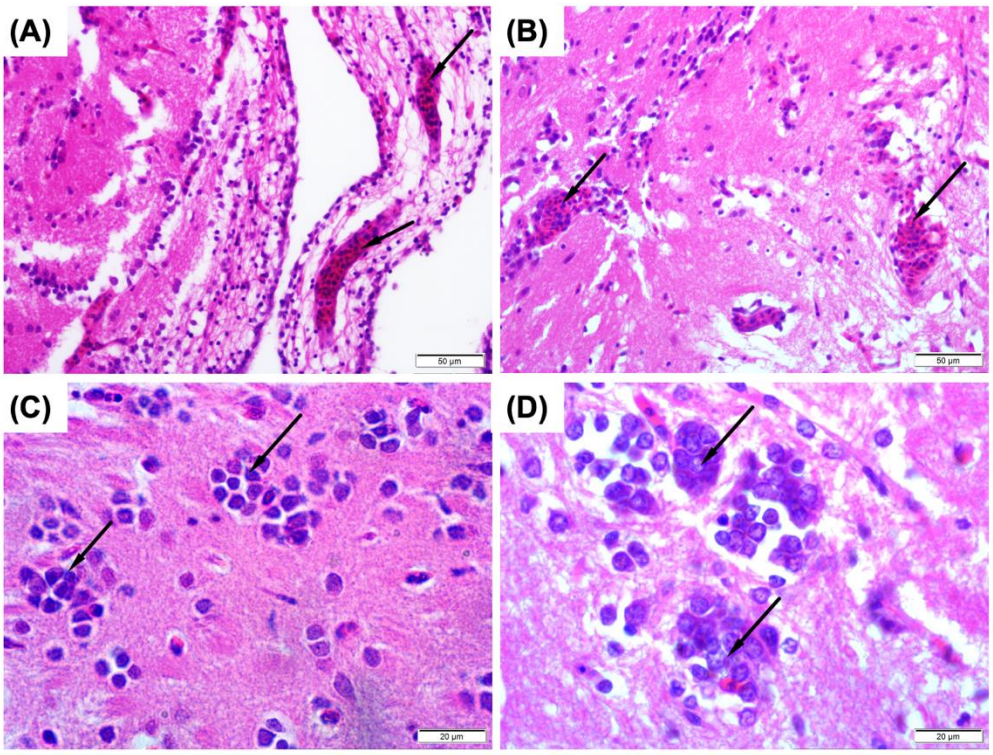

Figure 16. Photomicrograph of typical histopathology alteration in the infected brain. Congestion of blood vessels (arrow, A, B) and cells aggression (arrows, C, D) was found in various areas.

CHAPTER V

DISCUSSION

Localization of TiLV was initially investigated by ISH in a previous study, however, only one part of the brain resembling to optic tectum the midbrain was investigated (Dong et al., 2017). In the present study, the localization of TiLV in the whole-brain of infected Nile tilapia was comprehensively dissected for the first time, using two TiLV-specific probes in parallel for double confirmation. Although distribution of positive signals was detected throughout the brain, however, the signals appeared to be more concentrated in some particular areas of the forebrain and hindbrain. These findings suggest that these regions of the brain might contain more permissive cells for propagation of TiLV than that of the midbrain. With respect to disease diagnosis, these findings imply that the fore- and hindbrain might be the target tissues with predominant of the virus. Despite the fact that this study did not focus on functional study of the fish brain, basic science suggest that the brain is a central nervous system that control important living activities of the fish and different regions of the brain are responsible for different biological functions (Northcutt, 1981; Northcutt, 1995; Ferguson, 2006; Roberts, 2012; Baldisserotto et al., 2019). Therefore, heavy viral infection (indicated by density of ISH signals) in particular brain regions may result in impairment of the brain function and thus possibly explain for abnormal behavior observed in infected fish during the course of infection. The abnormal behavior such as lethargy, loss of appetite, erratic swimming, stop schooling was consistently recorded in this study and several previous studies (Hounmanou et al., 2018; Jansen et al., 2019; Surachetpong et al., 2020). Indeed, based on literature, the teleost brain regions in the fore-, mid-, and hindbrain are involved with specific physiological and behavioral outputs. Specifically, the forebrain contains the telencephalon and the diencephalon, that the telencephalon

encompasses control of sensory and motor as well as cognitive tasks like memory, learning, and emotion (Northcutt, 1981; Northcutt, 1995; Roberts, 2012; Baldisserotto et al., 2019), by detecting strong signals of TiLV in this region are implying that it could associate with some symptoms such as lethargy, stopping schooling, and swimming at the water surface. The diencephalon function mainly as correlation centers for sensory inputs such as gustation and olfaction (Muñoz-Cueto and College, 2001; Ferguson, 2006). With respect to the symptom of losing appetite and stopped eating, it is interesting to observe the intense TiLV signals in the hypothalamus, which controls feeding behavior, receives both olfactory and appetite information, and appears to be able to control movement over the jaw muscles involved in feeding (Roberts, 2012). Localization of TiLV in the middle brain (mesencephalon), the region is particularly involved in the reception and coordination of optic nerve inputs, such as interpretation of motor and visual signals (Roberts, 2012; Baldisserotto et al., 2019). Therefore, we speculate that neuron damage in this area may be related to behavioral disorders of food and prey seeking, navigating around obstacles in the environment, avoidance of approaching objects, and schooling. On the other hand, the cerebellum and medulla oblongata are located within the hindbrain (rhombencephalon) and are generally associated with the regulation of locomotion and balance stimuli as well as basal physiology (Roberts, 2012; Baldisserotto et al., 2019). Therefore, it was possible that the strong densities of TiLV in this area were compatible with erratic swimming or loss of balance. An earlier study also suggested that S. *agalactiae* infection caused damage in the cerebellum of tilapia and led to the erratic swimming symptoms (Palang et al., 2020). Taken together, localization of TiLV with high density detected in important regions of the brain may contribute to possible scientific explanations for an array of severe abnormal symptoms during the course of infection and resulted in high mortality.

Although TiLV was detected in the brain of infected fish (Bacharach et al., 2016; Dong et al., 2017), the mechanism of viral entry to the central nervous system remains unclear. Dong et al. (2020) proposed that TiLV is systemic infection and spreads of the virus to other organs perhaps via the circulatory system. In this study, the localization of the virus was found in the endothelial cells of the blood vessels, which provided quite persuasive evidence of the virus spread through a hematogenous route that results in systemic infection (Keller et al., 2003). One of the most important findings of the distribution of the virus in the brain was the gradual increase in the occurrence of virus-infected cells near the ventricle. It should be noted that the virus was detected near the periventricular region. In this area, more infected cells were detected close to the ventricle, while less infected cells were observed away from the ventricle. There have been no previous studies on the infection routes of TiLV into the CNS after intraperitoneal injection. Currently, the possibility of influenza virus entry into the brain via CSF by crossing the blood-CSF barrier has been suggested in ferrets (Yamada et al., 2012) and chickens (Chaves et al., 2011). They have reported that the influenza virus was found in CSF from infected animals. Either ependymal cells in the ventricle or choroid plexus epithelial cells were positive for viral antigen (Chaves et al., 2011; Yamada et al., 2012). Similar to the findings of previous studies in the influenza virus invasion, our data showed that the strong positive signals were dominantly observed in the periventricular regions including the ventricular ependymal cells and choroid plexus. This finding suggests that the virus may enter the brain through the ventricle and it is possible that CSF plays an important role in TiLV infection to the brain.

In conclusion, our investigation provided new information on the TiLV affinity distribution in the brain of experimentally infected Nile tilapia (*Oreochromis niloticus*). These findings contribute to the basic knowledge of the disease pathogenesis caused by TiLV and host-pathogen interactions. We also discussed the

possible link between TiLV-infected sites and behavioral changes. In addition, we suggested that the ventricles and cerebrospinal fluid (CSF) are important conduits of TiLV to the brain. However, we have not been able to accurately determine the infected cells and the spreading pathway. The pathogenesis and distribution of the virus in the central nervous system at the cellular levels and at various times points of post-infection are therefore needed to elucidate in further studies.

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APPENDIX

Buffer I (10X)

Tris - HCl	121.14 g
NaCl	87.66 g
Distilled H_2O	900 ml (qs to 1L)

Adjust pH to 7.5; autoclave to sterilize; store at RT. To make 1X Buffer I, dilute 100ml of 10X stock solution in 900ml distilled H_2O.

Buffer II (0.5% Blocking solution)

Blocking agent (Roche)	0.5 g
1X Buffer I	100 ml

Buffer III

Tris - HCl	12.114 g
NaCl	5.844 g
$MgCl_2 – 6H_2O$	10.16 g
Distilled H_2O	990 ml (qs to 1L)

Adjust pH to 9.5; autoclave to sterilize; store at RT.

Neutral buffered formalin (10%)

Formaldehyde solution (37%)	100 ml
NaH_2PO_4	4 g
Na_2HPO_4	6.5 g
Distilled H_2O	900 ml

SSC buffer (Saline Sodium Citrate) (20X)

NaCl	175.32 g
Sodium citrate	88.23 g
Distilled H_2O	900 ml

To make 2X SSC buffer, dilute 100ml of 20X stock solution in 900ml distilled H_2O. To make 1X SSC buffer, dilute 50ml of 20X stock solution in 950ml distilled H_2O. To make 0.5X SSC buffer, dilute 25ml of 20X stock solution in 975ml distilled H_2O.

TE buffer (10X)

Tris - HCl	12.114 g
EDTA	3.72 g
Distilled H_2O	990 ml (qs to 1L)

Adjust pH to 8.1; autoclave to sterilize; store at RT. To make 1X TE buffer, dilute 100ml of 10X stock solution in 900ml distilled H_2O.

TNE buffer (10X)

Tris - HCl	60.57 g
NaCl	5.844 g
EDTA	3.72 g
Distilled H_2O	990 ml (qs to 1L)

Adjust pH to 7.4; autoclave to sterilize; store at RT. To make 1X TNE buffer, dilute 100ml of 10X stock solution in 900ml distilled H_2O.

VITA

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