UNIVERSITI PUTRA MALAYSIA

ISOLATION AND CHARACTERIZATION OF VIRAL NERVOUS NECROSIS VIRUS ON NEWLY ESTABLISHED MARINE FISH CELL LINE AND ITS PATHOGENICITY IN NON-MARINE HOST

MAUIDA F. HASOON

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MAUIDA F. HASOON

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirement for the Degree of Doctor of Philosophy

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DEDICATION

To my parents: Fuad and NazhatHasoon, thank you for your unconditional support with my studies since I was preschool stage until this moment. I am honored to have you as my parents. Thank you for giving me a chance to prove and improve myself through all my walks of life. Hoping my quest to get this degree of higher education is making your dream a reality.

To my husband: Dr. Majed H. Mohammed, the most awesome person I ever know. To whom I owe my love and trust, to my husband who has supported and encouraged me through all of my desires and goals.

To my lovely sons: Ali and Mohammed. I give my deepest expression of love and appreciation for all the sacrifices you've made during this graduate program. Please accept my apology.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of
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ISOLATION AND CHARACTERIZATION OF VIRAL NERVOUS NECROSIS VIRUS ON NEWLY ESTABLISHED MARINE FISH CELL LINE AND ITS PATHOGENICITY IN NON-MARINE HOST

By

MAUIDA F. HASOON

December 2011

Chairman: Associate Professor Hassan Hj. Mohd. Daud, PhD

Faculty: Veterinary Medicine

In Southeast Asia, the intensification of aquaculture industry has led to the occurrence of various infectious diseases. Virus diseases in particular, affecting especially the larval and juvenile stages, are the most suppressing factors for sustainable production of high value fish species such as seabass *(Lates calcarifer*). Inevitable, because of lack of sensitive methods that could be used for diagnosis and the inadequacy of pathogen control, the aquaculture industry will continue to be plagued by these diseases. Currently, viral nervous necrosis (VNN) is one of fish diseases listed by the Office International des Epizooties (OIE) as a notifiable disease in the production of marine fish worldwide. Thus the main objective of the present study was to determine the role of VNN virus (VNNv) as a pathogen in the Malaysian aquaculture industry, with emphasis on

virus isolation in susceptible cell line, virus identification using polymerase chain reaction (PCR) and identification of characteristic microscopy lesions in VNNv infections.

A new cell line designated as Asian Seabass Brain (ASBB-1) was derived from the brain tissue of seabass cultured in Malaysia. This cell line was maintained in Leibovitz L-15 media supplemented with 10% fetal bovine serum (FBS). The ASBB-1 cell line was sub-cultured more than 60 times over a period of 15 months. This cell line consists predominantly of fibroblastic-like cells, which are able to grow at temperatures between 20°C and 30°C with an optimum temperature of 25°C. At 25°C, the growth rate of these cells increased as the proportion of FBS increased from 5 to 20% with optimum growth at 15% or 10% FBS.

The ASBB-1 cell line was characterized by karyotyping, and chromosome number distributions were different with subcultures P20 and P50 producing 48 and 46 chromosome peaks respectively. The ASBB-1 cells showed 90% viability after recovery from one year storage in liquid nitrogen. The ASBB-1 cells up on characterization by PCR using primer sets of microsatellite markers of Asian seabass *(Lates calcarifer)* were shown to be similar to the cells from tissues of seabass at 250 bp.

The VNNv isolate derived from seabass was tested positive for VNN by the IQ2000 Kit®. The isolate designated UPM08-1M was used in the cell line

infectivity study. The susceptibilities of ASBB-1 cell line isolate and the highly permissive commercial SSN-1 cells to UPM08-1M were compared. The results showed that ASBB-1 cell line was susceptible to VNNv (RGNNV genotype) with typical cytopathic effect (CPE) manifesting mainly as rounding-up at1 day post infection (dpi), severevacuolationwithin3-5dpi and complete detachment within 7dpi. The VNNv-induced CPE was further elucidated by electron microscopy (EM). Under EM, the ASBB-1cellsexhibitedvacuolated degeneration with presence of viral inclusion–like bodies. The CPE of VNNv on ASBB-1cells was producedat a virus titer of $10^{9.5}$ $TCID_{50}$/ml. This indicated that the ASBB-1 cell line is highly susceptible for use in the isolation of VNNv.

Biophysical and biochemical characterization of VNNv isolate was determined by heat treatment, UV irradiationand the stability under effect of chemical disinfectants. The VNNv isolate showed susceptibility to heat treatment at 60°C within 30 minutes with no viable virus after 1 hour. UV irradiation at an intensity of 440 mWrcm^2 resulted in a reduction in virus titer after 8 minutes. The virus appeared relatively resistant to changes in pH ranging between 2 to 11 after 1 hour incubation at 25°C. Treatment with 2% formalin was not totally effective even after 6 hours. Iodine did not inactivate the virus.

Molecular characterization of the tissue culture-propagated virus using RT-PCR and nested PCR, showed positive amplifications of genome size of 460 bp and 220 bp respectively. The 220 bp nucleotides from the T4 region of the coat

protein gene was sequenced and the phylogenetic analysis resultsshowed close resemblance between the UPM08-1M strain andSBNNV and GPNNV2strains of Malaysianisolates.The local isolate of UPM08-1M also showed phylogenetic similarity with other strains from South East Asia, while showed relatively distant similarity with other VNNv strains from the Middle East. Cold water VNNv strains isolated from Norway, Canada and USA were distant phylogenetic relations to the UPM08-1M strain.

Experimental infectivity study was also performed using fresh water guppy fish (*Poecilia reticulata)* as a model. Guppies were intranasally infected with 0.1 ml of the UPM08-1M strain at a titre of $10^{9.5}$ $TCID_{50}$/0.1ml. Although the artificial infection resulted in slight mortality (11%) occurring within 14 days post inoculation, the VNN-specific lesions such as necrosis and vacuolation in the target organs of brain and retina were evident. The presence of virus in infected brain and retina tissues was confirmed by transmission electron microscopy (TEM). The VNNv from experimentally infected guppy was successfully re-isolated in ASBB-1 cells inoculated with homogenate of brain and retinas of the survivors. There was gradual increment of virus titer in the guppy throughout the experimental period. The results indicated that guppy fish could be infected with VNNv isolated from diseased seabass.

In summary, the study successfully isolated VNNv using the ASBB-1cell line. Complete CPE of this cell line was observed within 5 – 7 dpi after infection with VNNv at producing considerable higher titer of $10^{9.5}TCID_{50}$/0.1ml. Thus ASBB-1

cells can be recommended for VNNv (RGNNV genotype) isolation and will be an important tool for future conduct of fish health assurance programs in the aquaculture industry. The study also showed that RT-PCR followed by nested-PCR is a sensitive technique for identification of VNNv isolated from seabass. It was also shown that guppy fish could be experimentally infected with VNNv, thus can be used as a virus-infection model.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMENCILAN DAN PENCIRIAN VIRUS NEKROSIS SARAF DALAM SEL TURUTAN IKAN MARIN YANG BAHARU DIBANGUNKAN DAN PATOGENISITINYA DALAM IKAN BUKAN MARIN

Oleh

MAUIDA F. HASOON

Disember 2011

Pengerusi: Profesor Madya Hassan Hj. Mohd. Daud, PhD

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Di Tenggara Asia, pengamatan industri akuakultur telah membawa kepada kemunculan pelbagai penyakit berjangkit. Penyakit virus, terutama sekali yang dikesan di peringkat larva dan juvenil ikan, merupakan faktor penghalang terhadap penghasilan mapan spesies ikan bernilai tinggi seperti ikan siakap (*Lates calcarifer*). Oleh sebab kekurangan kaedah peka yang boleh diguna untuk diagnosis dan pengawalan patogen yang tidak mencukupi, maka industry akuakultur akan terus diganggu oleh penyakit ini. Kini nekrosis saraf virus (VNN) merupakan satu daripada penyakit ikan yang tersenarai oleh Office International des Epizooties (OIE) sebagai penyakit kena-lapor dalam penghasilan ikan di seluruh dunia. Dengan demikian, objektif utama kajian ini ialah untuk

menentukan peranan virus VNN (VNNv) sebagai patogen dalam industri akuakultur Malaysia, dengan memberi tekanan kepada pemencilan virus dalam sel turutan yang susceptibel, pengenalpastian virus mengguna tindakbalas rantai polymerase (PCR) dan pengenalpastian ciri lesi mikroskopi dalam jangkitan VNNv.

Satu sel turutan baharu yang diberi nama *Asian Seabass* Brain (ASBB-1) telah diperolehi daripada tisu otak ikan siakap Malaysia. Turutan sel ini disenggarakan dalam mediam Leibovitz L-15 yang ditambah serum fetus bovin (FBS) 10%.Turutan sel ASBB-1 ini telah disubkultur lebih daripada 60 kali dalam tempoh 15 bulan. Turutan sel ini terdiri terutamanya daripada sel bentuk fibroblast yang berupaya untuk tumbuh pada suhu antara 20 hingga 30°C dengan suhu optimum 25°C. Pada suhu 25°C kadar pertumbuhan sel ini meningkat dengan peningkatan berkadar FBS daripada 5 hingga 20% dengan pertumbuhan optimum pada FBS 10% atau 15%.

Turutan sel ASBB-1 telah dicirikan melalui pengkariotipan dan taburan bilangan kromosom berbeza dengan subkultur 30 dan 50, masing-masing member puncak 48 dan 46 kromosom. Sel ASBB-1 ini menunjukkan kebolehhidupan 90% selepas satu tahun dipulih daripada simpanan dalam nitrogen cecair. Sel ASBB-1 apabila dicirikan melalui PCR dengan mengguna set primer penanda mikro satelit ikan siakap, telah didapati serupa dengan sel daripada tisu ikan siakap pada 250 bp.

Pencilan VNNv yang diperolehi daripada ikan siakap diuji positif untuk VNN apabila kit IQ2000® digunakan. Isolat yang diberi nama UPM08-1M ini telah diguna dalam kajian kejangkitan. Kepekaan turutan ASBB-1 dan sel SSN-1 komersial permisif tinggi terhadap isolat UPM08-1M telah dibandingkan. Hasilnya menunjukkan turutan sel ASBB-1 ini rentan terhadap VNNv (genotip RGNNV) dengan kesan sitopatik (CPE) ternyata terutama sekali sebagai pembulatan sel pada 1-2 hari pasca jangkitan (dpi), pengvakuolan teruk dalam tempoh 3-5 hari dan pemisahan sepenuhnya dalam tempoh 7 dpi. CPE teraruh VNNv kemudian dijelaskan lagi melalui mikroskopi electron (ME).Di bawah ME sel ASBB-1 ini menunjukkan penyahjanaan tervakuol dengan wujudnya jasad bak rangkuman virus. CPE VNNv terhadap sel ASBB-1 telah terhasilk dengan titer virus $10^{9.5}$ $TCID_{50}$/0.1ml. Ini menunjukkan, bahawa turutan sel ASBB-1 ini paling peka dan sesuai untuk diguna dalam pemencilan VNNv.

Pencirian biofizik dan biokimia pencilan VNNv telah dijalankan melalui perlakuan haba, pancaran UV dan kestabilan dibawah kesan disinfektan kimia. Pencilan VNNv ini menunjukkan sensitit terhadap perlakuan haba pada 60°C dalam tempoh 30 min dengan tiada virus yang masih hidup selepas 1 jam. Pancaran UV pada keamatan 440 $mWrcm^2$ menyebabkan kekurangan titer virus selepas 8 min. Virus ini agak tahan perubahan pH antara 2 hingga 11 selepas dieramkan pada 25°C. Perlakuan dengan 2% formalin tidak memberi kesan sepenuhnya walaupun selepas 6 jam. Iodin tidak mentakaktifkan virus ini.

Pencirian molekul virus terbiak kultur tisu mengguna RT-PCR diikuti PCR tersarang menunjukkan amplifikasi positif genom bersaiz 460 bp dan 220 bp. Nukleotida 220 bp daripada kawasan T4 pada gen protein selaput telah dijujukkan dan hasil analisis filogenetik menunjukkan persamaan rapat antara strain UPM08-1M dengan strain SBNNV dan GPNNV2 pencilan Malaysia. Pencilan tempatan UPM08-1M ini juga menunjukkan persamaan filogenetik dengan strain lain daripada Tenggara Asia, sambil menunjukkan persamaan agak jauh dengan strain VNNv daripada Negara Timur Tengah. Strain VNNv air sejuk yang dipencil di Norway, Kanada dan USA ada perhubungan jauh dengan strain UPM08-1M.

Kajian kejangkitan ujikaji telah dijalankan mengguna ikan gapi (*Poicelia reticulata)* sebagai model. Ikan gapi ini dijangkitkan secara intranasum dengan strain UPM08-1M pada titer $10^{9.5}$ $TCID_{50}$/0.1ml. Walaupun jangkitan tiruan ini menghasilkan sedikit sahaja mortaliti (15%) yang berlaku dalam tempoh 14 hari pasca inokulasi, lesi khusus VNN seperti nekrosis dan pengvakuolan dalam otak dan retina adalah jelas. Kewujudan virus dalam otak dan tisu retina terjangkit telah disahkan melalui mikroskopi electron pancaran (TEM). VNNv daripada ikan gapi terjangkit ujikaji telah berjaya dipencil semula dalam sel SSN-1 yang telah diinokulasi dengan homogenat otak dan retina daripada ikan yang mandiri. Titer virus meningkat secara beransur dalam ikan gapi dalam tempoh ujikaji ini. Hasil kajian ini menunjukkan ikan gapi boleh dijangkitkan dengan VNNv yang dipencil daripada ikan siakap terjangkit.

Ringkasannya, kajian ini telah berjaya memencilkan VNNv mengguna turutan sel ASBB-1. CPE menyeluruh sel ini telah dilihat dalam tempoh 5-7 hari selepas dijangkitkan dengan VNNv pada titer $10^{9.5}TCID_{50}$/ml. Justeru itu, sel ASBB-1 boleh dicadangkan untuk pemencilan VNNv (genotip RGNNV) dan ini akan menjadi suatu alat yang penting dalam program jaminan kesihatan ikan dalam industri akuakultur. Kajian ini juga menunjukkan RT-PCR diikuti dengan PCR tersarang merupakan suatu teknik peka untuk pengenalpastian VNNv yang dipencil daripada ikan siakap. Ikan gapi juga didapati boleh dijangkitkan secara ujikaji dengan VNNv dan diguna sebagai model jangkitan virus.

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Amen...

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

MAUIDA F. HASOON

Date: 20 December 2011

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LIST OF ABBREVIATIONS

ASBB-1	Asian sea bass brain-1 cell line
BFNNV	Barfin flounder nervous necrosis virus genotype
bp	Base pair
CNS	Central nervous system
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscope
FBS	Fetal bovine serum
GB	Grouper brain cell line
GF-1	Grouper fin-1 cell line
GS	Grouper spleen cell line
H&E	Haematoxylin and Eosin
kb	Kilo base pair
KDa	Kilo Dalton
LM	Light microscope
EM	Electron microscopy
TEM	Transmission electron microscopy
IHC	Immunohistochemistry
IFAT	Immunoflorescent antibody test
min	Minute
NaCl	Sodium chloride
HBSS	Hanks' balanced salt solution
CPE	Cytopathic effect
NE-PCR	Nested- polymerase chain reaction
NNV	Nervous necrosis virus
RT-PCR	Reverse-transcriptase polymerase chain reaction
nm	Nanometer

nt	Nucleotide
OD	Optical density
OIE	Office international des epizooties
ORF	Open reading frame
PBS	Phosphate-buffered saline
pH	Hydrogen ion exponent
pi	Post- infection, post-inoculation
%	Percentage
PCR	Polymerase chain reaction
RGNNV	Red-spotted grouper nervous necrosis virus
RNA	Ribonucleic acid
rpm	Revolution per minute
rt	Room temperature
SE	Sea bass fry cell line
SJNNV	Striped jack nervous necrosis virus genotype
SnRV	Type C retrovirus
SSN-1	Striped snakehead-1 cell line
ssRNA	Single stranded ribonucleic acid
TAE	Tris-acetate-EDTA
TF	Turbot fish cell line
TPNNV	Tiger puffer nervous necrosis virus genotype
UPM	Universiti Putra Malaysia
VER	Viral encephalopathy retinopathy
VNN	Viral nervous necrosis disease
VNNv	Viral nervous necrosis virus
µg	Micro gram
µl	Micro liter
µm	Micro meter
°C	Degree centigrade (Celsius)

CHAPTER 1

INTRODUCTION

In recent years, with the rapidly developing mariculture activities world over, outbreak of viral diseases has become a serious issue causing heavy economic losses (Seng and Colorni, 2002). Diseases due to viral etiology, such as the viral nervous necrosis virus (VNNv) have been reported from many parts of Asia with mariculture activities (Chang *et al.*, 2001).

Viral nervous necrosis viruses (VNNv) have emerged as major pathogens of a wide range of larval and juvenile marine finfish in aquaculture worldwide. Disease in larval and juvenile marine finfish attributable to a VNNv was first described by Yoshikoshi and Inoue (1990) in Japanese parrotfish (*Opelegnathus fasciatus*), where the disease was designated as viral nervous necrosis (VNN). Since that time similar diseases have been reported in a wide range of aquaculture species in both marine and fresh water, and from the sub-tropics to cold-temperate regions of the world (Glazebrook *et al.,* 1990; Bloch *et al.,* 1991; Breuil *et al.,* 1991; Chua *et al.,* 1995 and Hegde *et al.*, 2003). Some papers on VNN-like disease have described the disease as `barramundi picorna-like virus infection in barramundi (*Lates calcarifer*) (Glazebrook and Heasman 1992), `encephalomyelitis' in turbot (*Scophthalmus maximus*) (Bloch *et*

al., 1991), or `fish encephalitis' in sea bass (*Dicentrarchus labrax*) (Breuil *et al*., 1991).

Much remains to be learnt about the epidemiology of VNN as current knowledge is inadequate to provide a reasonable basis for development of control measures. From transmission studies, it appears that lateral transmission is a potent means of spread of infection. This is affected by stocking density (Arimoto *et al*., 1993), temperature and virulence of the particular VNNv for the exposed species (Tanaka *et al*., 1998). There is strong evidence for vertical transmission of infection in striped jack fish (Arimoto *et al*., 1992) and a lesser degree of evidence for European sea bass (Comps *et al*., 1996), Japanese flounder, barfin flounder and Atlantic halibut (Grotmol and Totland, 2000). It is tempting to consider that vertical transmission may occur in all susceptible species but at differing levels, thus influencing the relative importance of lateral transmission.

The emergence of neural diseases caused by viruses has highlighted the need for cell cultures from brain tissues of fish as hosts for tissue specific viruses (Chi *et al*., 2005; Parameswaran *et al*., 2006). Although more than 150 fish cell lines have been established, relatively few have been derived from tropical marine fish (Fryer and Lannon, 1994). Thus it becomes increasingly crucial to develop suitable cell lines for the isolation and study of viral diseases affecting fish in this region.

Cell culture methodologies are essential tools for the diagnosis of viral diseases in farmed fish (Wolf, 1988; Hetrick and Hedrick, 1993). Such techniques are mainly based on the use of fish cell lines sensitive to different viruses (Ahne, 1985; Hetrick and Hedrick, 1993).

Viral infection on cell culture is primarily demonstrated by the cytopathic effect (CPE) characterized by cell lysis or syncytial cell-cell fusion. Furthermore, viral *in vitro* infection of susceptible cell lines facilitates several serological techniques including the detection of fish antibodies against the pathogenic viruses by means of the viral neutralization test (OIE. 2000). The use of cell cultures is necessary to study the biology and pathogenic mechanisms of culturable viruses affecting aquaculture (Wolf, 1988).

Since the appearance of VNN, numerous attempts were made to isolate the virus using the principal existing cell lines (Watanabe and Yoshimuzu, 1999), but none of them were successful. In 1996, Frerichs *et al.* succeeded in replicating the virus in a cell line developed in 1991 from striped snakehead fish fry (*Ophiocephalus striatus*) called SSN-1. This cell line is fastidous to maintain and in addition it is persistently infected by a Type-C retrovirus known as SnRV (Frerichs *et al.*, 1991). In order to remediate the use of this contaminated cellular substrate, six cellular clones were created from SSN-1 known as A6, B7, C3, E2, E9, and E1. The cell line clones are characterized by their susceptibility to the four

official betanodavirus genotypes (SJNNV, RGNNV, TBNNV and BFNNV) (Iwamoto *et al.*, 2000). Although three of these (A6, E9, and E11) clones are found to be most permissive to the development of the CPE, but they are still co-infected with SnRV (Lee *et al.*, 2002).

Susceptible cell lines toward VNNv have subsequently been developed, such as GF-1 derived from fin tissues of *Epinephelus coioides* (Chi *et al.*, 1999), SF, derived from larvae of seabass (Chang *et al.*, 2001), GB, originating from brain of *E. awoara* (Lai *et al.*, 2003), TF from *Scophthalmus maximus* (Aranguren *et al.*, 2002), and GS, derived from the spleen of *E. coioides* (Qin *et al.*, 2006). A cell line known as BB originated from the brain of barramundi persistently infected with a VNN isolate has recently been developed (Chi *et al.*, 2005). The capacity of VNN strains isolated from sea bass to replicate in cell cultures has been studied by Delsert *et al.* (1997) whodemonstrated that; unlike insect nodaviruses, VNNv as belonging to betanodaviruses are not capable of replicating in many cell cultures.

The causative agent of Viral Nervous Necrosis disease (VNN) belongs to the genus *Betanodavirus* of the family *Nodaviridae*. It is non-enveloped and icosahedral in shape with a diameter of approximately 20 to 30 nm. Virions contain two segments (RNA1 and RNA2) of positive sense single-stranded RNA (ssRNA) (Mori *et al.,* 1992). RNA2 segment contains the sequence for the viral coat protein, and it is highly conserved among

isolates. This conserved region is the target for the detection of viral RNA by molecular methods.

There are four recognized genotypes: striped jack nervous necrosis virus (SJNNV); tiger puffer nervous necrosis virus (TPNNV); barfin flounder nervous necrosis virus (BFNNV) and red-spotted grouper nervous necrosis virus (RGNNV) (Nishizawa *et al.,* 1997). A possible fifth genotype; turbot betanodavirus (TNV) has recently been proposed (Johansen *et al.,* 2004).

Exclusion of infected animals would be the most certain means of control of VNN. At present, the elimination or segregation of viral nervous necrosis virus-carrying spawners, as detected by PCR, is the best choice to prevent disease in striped jack fish, even though a negative PCR does not mean complete absence of virus in the fish (Mushiake *et al.*, 1994). As an alternative control procedure, a basic investigation on vaccination has commenced in striped jack fish using a recombinant coat protein of SJNNV (Munday and Nakai, 1997).

It is apparent that nervous necrosis nodaviruses and their respective diseases are widespread in the Indo-Pacific region (Yoshikoshi and Inoue 1990; Mori *et al.,* 1991, 1992; Munday 1994). Such viruses have also been reported from the Mediterranean, France and Scandinavia (Grotmol *et al.,* 1995).

If marine aquaculture continues to be conducted under existing philosophies in the future, VNNv infections as well as other infectious diseases will further increase on both a geographic and a host species basis. However, as the establishment of infection generally depends on a balance between the amount of invading agent and the defense mechanisms of the host, the most important prophylactic method is to reduce various stress factors on the fish.

Further virological and molecular biological investigations on piscine nervous necrosis nodaviruses will be required to understand these diseases. In particular, there are very few reports of VNN nodavirus-associated mortality episodes in fish species from fresh water. Munday *et al.* (2002) and Bigarre *et al.* (2009) indicated in their studies, that tilapia larvae reared in freshwater were naturally susceptible to nodavirus infection belonging to the RGNNV genotype. Moreover, they are also susceptible to a bath infection with a nodavirus of the same genotype derived from sea water.

There is an urgent requirement to compare the infectivity among nodaviruses isolated from different sources. In addition, a cell line will be a useful tool comparable to rapid test of PCR, for epidemiological purposes in identifying the initial sources of infection in fish species, and the detection of infective virions in the environment (Maltese and Bovo, 2007).

The present study attempts to develop a new cell line for VNNv isolation and characterize this isolated virus to determine it's taxonomic identity with more detailed biochemical and biophysical analysis. The pathogenicity of the isolated virus for certain freshwater fish was determined by intranasal experimental infection and looking for viral replication and evidence of pathology. To accomplish these goals, it was first necessary to establish an appropriate cell line for the propagation and assay of viral isolate. Once this experiment was completed, more sophisticated sensitive techniques for utilizing tissue culture adapted virus were adopted. This would further facilitate the investigation of VNNv as a pathogen in Malaysian aquaculture, with emphasis on its capability to replicate in nonmarine host.

Objectives of this study were:

I. to establish a marine cell culture from the local Asian sea bass.

II. to isolate viral nervous necrosis virus (VNNv) using the established cells in comparison with commercial cell line.

III. to characterize the cell culture-adapted virus biophysically and biochemically.

IV. to determine the molecular characteristic of the cell culture-adapted virus.

V. to determine the pathogenicity in experimentally infected non-marine fish following VNNv infection.

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