

MOLECULAR CHARACTERIZATION OF CHANNEL
CATFISH VIRUS (CCV) ISOLATED FROM CAGE-
CULTURED *Pangasius hypophthalmus* FARMED IN
PAHANG RIVER, MALAYSIA

BY

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A thesis submitted in fulfilment of the requirement for the
degree of Master of Science (Biotechnology)

Kulliyyah of Science
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ABSTRACT

Pangasius hypophthalmus (Sauvage, 1878), is a freshwater fish originated from Mekong River, which currently cultivated in most Southeast Asia countries including Malaysia. In Malaysia, these species contributed more than 20% of the whole aquaculture production from freshwater culture system. However, viral infection in *Pangasius* may cause high mortality in cage-cultured farm. This study aims to characterize the local isolates of channel catfish virus (CCV) and determine the cytopathic effect (CPE) formation of local CCV in brown bullhead (BB) and channel catfish ovary (CCO) cell lines. Samples of liver, kidney and spleen were collected every two weeks in three selected *P. hypophthalmus* cage-cultured along Pahang River, Temerloh, Pahang. A total of 250 samples were collected from three cage-cultured farm which are 90 samples from Kuala Krau 1, 50 samples from Kuala Krau 2 and 110 samples from Kg Pangsenam. All of the samples were analysed using polymerase chain reaction (PCR) for CCV (ORF27 primers). The PCR analysis showed that 17.6% of the samples were CCV positive, where Farm Kg. Pangsenam placed first with 27.3% followed by Farm 2 Kuala Krau with 12.0% and lastly Farm 1 Kuala Krau with only 8.9% prevalence of CCV detected. It was found that CCV prevalence in Farm Kg. Pangsenam was significantly higher ($p < 0.01$) in the cage-cultured from Pahang River downstream because of the low pH value. In addition, the CCV prevalence was also significantly higher ($p < 0.01$) during dry season because CCV outbreaks occur at high water temperature (25-33°C). However, the CCV prevalence detected in this study show no significant difference ($p > 0.05$) with the fish bodyweight. Sequence analysis of the amplified band (590-bp) using NCBI-BLAST showed that local CCV sequence had 100% similarity with M75136.2 Ictalurid herpesvirus-1 (IcHV1) strain Auburn. Next, the local CCV-positive samples were inoculated in BB and CCO cells for CPE formation observation and susceptibility test. As a result, CPE progression of local CCV in CCO cells took 10 days of post inoculation (dpi) to achieve 80% CPE. The pyknotic cells and loosed network of syncytia were observed and monolayer cell start to detach from the vessel surface. While in BB cells, the normal cells start to rounded and granulated, lead to formation gigantic cell followed by cells detach from surface in 14 dpi. This study shows CPE formation occurs more rapidly in CCO cells than in BB cells. This finding can be applied for early detection and future disease surveillance of CCV infection in *P. hypophthalmus*.

خلاصة البحث

إن سمكة بانغاسيوس (*Pangasius hypophthalmus* (Sauvage, 1878)، هي من أسماك المياه العذبة التي تتكاثر أصلاً في نهر ميكونج (Mekong River)، وتزرع حالياً في معظم دول جنوب شرق آسيا بما في ذلك ماليزيا. تساهم ماليزيا بأكثر من 20٪ من إجمالي إنتاج هذا النوع من الاستزراع المائي من نظام المياه العذبة. ومع ذلك، قد تسبب العدوى الفيروسية في *Pangasius* ارتفاع معدل الوفيات في المزرعة. تهدف هذه الدراسة إلى توصيف العزلات المحلية لفيروس قناة سمك السلور (CCV) وتحديد التأثير الخلوي (CPE) لتشكيل CCV المحلي في خطوط خلايا رأس المبيض (BB) وخلايا قناة مبيض سمك السلور. تم جمع عينات من الكبد والكلية والطحال كل أسبوعين في ثلاثة أنواع مختارة من *P. hypophthalmus* المستزرعة على طول نهر بهنج (Pahang River) الذي يقع في تملوه، بهنج. تم جمع ما مجموعه 250 عينة من ثلاث مزارع مزروعة في أفاص، وهي 90 عينة من كوالا كراو 1، و 50 عينة من كوالا كراو 2 و 110 عينات من قرية بعسينم (Pangsenam). تم تحليل جميع العينات باستخدام تفاعل البلمرة المتسلسل (PCR) في CCV (الاشعال ORF27). وأظهر تحليل PCR أن 17.6٪ من العينات كانت CCV إيجابية، حيث احتلت مزرعة قرية بعسينم (Pangsenam) في المرتبة الأولى بنسبة 27.3٪، تليها عينة من كوالا كراو 2، بنسبة 12.0٪ وأخيراً عينة من كوالا كراو 1، مع 8.9٪ فقط من انتشار CCV المكتشفة. وقد وجد أن انتشار CCV في مزرعة قرية بعسينم (Pangsenam) أعلى بكثير ($p < 0.01$) في قفص المستزرعة من نهر بهنج أسفل المصب بسبب انخفاض قيمة الرقم الهيدروجيني. بالإضافة إلى ذلك، كان معدل انتشار CCV أعلى أيضاً ($p < 0.01$) خلال موسم الجفاف لأن فاشيات CCV تحدث عند ارتفاع درجة حرارة الماء (25-33 درجة مئوية). ومع ذلك، فإن انتشار CCV المكتشفة في هذه الدراسة لا يظهر أي فرق كبير ($P > 0.05$) مع وزن الجسم السمكي. أظهر تحليل تسلسل النطاق المضخم (590-bp) باستخدام NCBI-BLAST أن تسلسل CCV المحلي كان له تشابه 100٪ مع M75136.2 فيروس القوباء الحلقي (Ictalurid-1 (IcHV1)). وبعد ذلك، تم تلقيح العينات المحلية CCV الإيجابية في خلايا BB و CCO لملاحظة تشكيل CPE واختبار الحساسية. نتيجة لذلك، استغرق تقدم CPE المحلي CCV في خلايا CCO 10 أيام من التلقيح آخر (نقطة في البوصة) لتحقيق 80٪ CPE. وقد لوحظت خلايا الحويصلات والشبكة المفكوكة من المخلوقات وتبدأ الخلية أحادية الطبقة في الانفصال عن سطح الوعاء. بينما في خلايا BB، تبدأ الخلايا الطبيعية في التقريب والحبيبات، وتؤدي إلى تكوين خلية عملاقة تليها خلايا منفصلة عن السطح في 14 نقطة في البوصة. توضح هذه الدراسة أن تكوين CPE يحدث بشكل أسرع في خلايا CCO مقارنة بخلايا BB. يمكن تطبيق هذا الاكتشاف للكشف المبكر والمراقبة المستقبلية للأمراض من عدوى CCV في *P. hypophthalmus*.

APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology)

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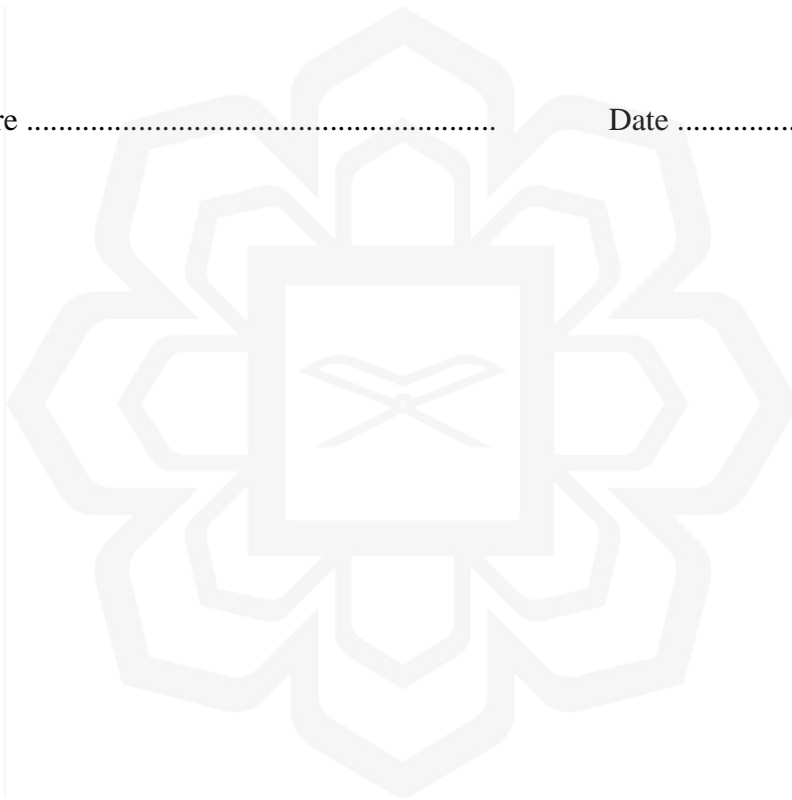
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LIST OF ABBREVIATIONS

(NH ₄) ₂ SO ₄	Ammonium sulphate
ATCC	American Type Culture Collection
ATPase	Adenosine Triphosphatase
BB	Brown bullhead cell line
BNP	Bacillary necrosis of Pangasius
bp	base pair
CCG	Channel catfish gonad cell line
CCO	Channel catfish ovary cell line
CCVD	Channel catfish virus disease
CPE	Cytopathic effect
DEPC	Diethyl pyrocarbonate
<i>E. coli</i>	<i>Escherichia coli</i>
FBS	Fetal Bovine Serum
gB	glycoprotein B
gD	glycoprotein D
gH/gL	glycoprotein H/L
HSV1	human Herpes Simplex Virus 1
IcHV-1	Ictalurid herpesvirus 1
ICTV	International Committee on Taxonomy of Virus
IFA	Indirect fluorescent antibody test
L-15	Leibovitz's medium
MS-222	Tricaine methanesulfonate
NGS	Next generation sequencing
Oct-1	Organic cation transporter 1
OIE	Office international des epizooties
pen/strep	Penicillin-Streptomycin
pH	Potential hydrogen
RT-PCR	Reverse transcription polymerase chain reaction
Taq	Thermus aquaticus
TBE	Tris-borate-EDTA
V	Voltage
ver.	version
VP16	Virus protein 16

CHAPTER ONE

INTRODUCTION

1.1 RESEARCH BACKGROUND

Pangasius hypophthalmus (Sauvage, 1878), commonly known as Sutchi catfish or locally called as 'patin' is a freshwater fish originated from Mekong River, which currently cultivated in most Southeast Asia countries (Thailand, Cambodia, Laos, Indonesia, and Malaysia). The optimum environmental livings for these species are within the ranges of pH 6.5 to 7.5 and a water temperature between 22 to 26°C (FAO, 2009). It is one of the largest and most outstanding aquaculture productions from freshwater in Malaysia after catfish and red tilapia.

P. hypophthalmus are widely cultured in Perak and Pahang, where Pahang ranked first for both wholesale and retail compared to other twelve states in Malaysia (DOF, 2018). The annual aquaculture production recorded by Department of Fisheries Malaysia (DOF) shows the production of *P. hypophthalmus* valued about RM 129 million wholesale and RM 204 million retails in 2017 (no recent update on 2018 aquaculture production; web page last updated was on August 2018). These species contributed more than 20% of the whole aquaculture production from freshwater culture system (DOF, 2018). According to Syakeera, in 2011 the production of *P. hypophthalmus* in Pahang River was reported to face multiple bacterial and viral infection problem causing 30% mortality (Syakeera *et al.*, 2019). The estimated losses in that year was about RM 100 million (DOF, 2018).

The food and aquaculture organization of the United Nations (FAO) reported *P. hypophthalmus* severe outbreak was only caused by bacterial and parasite infection.

According to Camus, the susceptibility of *Pangasius* species to viral infection is unknown (Camus, 2004). However, the epidemiology study reported that there was focal encephalitis with infiltration of mononuclear cell around the ventricle and blood vessels, suggesting viral infection which was CCV infection (Zahrah *et al.*, 2013). The present of channel catfish virus (CCV) was proven as the causative agent of the *P. hypophthalmus* outbreak cases. They managed to prove the present of channel catfish virus (CCV) through molecular detection method polymerase chain reaction, (PCR) using OIE primers (Zahrah *et al.*, 2013). OIE stands for the World Organization for Animal Health which was formerly known as the Office International des Epizooties.

Channel catfish virus (CCV) was identified as a causative agent that caused high mortalities were reported in fingerlings and fry channel catfish in 1960s in the United States (Fijan *et al.*, 1969; Camus, 2004; Hanson, Dishon & Kotler, 2011). CCV is a pathogenic herpesvirus that causes economically severe disease outbreaks in the channel catfish aquaculture industry (Wolf & Darlington, 1971).

Herpesviruses can be recognized from their distinct characteristic morphologies which are icosahedral capsid, linear double-stranded DNA genomes of 125 to 245 kbp and envelope virus (Davison, 2002; Hanson *et al.*, 2011). The clinical signs are erratic swimming, exophthalmia, distended abdomen and haemorrhages at the base fins. Due to high mortality occurrence, a better understanding of CCV from local *P. hypophthalmus* is important to propose a better treatment and prevention measure of this disease. The objective of this study is to compare the genetic distance of CCV Malaysia isolate with CCV reference strain, which is from the Auburn strain. This study also aims to observe and determine the formation of cytopathic effect (CPE) in brown bullhead (BB) and channel catfish ovary (CCO) cell lines caused by CCV Malaysia isolate.

1.2 PROBLEM STATEMENT

CCV is considered as host specific to channel catfish which are native to temperate environments within North America, including southern Canada to northern Mexico (FAO, 2007). Susceptibility of catfish species to CCV only been reported in channel catfish (Nusbaum & Grizzle, 1987), blue catfish (Camus, 2004), channel catfish (Hanson, Rudis, & Petrie-Hanson, 2004) but there no report in *P. hypophthalmus* until recently Zahrah *et al.* reported in 2013.

In 2013, Zahrah *et al.*, revealed the presence of CCV in *P. hypophthalmus* by polymerase chain reaction (PCR) detection method using OIE primers. However, the studies did not discuss further on the genetic distance of CCV isolated from Malaysia compared to CCV reference strains.

Thus, this study is crucial need to screen CCV by using molecular method (PCR) from a whole life cycle of cage-cultured *P. hypophthalmus* to acquire baseline data of CCV infection in Malaysia. This study may conclude what bodyweight ranges that were prone to CCV outbreaks. The finding of the study can be applied for early detection of CCV infection in *P. hypophthalmus* in Malaysia.

1.3 RESEARCH OBJECTIVES

1. To characterize the CCV local strain from *P. hypophthalmus* using molecular detection method.
2. To determine the cytopathic effect (CPE) formation of CCV local strain from *P. hypophthalmus* in brown bullhead (BB) and channel catfish ovary (CCO) cell lines.

1.4 RESEARCH QUESTIONS

1. How similar is the local CCV DNA sequences from *P. hypophthalmus* compared to the references strain (CCV-ATCC VR665)?
2. What are the CPE pattern on BB and CCO cell lines caused by CCV from *P. hypophthalmus* compared to CCV-ATCC VR665?

1.5 RESEARCH HYPOTHESES

1. There are dissimilarities in nucleotide sequences of CCV local strain compared to CCV-ATCC VR665.
2. There are different CPE pattern on BB and CCO cell lines produced by CCV local strain compared to CCV-ATCC VR665.

1.6 SIGNIFICANCE OF THE STUDY

This study intended to determine the genetic distance of isolated CCV from local *P. hypophthalmus* compared to CCV Auburn stain isolated from *Ictalurus punctatus*. Furthermore, this study was conducted to provide information on the CPE patterns formed by local CCV on CCO and BB cell lines. At the end of the study, better understanding of local CCV strains can be applied for early detection and future disease surveillance such as production of CCV detection kits based on ORF27 genes.

CHAPTER TWO

LITERATURE REVIEW

2.1 *P. hypophthalmus*

P. hypophthalmus or commonly known as striped catfish, Sutchi catfish and iridescent shark catfish is one of the most valuable freshwaters fish cultured in Southeast Asia including Malaysia. The common names related to the physical features of the *P. hypophthalmus* which are striped, shiny and iridescent colour. This fish is also known as silure requin and Tiburon pangasio in French and Spanish respectively (FAO, 2009). While, in Malaysia this fish was called as ‘patin’ which was used as the main ingredient in signature dishes of Pahang state. This freshwater fish prefer water with a 6.5-7.5 pH and a temperature range of 22-26 °C (FAO, 2009). This species was classified in Pangasiidae family as shown in Figure 2.1 (Vidthayanon & Hogan, 2013).

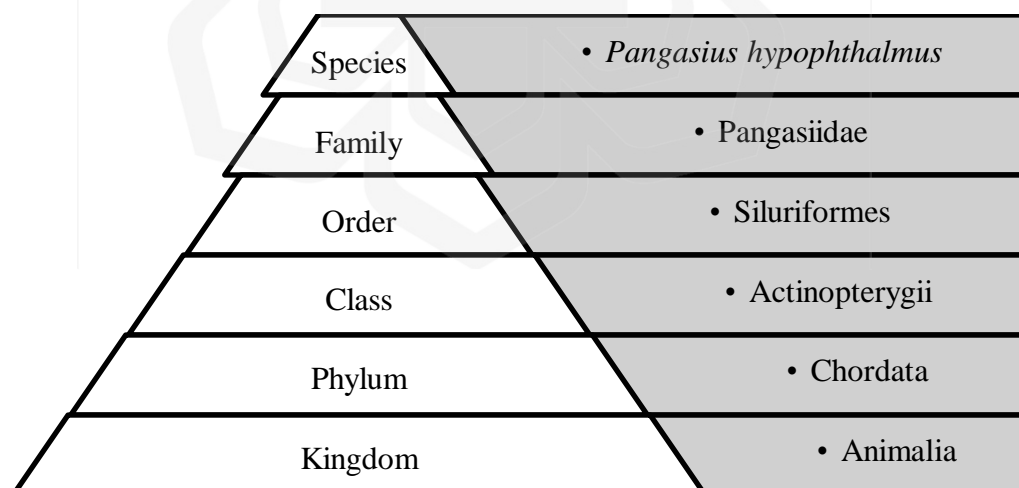


Figure 2.1 The Taxonomy Order of *P. hypophthalmus* (Sauvage, 1878)

2.1.1 Biological Features

P. hypophthalmus has long body with no scales and relatively flattened small head but large eyes. This species has broad mouth with small and sharp teeth. The fins colour was darker than body, either in dark grey or black. This fish gill rakers developed as it grows which functioning as the food particles retention. The most obvious features of this fish are two pairs of barbels which the upper pair is shorter than lower. Meanwhile, young fish of *P. hypophthalmus* have black stripe along lateral line and another stripe below it but not for adult fish (FAO, 2009). Adult fish body usually appeared in grey but sometimes with greenish tint or silvery. *P. hypophthalmus* is omnivorous which can reach maximum length of 130 cm and weight up to 44 kg (FAO, 2009).



Figure 2.2 The *Pangasius hypophthalmus*

2.1.2 Life Cycle

Males *P. hypophthalmus* took two years to reach sexual maturity while females required a year more to mature. These fish can grow up to one kilogram a year (FAO, 2009).

Matured female (>4 years old) was estimated able to produce 112,000 to 138,000 eggs per kilogram body weight and the maximum fecundity was two million eggs (Vidthayanon & Hogan, 2013). Maximum life span of *P. hypophthalmus* was 20 years as displayed by pangasiids at the Steinhart Aquarium in San Francisco, U.S.A (Vidthayanon & Hogan, 2013) . The life cycle of *P. hypophthalmus* was closely related to the monsoon season in Vietnam where spawning takes place in May- June (FAO,2009).

2.1.3 Geographic Range

P. hypophthalmus was originated from the Chao Phraya and Mekong rivers before being introduced widely outside of its native range for aquaculture. The *P. hypophthalmus* were reported to be native occurrence in Cambodia, Laos, Thailand and Vietnam countries. Vietnam was the largest producer of *P. hypophthalmus* that had produced more than 1.1 mil. In 2008 (FAO, 2009). Then, Vietnam introduced the *P. hypophthalmus* aquaculture to another Asian countries like Bangladesh, China, India, Indonesia, Malaysia and Myanmar (FAO, 2009; Vidthayanon & Hogan, 2013).

2.1.4 *P. hypophthalmus* Swimming Patterns

Like other Pangasiid species, *P. hypophthalmus* also capable to make long distance migration (potamodromous). This had been proven by Hogan *et al.* in 2007 by using ultrasonic and conventionally tagging a *P. hypophthalmus* before releasing it in the Tonle Sap River on 30th November 2001. Then, the tagged fish was detected in the Mekong River which was 320 km upstream from the released point. In addition, *Pangasius krempfi* was reported able to migrate over 700 km that support the alibi of Pangasiid species were capable of long-distance migration. These characteristics might

be the cause of *P. hypophthalmus* distribution to other location through spawning during migration (Vidthayanon & Hogan, 2013).

2.1.5 Emerging Diseases in *P. hypophthalmus*

This fish has been reported to be susceptible to diseases such as aeromonad infection caused by *Aeromonas hydrophilia* (Ferguson, 2001), bacillary necrosis of Pangasius (BNP) caused by *Edwardsiella ictaluri* (Crumlish *et al.*, 2002), channel catfish virus disease (CCVD) infection caused by CCV (Zahrah *et al.*, 2013) and epitheliocystis caused by Chlamydiale bacteria (Sood *et al.*, 2017). According to Zahrah *et al.* the presence of CCV in *P. hypophthalmus* was detected through molecular detection suggested by OIE (23 out of 42 samples). Most fish that survive an outbreak still carry CCV in a dormant form (Zahrah *et al.*, 2013).

2.2 CHANNEL CATFISH VIRUS (CCV)

CCV was first isolated by Fijan *et al.* in 1960s from outbreak samples of juvenile channel catfish in Alabama, Arkansas and Kentucky, US (Fijan *et al.*, 1969; Hanson *et al.*, 2011; Wolf & Darlington, 1971). Channel catfish virus disease (CCVD) severely influenced by environmental stress such as pH, temperature and crowding. The CCVD may cause high mortality, reduced growth and predisposition to bacterial diseases in fish population (Camus, 2004; Hanson *et al.*, 2011). The virus is most abundant in kidney, spleen, intestine and liver of an infected fish. CCV also known as *Ictalurid herpesvirus 1* (IcHV1) which was classified in Alloherpesviridae family (Figure 2.3). There were no changes to the taxonomy of IcHV1 species (Adam *et al.*, 2017).

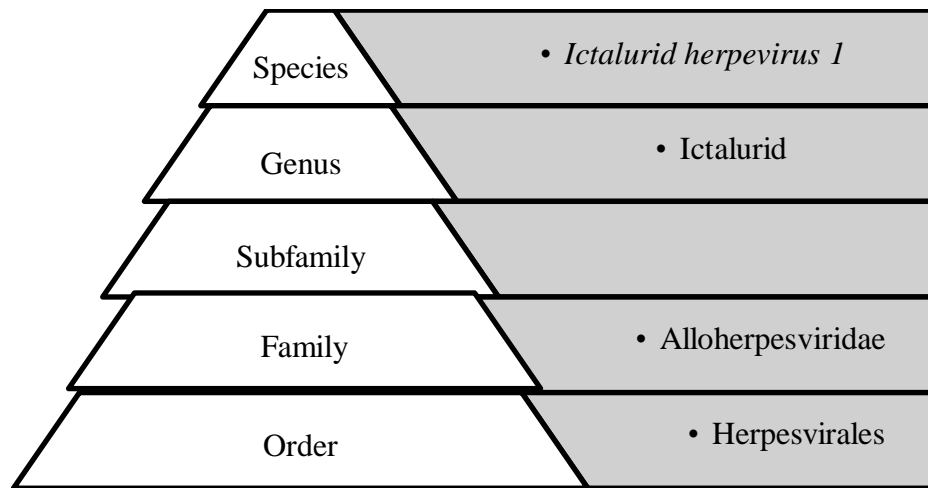


Figure 2.3 The Taxonomy Order of IcHV1.

Note. Adapted from International Committee on Taxonomy of Virus (ICTV) 2017 Master Species List (Adam *et al.*, 2017).

The IcHV1 genome size is 134 kbp, which is among the smallest size in herpesviruses. Although, there are few others CCV strain that has been fully sequenced such as strain S98-675 and strain 760/94. But, the IcHV1 genome or Auburn strain from GeneBank will be used as a reference sequences for this study because it has been isolated from CCV outbreak that caused high mortality in *Ictalurus punctatus* aquaculture. This study is important to trigger a better treatment and prevention measure of this disease in the *P. hypophthalmus* aquaculture industry. The best growth of CCV was seen by from 25-33°C (Wolf and Darlington, 1971). The highest propagation of CCV was 30 °C (Bowser & Plumb, 1980).

2.2.1 Susceptibility of Catfish Species to CCV Infection

The CCV infection was initially detected in most Southern States and from culture systems in most other areas of the United States where channel catfish are grown (Plumb, 1978). It has also been isolated from fry shipped from the United States to

Honduras, Central America (McCraen *et al.*, 1989). The CCV infection spread to the North USA (Mexico) on the early summer of 2005 which has significantly high mortality and a typical external symptoms of virus infection (Sanchez *et al.*, 2007).

The study of the CCV-sperm adherence in channel catfish reported that the attachment of CCV to channel catfish leukocytes was higher than CCV adhered to channel catfish sperm (Nusbaum & Grizzle, 1987). The resistance of African catfish (*Clarias gariepinus*) and the Asian catfish (*Clarias batrachus*) to CCV was observed (Boon *et al.*, 1988). The result shows that only eight from thirty African catfish were exposed to the CCV at high concentration (3×10^5 TCID₅₀/g) but none was detected in Asian catfish. This study suggested that CCV was not a major treat to the introduced cultured of African and Asian catfish in The Netherlands (Boon *et al.*, 1988).

The National Aquaculture Research Centre in Stuttgart, USA has observed the CCVD experimental immersion challenge in juvenile channel catfish (5-7 cm) (Gray *et al.*, 1999). The study reported that more than 70% of infected fish were died between day three and ten of post-infection. The fish were immersed at two different p.f.u (1×10^7 or 1×10^8 p.f.u CCV) at 28 °C for 30 min. The acutely infected fish expressed the clinical signs of CCVD (Gray *et al.*, 1999). While, the surviving fish (22%) from the primary CCV infection were sustained until day 140 post-infection for detection of CCV in latently infection. This experiment found out a latent infection of CCV in channel catfish through PCR analysis (Gray *et al.*, 1999). According to Hanson, the susceptibility of fry channel catfish to CCVD increases with age (Hanson *et al.*, 2004).

The susceptibility of catfish species was varied among species, the inbred strains were commonly more susceptible to infection compared to outbred strains (Camus, 2004). The channel catfish x blue catfish (*Ictalurus furcatus*) hybrids reported to be susceptible to CCV infection as the parental was the channel catfish (Camus, 2004).

The Bullhead, European, African and Asian catfish were reported to be resistant to CCV infection but the *Pangasius* species is unknown (Camus, 2004). Later, about 54.7% prevalence of CCV were detected in *P. hypophthalmus* isolated from cage-cultured in Pahang, Malaysia (Zahrah *et al.*, 2013). The study concluded the occurrence of CCV in *P. hypophthalmus* was most likely influenced by the importation of *Pangasius* brood stocks from Thailand and Vietnam which were currently channel catfish was cultured. Otherwise, the significance of the finding is unknown because the clinical symptoms observed resembled *A. hydrophilia* and *E. ictaluri* infection (Zahrah *et al.*, 2013). In addition, CCV infection commonly found concurrent with bacterial infection (Plumb, 1986). Thus, suggesting that CCV may be present in the fish in dormant state (latent) and lead to secondary bacterial infection (Gray *et al.*, 1999).

The CCVD typically accounts for only 1 to 2 percent of total disease losses in catfish. For example, in the Mississippi Delta, CCV accounted for 1.8 to 5.8 percent of cases received by the Aquatic Diagnostic Laboratory at the Thad Cochran National Warmwater Aquaculture Centre in Stoneville, Mississippi, from 1997 to 2002 (Camus, 2004). Although CCV has a small overall impact on the industry, the effects on individual farms can be significant, with mortalities approaching 100 percent in individual production units.

2.2.2 CCVD Clinical Signs

The fish that has been infected by CCV will swim erratically; horizontally or vertically. In details, the fish were rotating about the longitudinal axis or holding their head up in the water. On the external observation, diseased fish show exophthalmia (protrude eye), distended abdomen, discolouration or haemorrhagic gills, at the base of fins (Fig 4.4)