GENOME ANALYSIS AND EXPERIMENTAL EVIDENCE OF HORIZONTAL TRANSMISSION OF NERVOUS NECROSIS VIRUS (NNV) INFECTING HATCHERY STOCKS OF MARINE FISH

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2011

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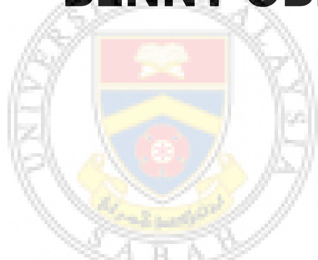

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THESIS SUBMITTED IN FULFILLMENT FOR THE DEGREE OF MASTER OF SCIENCE

**BORNEO MARINE RESEARCH INSTITUTE
UNIVERSITI MALAYSIA SABAH**

2011

DECLARATION

I hereby declare that the material in this thesis is my own except for quotations, excerpts, equations, summaries and references, which have been duly acknowledged.

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DEGREE : **MASTER OF SCIENCE (AQUACULTURE)**

VIVA DATE : **18 OCTOBER 2011**

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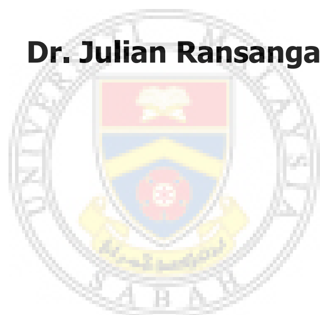

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ACKNOWLEDGEMENTS

First of all, I wish to thank our heavenly Father through His beloved son Jesus Christ, Who has guided me during the course of my study, giving me strength physically and spiritually. Without His great help, this work would never been done.

I would like to thank my supervisor, Dr. Julian Ransangan for sharing his great ideas, knowledge, discussion and guiding me in doing the experiments and also during the process of writing this thesis. Thank you also for spending so much time to read and correcting this thesis. I feel that you are the best supervisor.

I also would like to thank the current and former director of Borneo Marine Research Institute for their supports and providing space and facilities to do the experiments. And special thanks to Prof. Dr. Saleem Mustafa for reading through this thesis and valuable comments.

Many thanks also to the UMS hatchery staffs, Mr Kien Chee Lu and his staffs at Seed Production Centre of Sabah Fisheries and Dr Azila Abdullah at National Fish Health Research Centre for providing fish specimens for this study.

I am grateful to all my friends especially Veronica Ginus, Mohd Tamrin, Chong Yen Thing and Veronica Albert for supporting and helping me during the experiments and throughout the period of my study. Special thanks also to Catherine May Godon for your love and support and also for sharing good and bad times with me.

I would also like to show my appreciation toward the Ministry of Higher Education (MOHE) for providing scholarship and to Universiti Malaysia Sabah (UMS) for providing allowance during the period of my study. This study was funded by the Ministry of Science, Technology and Innovation (MOSTI) under research grant no. 05-06-ABI-AB12.

Last but not least, special thanks also to my parents for their support, understanding and encouragement for me to explore new area in my life and to become a better person.

ABSTRACT

GENOME ANALYSIS AND EXPERIMENTAL EVIDENCE OF HORIZONTAL TRANSMISSION OF NERVOUS NECROSIS VIRUS (NNV) INFECTING HATCHERY STOCKS OF MARINE FISH

High stocking density in larviculture is important for successful production of marine fish species in hatchery. However, mass mortality due to nervous necrosis virus (NNV) often occurred in newly hatched fish larvae. This reduces the marine fish seed production in hatcheries throughout the country. Hence, this study was conducted to analyze the genomes of NNV and examine the possibility of horizontal transmission of the virus in fish larvae through viral-contaminated fish. In the first experiment, NNV was detected using RT-PCR and histopathological methods from twenty sampling sets of fish larvae collected from different hatcheries and aquaculture farms. The collected fish specimens represented the four most cultured marine fish species in the South East Asian region. The RT-PCR analysis revealed that 60.98% of the fish specimens were infected by NNV. The designed PCR primers in this study were suitably used for the purpose of detection of NNV in marine fish species. The histopathological study showed that cell vacuolation has been observed in brain and retina tissues of infected fish. In the second experiment, the complete coding sequence of both genomes (RdRp and Cp genes) in NNV was successfully PCR amplified and sequenced. The complete coding sequence of RdRp gene consisted of 3024 nucleotides and 982 amino acids. Meanwhile the complete coding sequence for Cp gene consisted of 1363 nucleotides and 338 amino acids. The analysis of nucleotide sequences revealed that both RdRp and Cp genes in NNV isolates in Malaysia had 94.5 – 99.7% and 95.9 – 99.8% similarity to RGNNV genotype, respectively. This shows that only RGNNV genotype of the virus exists in Malaysia. In the third experiment, amplified fragments of RdRp and Cp genes of NNV were restricted using six different restriction enzymes. The result showed that RdRp and Cp genes produced five and seven different RFLP-PCR profiles, respectively. The phylogenetic tree further showed that the RGNNV genotype in Malaysia could be clustered into five main clusters based on RdRp gene and seven main clusters based on Cp gene. In the last experiment, clinically healthy Asian seabass, *Lates calcarifer* and tiger grouper, *Epinephelus fuscoguttatus* larvae were exposed to pure culture of GPNNV and tissue homogenates of infected fish. The transmission of NNV was evaluated using RT-PCR, histopathological, RFLP-PCR and DNA sequencing methods. The results showed that horizontal transmission of NNV has successfully occurred in the exposed fish groups except for groups D2 and E2, respectively. The exposed fish specimens showed cell vacuolations in brain and retina tissues similar to those observed in naturally infected fish. The cDNA of Cp gene in NNV isolated from the exposed fish showed 100% similarity in RFLP-PCR profiles and nucleotide sequence to the viral sources. Overall, this study has provided scientific evidence that aquaculture industry in Malaysia is threatened by NNV infection. This requires holistic management approaches to be adopted in both hatcheries and aquaculture farms to prevent spreading of the pathogen thus ensuring the sustainability of sea food security in the country.

ABSTRAK

Penstokan pada kepadatan yang tinggi semasa peringkat larvikultur adalah sangat penting untuk produksi yang berjaya bagi spesis ikan marin di hatceri. Walau bagaimanapun, kematian yang tinggi disebabkan oleh nervous necrosis virus (NNV) sering terjadi pada anak ikan yang baru menetas. Ini telah mengurangkan pengeluaran benih ikan marin di semua hatceri di seluruh negara. Maka, kajian ini dijalankan untuk menganalisis kedua-dua genom NNV dan kemungkinan berlakunya jangkitan secara melintang virus tersebut pada anak ikan melalui ikan yang telah dijangkiti. Dalam eksperimen pertama, NNV telah dikesan dengan menggunakan kaedah RT-PCR dan kaedah histopatologi dari dua puluh set sampel anak ikan yang diperolehi secara berasingan dari pusat-pusat hatceri dan ladang-ladang akuakultur. Ikan-ikan yang dikumpul mewakili empat daripada spesis ikan marin yang paling banyak dikultur di Asia Tenggara. Analisis RT-PCR mendapati bahawa 60.98% daripada spesimen-spesimen ikan tersebut telah dijangkiti oleh NNV. PCR primer yang direka dalam eksperimen ini adalah sesuai untuk tujuan pengesanan NNV pada ikan marin. Kajian histopatologi menunjukkan bahawa vakulasi sel diperhatikan di dalam tisu-tisu otak dan retina ikan yang telah dijangkiti. Dalam eksperimen kedua, jujukan pengekodan lengkap bagi kedua-dua genom (gen RdRp dan gen Cp) dalam NNV telah berjaya diamplifikasikan dan dijujukkan. Jujukan pengekodan lengkap bagi gen RdRp terdiri daripada 3024 nukleotida dan 982 asid amino. Sementara itu, jujukan pengekodan lengkap bagi gen Cp mengandungi 1363 nukleotida dan 338 asid amino. Analisis jujukan nukleotida mendedahkan bahawa kedua-dua gen RdRp dan gen Cp daripada NNV yang dipencilkan di Malaysia masing-masing adalah 94.5-99.7% dan 95.9-99.8% menyerupai genotip RGNNV. Ini menunjukkan bahawa hanya genotip RGNNV bagi virus tersebut wujud di Malaysia. Dalam eksperimen ketiga, gen RdRp dan gen Cp yang diamplifikasi dari NNV telah dibatasi menggunakan enam jenis enzim pembatasan yang berbeza. Keputusan menunjukkan bahawa gen RdRp menghasilkan lima dan gen Cp menghasilkan tujuh profil RFLP-PCR yang berbeza. Pokok filogenetik seterusnya menunjukkan bahawa genotip RGNNV di Malaysia boleh dirumpunkan ke dalam lima rumpun utama berdasarkan gen RdRd dan tujuh rumpun utama berdasarkan gen Cp. Dalam eksperimen terakhir, anak ikan siakap, <u>Lates</u> <u>calcarifer</u> dan anak ikan kerapu harimau, <u>Epinephelus</u> <u>fuscoguttatus</u> yang secara klinikalnya sihat didedahkan kepada kultur tulen GPNNV dan homogenat tisu-tisu ikan yang dijangkiti. Transmisi NNV dianalisa dengan menggunakan kaedah-kaedah RT-PCR, histopatologi, RFLP-PCR dan jujukan DNA. Keputusan menunjukkan bahawa transmisi NNV secara melintang telah berlaku dengan jayanya kecuali kumpulan D2 dan E2. Spesimen-spesimen ikan yang terdedah menunjukkan vakulasi sel di dalam tisu-tisu otak dan retina sama seperti yang diperhatikan pada ikan yang dijangkiti secara semulajadi. cDNA bagi gen Cp dalam NNV yang dipencilkan semula dari ikan yang terdedah menunjukkan bahawa 100% persamaan dalam profil RFLP-PCR dan jujukan DNA dengan sumber virus. Pada keseluruhannya, kajian ini menyediakan bukti saintifik bahawa industri akuakultur di Malaysia sedang dibebani dengan jangkitan NNV. Ini memerlukan adaptasi pengurusan holistik di hatceri-hatceri dan ladang-ladang akuakultur bagi menghalang penyebaran patogen tersebut dan memastikan kemampanan sumber makanan laut negara.

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Figure 2.4: Denaturing TAE agarose gel electrophoretic analysis of extracted RNA from sampling sets 14 – 20. Lane M: RNA Marker (Promega); Lane 40 – 42: TG 0809 Tuaran Malaysia; Lane 43 – 45: SB 0909 Sandakan Malaysia; Lane 46 – 48: SB 1009 KK Malaysia; Lane 49 – 51: SB 1109 Tuaran Malaysia; Lane 52 – 54: TG 1109 Tuaran Malaysia; Lane 55 – 57: SB 1209 Tuaran Malaysia; Lane 58 – 60: TG 1209 Tuaran Malaysia. 34

Figure 2.5: Combinations of primers used to amplify target fragment of RdRp and Cp genes in GPNNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: JRNV1F1 + JRNV1R1 (1122bp); Lane 2: JRNV1F2 + JRNV1R2 (1032bp); Lane 3: JRNV1F3 + JRNV1R3 (1099bp); Lane 4: JRNV2F1 + JRNV2R1 (1363bp); Lane 5: JRNV2F2 + JRNV2R2 (1026bp); Lane 6: Control (No primers). 35

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Figure 2.6: Agarose gel electrophoresis of PCR 1a. The primer (JRNV1F1 + JRNV1R1) was tested for three different concentrations of $MgCl_2$ and three different annealing temperatures. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 1.5mM $MgCl_2$ at 56°C; Lane 2: 1.7mM $MgCl_2$ at 56°C; Lane 3: 1.9mM $MgCl_2$ at 56°C; Lane 4: 1.5mM $MgCl_2$ at 58°C; Lane 5: 1.7mM $MgCl_2$ at 58°C; Lane 6: 1.9mM $MgCl_2$ at 58°C; Lane 7: 1.5mM $MgCl_2$ at 60°C; Lane 8: 1.7mM $MgCl_2$ at 60°C; Lane 9: 1.9mM $MgCl_2$ at 60°C; Lane 10: Control (Nuclease free water). 40

Figure 2.7: Agarose gel electrophoresis of PCR 1b. The primer (JRNV1F2 + JRNV1R2) was tested for three different concentrations of $MgCl_2$ and three different annealing temperatures. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 1.5mM $MgCl_2$ at 56°C; Lane 2: 1.7mM $MgCl_2$ at 56°C; Lane 3: 1.9mM $MgCl_2$ at 56°; Lane 4: 1.5mM $MgCl_2$ at 58°C; Lane 5: 1.7mM $MgCl_2$ at 58°C; Lane 6: 1.9mM $MgCl_2$ at 58°C; Lane 7: 1.5mM $MgCl_2$ at 60°C; Lane 8: 1.7mM $MgCl_2$ at 60°C; Lane 9: 1.9mM $MgCl_2$ at 60°C; Lane 10: Control (Nuclease free water). 41

Figure 2.8: Agarose gel electrophoresis of PCR 1c. The primer (JRNV1F3 + JRNV1R3) was tested for three different concentrations of $MgCl_2$ and three different annealing temperatures. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 1.5mM $MgCl_2$ at 56°C; Lane 2: 1.7mM $MgCl_2$ at 56°C; Lane 3: 1.9mM $MgCl_2$ at 56°; Lane 4: 1.5mM $MgCl_2$ at 58°C; Lane 5: 1.7mM $MgCl_2$ at 58°C; Lane 6: 1.9mM $MgCl_2$ at 58°C; Lane 7: 1.5mM $MgCl_2$ at 60°C; Lane 8: 1.7mM $MgCl_2$ at 60°C; Lane 9: 1.9mM $MgCl_2$ at 60°C; Lane 10: Control (Nuclease free water). 41

Figure 2.9: Agarose gel electrophoresis of PCR 2a. The primer (JRNV2F1 + JRNV2R1) was tested for three different concentrations of $MgCl_2$ and three different annealing temperatures. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 1.5mM $MgCl_2$ at 56°C; Lane 2: 1.7mM $MgCl_2$ at 56°C; Lane 3: 1.9mM $MgCl_2$ at 56°; Lane 4: 1.5mM $MgCl_2$ at 58°C; Lane 5: 1.7mM $MgCl_2$ at 58°C; Lane 6: 1.9mM $MgCl_2$ at 58°C; Lane 7: 1.5mM $MgCl_2$ at 60°C; Lane 8: 1.7mM $MgCl_2$ at 60°C; Lane 9: 1.9mM $MgCl_2$ at 60°C; Lane 10: Control (Nuclease free water). 41

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Figure 2.10: Agarose gel electrophoresis of PCR 2b. The primer (JRNV2F2 + JRNV2R2) was tested for three different concentrations of $MgCl_2$ and three different annealing temperatures. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 1.5mM $MgCl_2$ at 56°C; Lane 2: 1.7mM $MgCl_2$ at 56°C; Lane 3: 1.9mM $MgCl_2$ at 56°; Lane 4: 1.5mM $MgCl_2$ at 58°C; Lane 5: 1.7mM $MgCl_2$ at 58°C; Lane 6: 1.9mM $MgCl_2$ at 58°C; Lane 7: 1.5mM $MgCl_2$ at 60°C; Lane 8: 1.7mM $MgCl_2$ at 60°C; Lane 9: 1.9mM $MgCl_2$ at 60°C; Lane 10: Control (Nuclease free water). 42

Figure 2.11: Agarose gel electrophoresis of PCR 1a (JRNV1F1 + JRNV1R1) subjected to different gene copies of RdRp in GPNNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 4.6 X 10^7 gene copies; Lane 2: 4.6 X 10^6 gene copies; Lane 3: 4.6 X 10^5 gene copies; Lane 4: 4.6 X 10^4 gene copies; Lane 5: 4.6 X 10^3 gene copies; Lane 6: 4.6 X 10^2 gene copies; Lane 7: 4.6 X 10^1 gene copies; Lane 8: 4.6 gene copies; Lane 9: Control (Nuclease free water). 43

Figure 2.12: Agarose gel electrophoresis of PCR 1b (JRNV1F2 + JRNV1R2) subjected to different gene copies of RdRp in GPNNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 4.6 X 10^7 gene copies; Lane 2: 4.6 X 10^6 gene copies; Lane 3: 4.6 X 10^5 gene copies; Lane 4: 4.6 X 10^4 gene copies; Lane 5: 4.6 X 10^3 gene copies; Lane 6: 4.6 X 10^2 gene copies; Lane 7: 4.6 X 10^1 gene copies; Lane 8: 4.6 gene copies; Lane 9: Control (Nuclease free water). 43

Figure 2.13: Agarose gel electrophoresis of PCR 1c (JRNV1F3 + JRNV1R3) subjected to different gene copies of RdRp in GPNNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 4.6 X 10^7 gene copies; Lane 2: 4.6 X 10^6 gene copies; Lane 3: 4.6 X 10^5 gene copies; Lane 4: 4.6 X 10^4 gene copies; Lane 5: 4.6 X 10^3 gene copies; Lane 6: 4.6 X 10^2 gene copies; Lane 7: 4.6 X 10^1 gene copies; Lane 8: 4.6 gene copies; Lane 9: Control (Nuclease free water). 44

Figure 2.14: Agarose gel electrophoresis of PCR 2a (JRNV2F1 + JRNV2R1) subjected to different gene copies of Cp in GPNNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 4.6 X 10^7 gene copies; Lane 2: 4.6 X 10^6 gene copies; Lane 3: 4.6 X 10^5 gene copies; Lane 4: 4.6 X 10^4 gene copies; Lane 5: 4.6 X 10^3 gene copies; Lane 6: 4.6 X 10^2 gene copies; Lane 7: 4.6 X 10^1 gene copies; Lane 8: 4.6 gene copies; Lane 9: Control (Nuclease free water). 44

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Figure 2.15: Agarose gel electrophoresis of PCR 2b (JRNV2F2 + JRNV2R2) subjected to different gene copies of Cp in GPNNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: 4.6 X 10^7 gene copies; Lane 2: 4.6 X 10^6 gene copies; Lane 3: 4.6 X 10^5 gene copies; Lane 4: 4.6 X 10^4 gene copies; Lane 5: 4.6 X 10^3 gene copies; Lane 6: 4.6 X 10^2 gene copies; Lane 7: 4.6 X 10^1 gene copies; Lane 8: 4.6 gene copies; Lane 9: Control (Nuclease free water). 44

Figure 2.16: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in SB 0207 KK Malaysia. Twelve Asian seabass specimens were examined. All specimens were infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 13: SB 0207 KK Malaysia; Lane 14: Negative control (Nuclease free water). 45

Figure 2.17: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in SB 0607 KK Malaysia. Fourteen Asian seabass specimens were examined. All specimens were infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 15: SB 0607 KK Malaysia; Lane 16: Negative control (Nuclease free water). 45

Figure 2.18: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in MG 0708 Gondol Indonesia. Twenty humpback grouper specimens were examined. Seventeen specimens were infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 21: MG 0708 Gondol Indonesia; Lane 22: Negative control (Nuclease free water). 46

Figure 2.19: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in TG 0708 Gondol Indonesia. Twenty tiger grouper specimens were examined. All specimens were infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 21: TG 0708 Gondol Indonesia; Lane 22: Negative control (Nuclease free water). 46

Figure 2.20: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in SB 0808 KK Malaysia and GP 1108 P Langkawi Malaysia. Twelve Asian seabass specimens and six specimens of golden pompano were examined. All specimens were infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 13: SB 0808 KK Malaysia; Lane 14 – 19: GP 1108 P Langkawi Malaysia; Lane 20: Negative control (Nuclease free water). 46

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Figure 2.21: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in SB 1108 KK Malaysia. Sixteen Asian seabass specimens were examined. All specimens were infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 17: SB 1108 KK Malaysia; Lane 18: Negative control (Nuclease free water). 47

Figure 2.22: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in TG 1208 Tuaran Malaysia and TG 0109 P Langkawi Malaysia. Five tiger grouper specimens from Tuaran and ten specimens from Langkawi Island were examined. Four specimens of tiger grouper from Tuaran and six specimens of tiger grouper from Langkawi Island were infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 6: TG 1208 Tuaran Malaysia; Lane 7 – 16: TG 0109 P Langkawi Malaysia; Lane 17: Negative control (Nuclease free water). 47

Figure 2.23: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in MG 0409 Gondol Indonesia. Fifteen humpback grouper specimens were examined. All specimens were not infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 16: MG 0409 Gondol Indonesia; Lane 17: Negative control (Nuclease free water). 47

Figure 2.24: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in TG 0409 Gondol Indonesia. Fifteen tiger grouper specimens were examined. All specimens were not infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 16: TG 0409 Gondol Indonesia; Lane 17: Negative control (Nuclease free water). 48

Figure 2.25: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in SB 0709 KK Malaysia and SB 0809 Tuaran Malaysia. Thirteen Asian seabass specimens from Kota Kinabalu and five specimens from Tuaran were examined. Nine specimens from Kota Kinabalu were infected by NNV. However, no specimens from Tuaran were infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 14: SB 0709 KK Malaysia; Lane 15 – 19: SB 0809 Tuaran Malaysia; Lane 20: Negative control (Nuclease free water). 48

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Figure 2.26: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in TG 0809 Tuaran Malaysia. Thirty eight tiger grouper specimens were examined. Twenty four specimens were infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lanes 1 and 24: Positive control (GPNNV); Lanes 2 – 22 and 25 - 41: TG 0809 Tuaran Malaysia; Lanes 23 and 42: Negative control (Nuclease free water). 49

Figure 2.27: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in SB 0909 Sandakan Malaysia and SB 1009 KK Malaysia. Ten Asian seabass specimens from Sandakan and six specimens from Kota Kinabalu were examined. All specimens from Sandakan were infected by NNV. However, no specimens from Kota Kinabalu were infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 11: SB 0909 Sandakan Malaysia; Lane 12 – 17: SB 1009 KK Malaysia; Lane 18: Negative control (Nuclease free water). 49

Figure 2.28: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in SB 1109 Tuaran Malaysia. Fourteen Asian seabass specimens were examined. All specimens were not infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 15: SB 1109 Tuaran Malaysia; Lane 16: Negative control (Nuclease free water). 50

Figure 2.29: PCR amplifications of A) RdRp gene fragment and B) Cp gene fragment NNV in TG 1109 Tuaran Malaysia, SB 1209 Tuaran Malaysia and TG 1209 Tuaran Malaysia. Seven tiger grouper specimens and eight Asian sea bass specimens were examined. All specimens were not infected by NNV. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Positive control (GPNNV); Lane 2 – 4: TG 1109 Tuaran Malaysia; Lane 5 – 12: SB 1209 Tuaran Malaysia; Lane 13 – 16: TG 1209 Tuaran Malaysia; Lane 17: Negative control (Nuclease free water). 50

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Figure 2.30: Agarose gel electrophoresis of PCR 1a (JRNV1F1 + JRNV1R1) subjected to five different viruses and ten bacterial species. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Infectious hematopoietic necrosis virus (IHNV); Lane 2: Iridovirus; Lane 3: Koi herpes virus (KHV); Lane 4: Lymphocystis virus; Lane 5: Nervous necrosis virus (NNV); Lane 6: *Aeromonas caviae* ATCC 15947; Lane 7: *Aeromonas hydrophila* ATCC 7965; Lane 8: *Aeromonas salmonicida subsp salmonicida* ATCC 33658; Lane 9: *Edwardsiella tarda* ATCC 15947; Lane 10: *Listonella anguillarum* ATCC 19264; Lane 11: *Pseudomonas fluorescens* ATCC 13525; Lane 12: *Vibrio alginolyticus* ATCC 17749; Lane 13: *Vibrio harveyi* ATCC 35084; Lane 14: *Vibrio parahaemolyticus* ATCC 17802; Lane 15: *Yersinia ruckeri* ATCC 29473; Lane 16: Negative control (Nuclease free water). 51

Figure 2.31: Agarose gel electrophoresis of PCR 1b (JRNV1F2 + JRNV1R2) subjected to five different viruses and ten bacterial species. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Infectious hematopoietic necrosis virus (IHNV); Lane 2: Iridovirus; Lane 3: Koi herpes virus (KHV); Lane 4: Lymphocystis virus; Lane 5: Nervous necrosis virus (NNV); Lane 6: *Aeromonas caviae* ATCC 15947; Lane 7: *Aeromonas hydrophila* ATCC 7965; Lane 8: *Aeromonas salmonicida subsp salmonicida* ATCC 33658; Lane 9: *Edwardsiella tarda* ATCC 15947; Lane 10: *Listonella anguillarum* ATCC 19264; Lane 11: *Pseudomonas fluorescens* ATCC 13525; Lane 12: *Vibrio alginolyticus* ATCC 17749; Lane 13: *Vibrio harveyi* ATCC 35084; Lane 14: *Vibrio parahaemolyticus* ATCC 17802; Lane 15: *Yersinia ruckeri* ATCC 29473; Lane 16: Negative control (Nuclease free water). 51

Figure 2.32: Agarose gel electrophoresis of PCR 1c (JRNV1F3 + JRNV1R3) subjected to five different viruses and ten bacterial species. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Infectious hematopoietic necrosis virus (IHNV); Lane 2: Iridovirus; Lane 3: Koi herpes virus (KHV); Lane 4: Lymphocystis virus; Lane 5: Nervous necrosis virus (NNV); Lane 6: *Aeromonas caviae* ATCC 15947; Lane 7: *Aeromonas hydrophila* ATCC 7965; Lane 8: *Aeromonas salmonicida subsp salmonicida* ATCC 33658; Lane 9: *Edwardsiella tarda* ATCC 15947; Lane 10: *Listonella anguillarum* ATCC 19264; Lane 11: *Pseudomonas fluorescens* ATCC 13525; Lane 12: *Vibrio alginolyticus* ATCC 17749; Lane 13: *Vibrio harveyi* ATCC 35084; Lane 14: *Vibrio parahaemolyticus* ATCC 17802; Lane 15: *Yersinia ruckeri* ATCC 29473; Lane 16: Negative control (Nuclease free water). 52

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Figure 2.33: Agarose gel electrophoresis of PCR 2a (JRNV2F1 + JRNV2R1) subjected to five different viruses and ten bacterial species. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Infectious hematopoietic necrosis virus (IHNV); Lane 2: Iridovirus; Lane 3: Koi herpes virus (KHV); Lane 4: Lymphocystis virus; Lane 5: Nervous necrosis virus (NNV); Lane 6: *Aeromonas caviae* ATCC 15947; Lane 7: *Aeromonas hydrophila* ATCC 7965; Lane 8: *Aeromonas salmonicida subsp salmonicida* ATCC 33658; Lane 9: *Edwardsiella tarda* ATCC 15947; Lane 10: *Listonella anguillarum* ATCC 19264; Lane 11: *Pseudomonas fluorescens* ATCC 13525; Lane 12: *Vibrio alginolyticus* ATCC 17749; Lane 13: *Vibrio harveyi* ATCC 35084; Lane 14: *Vibrio parahaemolyticus* ATCC 17802; Lane 15: *Yersinia ruckeri* ATCC 29473; Lane 16: Negative control (Nuclease free water). 52

Figure 2.34: Agarose gel electrophoresis of PCR 2b (JRNV2F2 + JRNV2R2) subjected to five different viruses and ten bacterial species. Lane M: 1.0 kb DNA ladder (Promega); Lane 1: Infectious hematopoietic necrosis virus (IHNV); Lane 2: Iridovirus; Lane 3: Koi herpes virus (KHV); Lane 4: Lymphocystis virus; Lane 5: Nervous necrosis virus (NNV); Lane 6: *Aeromonas caviae* ATCC 15947; Lane 7: *Aeromonas hydrophila* ATCC 7965; Lane 8: *Aeromonas salmonicida subsp salmonicida* ATCC 33658; Lane 9: *Edwardsiella tarda* ATCC 15947; Lane 10: *Listonella anguillarum* ATCC 19264; Lane 11: *Pseudomonas fluorescens* ATCC 13525; Lane 12: *Vibrio alginolyticus* ATCC 17749; Lane 13: *Vibrio harveyi* ATCC 35084; Lane 14: *Vibrio parahaemolyticus* ATCC 17802; Lane 15: *Yersinia ruckeri* ATCC 29473; Lane 16: Negative control (Nuclease free water). 53

Figure 2.35: Histopathological sections of B) brain and E) retina tissues of SB 1108 KK Malaysia. Cell vacuolation was observed in both organs or in one of the organ (black arrows) of infected fish specimen. 54

Figure 2.36: Histopathological sections of B) brain and E) retina tissues of TG 1208 Tuaran Malaysia. Cell vacuolation was observed in both organs or in one of the organ (black arrows) of infected fish specimen. 54

Figure 2.37: Histopathological sections of B) brain and E) retina tissues of SB 0709 KK Malaysia. Cell vacuolation was observed in both organs or in one of the organ (black arrows) of infected fish specimen. 54

Figure 2.38: Histopathological sections of B) brain and E) retina tissues of TG 0809 Tuaran Malaysia. No cell vacuolation was observed in both organs of non-infected fish specimens. 55

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Figure 2.39: Histopathological sections of B) brain and E) retina tissues of SB 0909 Sandakan Malaysia. Cell vacuolation was observed in both organs or in one of the organ (black arrows) of infected fish specimen. 55

Figure 2.40: Histopathological sections of B) brain and E) retina tissues of SB 1009 KK Malaysia. No cell vacuolation was observed in both organs of non-infected fish specimens. 55

Figure 2.41: Histopathological sections of B) brain and E) retina tissues of SB 1109 Tuaran Malaysia. No cell vacuolation was observed in both organs of non-infected fish specimens. 56

Figure 2.42: Histopathological sections of B) brain and E) retina tissues of TG 1109 Tuaran Malaysia. No cell vacuolation was observed in both organs of non-infected fish specimens. 56

Figure 2.43: Histopathological sections of B) brain and E) retina tissues of SB 1209 Tuaran Malaysia. No cell vacuolation was observed in both organs of non-infected fish specimens. 57

Figure 2.44: Histopathological sections of B) brain and E) retina tissues of TG 1209 Tuaran Malaysia. No cell vacuolation was observed in both organs of non-infected fish specimens. 57

Figure 3.1: Schematic diagram shows the amplification strategy for partial sequence of RdRp gene in NNV. 73

Figure 3.2: Schematic diagram shows the amplification strategy for partial sequence of Cp gene in NNV. 73

Figure 3.3: Denaturing TAE agarose gel electrophoretic analysis of extracted RNA. Lane M: RNA ladder (Promega); Lane 1: SB 0207 KK Malaysia 1; Lane 2: SB 0207 KK Malaysia 2; Lane 3: SB 0207 KK Malaysia 3; Lane 4: SB 0607 KK Malaysia 4; Lane 5: SB 0607 KK Malaysia 5; Lane 6: MG 0708 Gondol Indonesia 3; Lane 7: MG 0708 Gondol Indonesia 8; Lane 8: MG 0708 Gondol Indonesia 9; Lane 9: TG 0708 Gondol Indonesia 2; Lane 10: TG 0708 Gondol Indonesia 4; Lane 11: TG 0708 Gondol Indonesia 6; Lane 12: SB 0808 KK Malaysia 1; Lane 13: SB 0808 KK Malaysia 4; Lane 14: GP 1108 P. Langkawi 1; Lane 15: GP 1108 P. Langkawi 2; Lane 16: GP 1108 P. Langkawi 3. 86

Figure 3.4: Denaturing TAE agarose gel electrophoretic analysis of extracted RNA. Lane M: RNA ladder; Lane 17: SB 1108 KK Malaysia 1; Lane 18: SB 1108 KK Malaysia 2; Lane 19: SB 1108 KK Malaysia 3; Lane 20: TG 1208 Tuaran Malaysia 1; Lane 21: TG 1208 Tuaran Malaysia 2; Lane 22: TG 0109 P. Langkawi Malaysia 1; Lane 23: TG 0109 P. Langkawi Malaysia 2; Lane 24: TG 0109 P. Langkawi Malaysia 10; Lane 25: SB 0709 KK Malaysia 4; Lane 26: SB 0709 KK Malaysia 5; Lane 27: TG 0809 Tuaran Malaysia 1; Lane 28: TG 0809 Tuaran Malaysia 2; Lane 29: TG 0809 Tuaran Malaysia 3; Lane 30: SB 0909 Sandakan Malaysia 3; Lane 31: SB 0909 Sandakan Malaysia 5; Lane 32: SB 0909 Sandakan Malaysia 8. 86

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