UNIVERSITI PUTRA MALAYSIA

ISOLATION AND IDENTIFICATION OF NERVOUS NECROSIS DISEASE VIRUS IN WILD GOLDEN GREY MULLET (Liza auratus Risso) FROM IRANIAN COASTAL WATERS OF CASPIAN SEA

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By

ALIREZA NAZARI

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

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Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the Doctor of Philosophy

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Chairman: Associate Professor Hassan Hj. Mohd Daud, PhD

Faculty: Veterinary Medicine

A study was conducted to determine the etiological agents of serious mortality which occurred in Golden grey mullet (*Liza auratus)* in Iranian water of south of Caspian Sea. This study was apart of Iranian national research project supported by Iranian Fisheries Research Organization (IFRO). Three hundreds and twelve moribund Grey mullet specimens were collected from 2006 until 2009 in the costal waters of three Northern Provinces of Iran i.e. Gilan, Mazandaran and Golestan. Infected fishes manifested marked clinical signs such as erratic swimming, belly-up at rest, swim bladder distension, exophthalmia and hemorrhages in the skin. Internal organs such as kidney, swim bladder, intestines, eyes and brain were taken for cell culture isolation, serology, and histopathology. Twenty-one VNN-PCR-positive samples obtained from Gilan Province were further examined using cell culture. Cytopathic effects were visualized in SSN-1 cell line which was inoculated with brain and eyes

filtrate. The CPEs were characteristic of VNN virus, showing severe cytoplasmic vacuolation and eventually total detachment of the monolayer. Negative staining of infected monolayer cells showed icosahedral particles with a size of 30 nm in diameter. The TEM morphology of these particles was similar to nodavirus particles. IFAT examination of smears prepared from infected cells using MAbs and AAbs against VNNV showed positive results.

Histopathological examination of the brain and retina that manifested CPE in cell culture showed necrosis and vacuolation in the brain and retina. TEM examination showed the presence of intracytoplasmic virus particles with 30 nm in diameter in the affected tissues.

For confirmation of these results, infectivity study was conducted. Due to difficulty in keeping Grey mullet in aquarium for a long time, an ornamental freshwater fish, guppy (*Poecilia reticulata*), was chosen and bath challenge using the media of infected cell line. After 15 days post challenge, the fishes showed clinical signs similar to Grey mullet in natural infection. Cell culture isolation, TEM, histopathology and IHC were done for brain and retina from the infected guppy. Similar findings were obtained in infected guppy tissues when compared to naturally infected Grey mullet.

In conclusion, with distinguishable clinical signs, TEM and cell culture observation, IFAT and IHC findings from naturally infected Grey mullet and also from experimentally infected guppy, it was thus proved that a viral disease was the causative agent of mortality in the Grey mullet. From the current study it was

concluded that Viral Nervous Necrosis Virus was the etiological agent of mortality in Gray mullet in Iranian water of Caspian Sea.

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

PEMENCILAN DAN PENGENALPASTIAN VIRUS PENYAKIT NEKROSIS SARAF DALAM IKAN BELANAK KELABU EMAS (*Liza auratus* Risso) DARI PERSISIRAN PANTAI IRAN, LAUT KASPIAN

Oleh

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Pengerusi: Profesor Madya Hassan Hj. Mohd Daud, PhD

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Satu kajian telah dijalankan untuk menentukan agen etiologik kejadian kematian yang serius dalam ikan Belanak kelabu keemasan (*Liza auratus)* di perairan selatan Laut Caspian, Iran. Kajian yang dijalankan adalah sebahagian dari projek penyelidikan kebangsaan Iran yang di sokong oleh Organisasi Penyelidikan Perikanan Iran (IFRO). Tiga ratus dua belas ekor spesimen ikan belanak kelabu telah dikutip dari tahun 2006 hingga 2009 di persisiran pantai tiga wilayah utara Iran iaitu Gilan, Mazandaran dan Golestan. Ikan yang dijangkiti menunjukkan petanda klinikal yang nyata seperti berenang yang tidak menentu, perut ke atas semasa berehat, pundi renang mengembung, eksoptalmia dan hiperemia di kulit. Sampel mata dan otak diperiksa secara pemencilan kultur sel, serologi dan histopatologi. Dua puluh satu sampel positif PCR-VNS yang di ambil dari wilayah Gilan telah diberi pemeriksaan lanjut dengan inokulasi dalam kultur sel.

Kesan sitopatik (KSP) telah dilihat dalam sel turutan SSN-1 yang diinokulat dengan filtrat otak dan mata. KSP yang dilihat adalah sama dengan KSP virus VNS, menunjukkan vakulasi sitoplasmik yang teruk dan akhirnya keluruhan seluruh ekalapis. Pewarnaan negatif sel ekalapis yang dijangkiti menunjukkan partikel virus berdiameter purata 30 nm. Morfologi TEM partikel ini adalah sama seperti partikel nodavirus. Pemeriksaan IFAT pada smer yang disediakan dari sel yang dijangkiti menggunakan MAbs dan AAbs terhadap VNS menunjukkan keputusan positif. Pemeriksaan histopatologikal tisu ikan yang telah menunjukkan KSP dalam k ultur sel mempamerkan perubahan vakulatif dalam otak dan retina. Pemeriksaan TEM pada sampel tersebut juga memperlihatkan kehadiran partikel secara intrasitoplasmik virus berdiameter 30 nm.

Untuk pengesahan keputusan ini, jangkitan eksperimen telah dijalankan. Olehkerana ketidakbolehan untuk menyimpan ikan belanak kelabu dalam akuarium pada jangkamasa yang panjang, sejenis ikan perhiasan airtawar iaitu ikan gupi (*Poecilia reticulata*), telah dipilih dan prosedur cabaran mandian menggunakan media dari sel turutan yang terdahulu dijangkiti digunakan. Selepas 15 hari jangkitan, ikan gupi menunjukkan petanda klinikal serupa seperti jangkitan semulajadi dalam ikan belanak kelabu. Propagasi dalam kultur sel, TEM, histopatologi dan IHC telah ulang untuk sampel yang diambil dari gupi yang dijangkiti. Keputusan yang sama juga ditemui. Sebagai kesimpulan, melihatkan persamaan petanda klinikal yang nyata, pemerhatian TEM dan kultur sel, dan keputusan IFAT dan IHC dari jangkitan semulajadi ikan belanak kelabu dan dari cabaran eksperimen dalam ikan gupi, adalah bukti bahawa satu agen virus adalah penyebab kematian dalam ikan belanak kelabu. Dari kajian semasa juga dapat dibuat kesimpulan bahawa Virus Nekrosis Saraf

adalah agen etiologik kematian yang serius dalam ikan Belanak kelabu emas di persisiran Laut Caspian, Iran.

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I believe without help of ALLAH I would never able to finish my dissertation

In 2006, Universiti Putra Malaysia in cooperation with Iranian Fishery Research and Organization (IFRO) gave me opportunity to caring my PhD Thesis. I am grateful for your trust and confidence in me. This thesis would never have been completed without your support.

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I also thank my colleagues in Aquatic Animal Health Unit, Virology and Histopathology laboratory in Faculty of Veterinary Medicine, UPM.

I certify that an Examination Committee has met on **August 2011** to conduct the final examination of **Alireza Nazari** on his thesis entitled "**Isolation and Identification of Nervous Necrosis Virus in Golden grey mullet (*Liza auratus*) captured in Iranian water of Caspian Sea**" in accordance with the Universiti and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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 other institutions.

ALIREZA NAZARI

Date: 10 August 2011

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LIST OF ABBREVIATIONS

ABV	Aquabirnavirus
BFNNV	Barfin Flounder Nervous Necrosis Virus
CPE	Cytopathic effect
CNS	Central Nervous System
IFAT	Indirect immunofluorescence antibody test
FAT	Immunofluorescence antibody test
ELISA	Enzyme-linked immunosorbent assay
EM	Electron Microscopy
EMEM	Eagle Minimum Essential Medium
EGC	Eosinophilic Granular Cells
FAO	Food and Agriculture Organization
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate Conjugate
GMVNN	Grey mullet Viral Nervous Necrosis
HBSS	Hank's Balance Salt Solution
IHC	Immunohistochemistry
I.R.IRAN	Islamic Republic of Iran

IFRO	Iranian Fisheries Organization
IgG	Immunoglobulin G
iRGNNV	inactivated Red-spotted Grouper Nervous Necrosis Virus
H&E	Haematoxylin and Eosin
LM	Light Microscopy
MAb	Monoclonal Antibody
MEM	Minimal Essential Medium
NNV	Nervous Necrosis Virus
OIE	*Office International des Epizooties*
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
RT-qPCR	Reverse Transcriptase quantitive Polymerase Chain Reaction
RGNNV	Red-spotted Grouper Nervous Necrosis Virus
RNA	Ribonucleic acid
SC	Spinal Cord
SJNNV	Striped Jack Nervous Necrosis Virus
SSN-1	Striped snakehead
TBL	Total Body Length
$TCID_{50}$	Tissue Culture Infective Dose at 50% end-point

TEM	Transmission Electron Microscopy
TPNNV	Tiger Puffer Nervous Necrosis Virus
UV	Ultra violet
VER	Viral Encephalopathy and Retinopathy
VLPs	Virus-like particles
VNN	Viral Nervous Necrosis
VNNV	Viral Nervous Necrosis Virus

CHAPTER ONE

INTRODUCTION

1.1 Caspian Sea

The Caspian Sea, the largest lake in the world, is located in north of Islamic Republic of Iran (I.R.Iran). It is an inland depression located between 47°07′ and 36°33′N and 45°43′ and 54°50′E (Figure 1.1).

Fig. 1.1: Map of Caspian Sea and regions in North of Iran. (www.wikipedia.org, July 2010).

The Caspian Sea is thousands of kilometers away from the world's open waters. Thus, it could be called a lake. In the other hand, it has many marine characteristics such as a large water surface area and volume, a specific hydro chemical regime, horrible gales and etc. More than 40% of the global lacustrine waters are in the Caspian Sea. The Caspian Sea area is 386,400 km² which may vary by 10-20% in the sea tide. The coastline of Caspian Sea is about 7250 km which extended around 1,200 km from north to south, and is 200-450 km wide. The Caspian Sea in the north is relatively shallow and the average depth is 5-6 m and the maximum depth is 15-20 m. The Middle of the lake has the maximum depth of 788 m and average depth of 190 m while in the South the maximum depth is 1025 m. The average depth of the shelf of the Caspian Sea is about 180 m (Wikipedia, 2010). The coastline of Iranian side is approximately 1000 km long (15% of the total coastline) which included the three littoral provinces of Gilan (center Rasht), Golestan (center Gorgan) and Mazanderan (center Sari) (Gorgani, 2004).

1.2 Fish species in Caspian Sea

There are over 120 fish species in the southern part of the Caspian Sea, which 23 species are important than others and commercially divided in two groups, i.e. sturgeons and bony fishes. The bony fishes are also divided into kilka and other species. The main commercial species are as follows: Beluga (*Huso huso*), Russian sturgeon (*Acipenser guldenstadti*), Iranian sturgeon (*A. persicus*), and Sevruga (A.stellatus), Kilka (*Clupeonella delicatula*; *C.engrauliformis; C.grimmi*) while other bony fishes, includes Kutum (*Rutilus frisii kutum*), Mullets (*Mugil auratus; M.saliens*), Carp (*Cyprinus carpio*), Bream (*Abramis brama*), Pike-perch

(*Lucioperca lucioperca*), Roach (*Rutilus rutilus*) and Rainbow trout (*Oncorhynchus mykiss*) (Coad, 2008). Some pictures of the important species in the Caspian Sea are shown in the Appendices A.

1.3 Golden grey mullet (*Liza auratus*)

1.3.1 General biology and taxonomy

The family Mugilidae belongs to order Mugiliformes, is very famous family in this order. Golden grey mullet (*Liza aurata*), flathead mullet (*Mugil cephalus*), and Leaping mullet (*Liza saliens*) are three species in this family. The mullets or Grey mullets are living in temperate to tropical coastal waters readily enter estuaries and even found in freshwaters. There were three species introduced to the Caspian Sea but only two species have been successfully adapted to Caspian Sea, *Liza auratus* and *Liza salience Risso 1810* (Coad, 2008).

The Grey mullet body color is a dusky grey to blue-grey with a silvery flank and belly. The back and upper flank have a series of dark to golden stripes. There is a golden blotch on the operculum. The peritoneum is dark brown to black. Golden grey mullet is recognized from its head and anterior-dorsal flank scale having only one pit or groove (occasionally double), scales on the snout ending anteriorly as a single row of small scales, and the oral edge of the preorbital bone is moderately concave. The upper jaw reaches posteriorly to a level with the posterior nostril, the only species in the genus with this character. The young fish at the origin of the caudal fin rays have two vertical dark lines and on the flanks, a herring bon pattern (Coad, 2008). In

Iranian coast of Caspian Sea, Grey mullet reaches weight of 0.3-0.8 kg and sometimes as much as 1.5-1.8 kg and maximum length of 54 cm. Its life cycle is up to 12 years and maturity is attained at 38-45 cm and age of 3-4 years for males and 5-6 years for females (Code, 2008).

1.3.2 Distribution and habitat

Grey mullets found worldwide in the Mediterranean and Black Seas and from the British Isles to South Africa. *Liza auratus* and *Liza saliens* were introduced to Caspian Sea Since more than 70 years ago and became acclimated to this environment. Grey mullet is found in the lower reaches of rivers along the Caspian coastline of Iranin the Anzali lagoon and its outlets where numbers will probably increase with increasing salinity, i.e. in the Sefid River, Gorgan Bay, southeast of Caspian Sea, southwest Caspian Sea and south-central Caspian Sea (Coad, 2008).

Grey mullet lives in the sea and enters the lower reaches of rivers, and is sometimes found in close to lakes with depths of 5-700 m. Their eggs develop in the open sea and larvae migrate to the coast and young feed along the shore and in bays. In the winter which is beginning from the March, Grey mullet migrate in surface water along the Iranian shore in coastal waters, for feeding in shallow areas of the middle Caspian and, by September-October, mature fish migrates over deeper water (300-700 m) of the middle and south Caspian to spawn. Its feeding will be stopped at 6-8ºC and death occurs below 1.5ºC. The optimum temperature is 23-25ºC but the young fish can be found in shallow water at 37.5ºC. It occurs from fresh water to salinities of 57 p.p.t. with mass mortalities recorded at 65 p.p.t (Coad, 2008).

Grey mullet is a benthonic feeder and feed small benthic invertebrates and detritus with some insects and plankton. Most stomach contents contain numerous sand grains. The adult fish graze periphyton from rocks, silt and artificial structures (Coad, 2008).

1.3.3 Viral Nervous Necrosis (VNN) in Grey mullet

Some morbidities and mortalities in Grey mullets were observed in southern coastline of Caspian Sea for the first time in central zone of Mazandaran province (Fereydunkenar and Babolsar) in 2002 (IFRO, 2002). Moribund fish showed clinical signs such as lethargy, darting swimming, exophthalmia, abdominal swollen and hemorrhage in skin. In autopsy, gas accumulation in swim bladder and full gall bladder were observed. These fishes were thought to be malnourished for long time. Also, empty digestive system and sand aggregation in the stomach was seen in some unhealthy fish.

In 2003, 2004 and 2005 similar occurrence of moribund Grey mullets were reported in different zone of other provinces in south of Caspian Sea.

1.4 Importance of study

1- Characterization of the causative agent causing mortality with the aim to eliminate the sources of disease and establishing a control and prevention programme against the disease.

2- To reduce fish mortality in Caspian Sea in order to increase fish production and supply of fresh protein for people.

3- Results obtained from this study would be useful for Iranian government and other countries bordering the Caspian Sea for future development of fisheries industry.

1.5 Objectives of the study

1- To assess tissue damage in naturally infected fish by light microscopy (LM) and transmission electron microscopy (TEM).

2- To isolate the causative agent of morbidity and mortality in Grey mullet in Caspian Sea.

3- To identify and characterize the causative agent of disease.

4- To confirm the causative agent of mortality using *in vivo* assay (Infectivity challenge).

1.6 Hypothesis

VNN virus (VNNV) could be the main causative agent of morbidity and mortality in Golden grey mullet *Liza auratus* in Iranian coastal water of South Caspian Sea.

1.7 Research methods

In the present research a multidisciplinary and complete approach were planned and several useful methods were engaged to reach the stated objectives. Six basic fundamental diagnostic methods were used viz., virus isolation in cell culture, TEM, serological assay, histopathology assessment and pathogenicity confirmation by

challenge test. Procedures for sampling and laboratory approaches are showed in schematic diagrams in Figures 1.4 and 1.5.

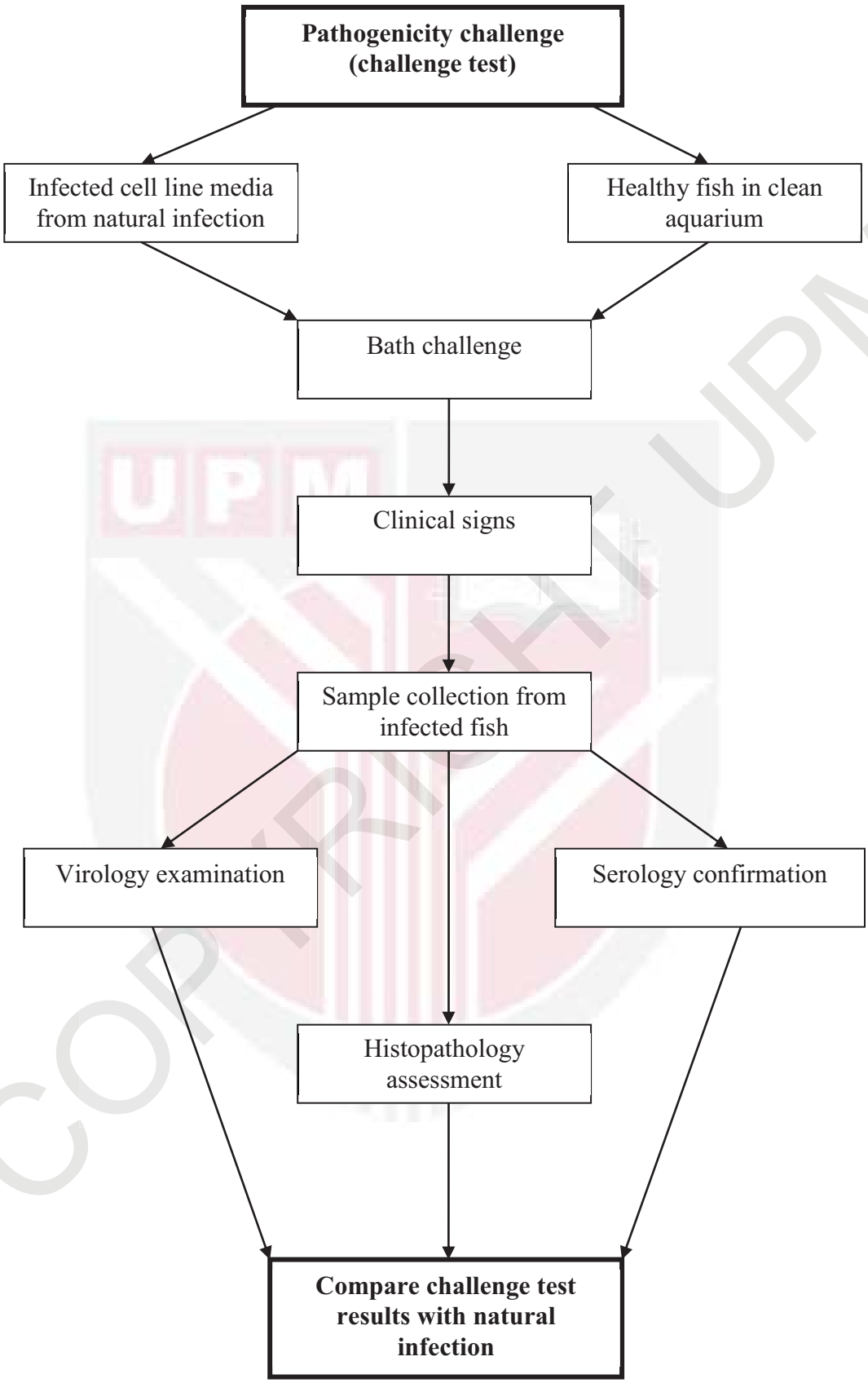

Fig.1.2: Schematic diagram of challenge test carried out in this study.

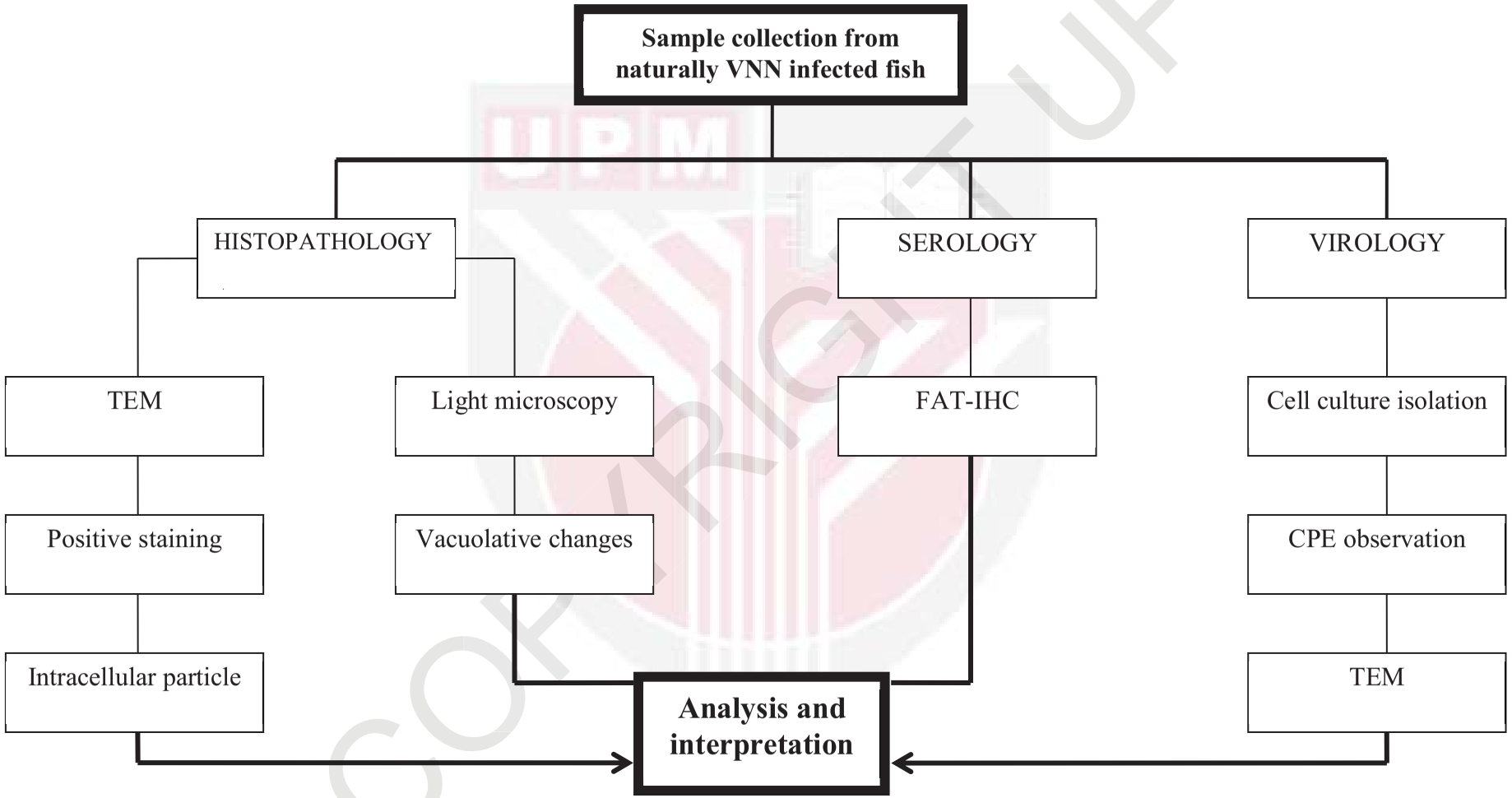

Fig.1.3: Schematic diagram of steps used in this study.

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