



UNIVERSITI PUTRA MALAYSIA

**IMMUNOCHEMISTRY AND MOLECULAR APPROACHES TOWARDS
IDENTIFICATION OF MALAYSIAN CYPRINID HERPESVIRUS**

SAMSON SOON MIN NGEN

FPV 2001 13

**IMMUNOCHEMISTRY AND MOLECULAR APPROACHES TOWARDS
IDENTIFICATION OF MALAYSIAN CYPRINID HERPESVIRUS**

By

SAMSON SOON MIN NGEN

**Thesis Submitted in Fulfilment of the Requirement for the Degree
of Doctor of Philosophy in the Faculty of Veterinary Medicine
Universiti Putra Malaysia**

May 2001



Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

IMMUNOCHEMISTRY AND MOLECULAR APPROACHES TOWARDS IDENTIFICATION OF MALAYSIAN CYPRINID HERPESVIRUS

By

SAMSON SOON MIN NGEN

May 2001

Chairman: Dr. Hassan Hj. Mohd. Daud, Ph.D

Faculty : Veterinary Medicine

Immunochemistry and molecular approaches were used to identify a Malaysian cyprinid herpesvirus responsible for papilloma among Koi carps (*Cyprinus carpio* L.) and goldfish (*Carassius auratus* L.) in Malaysia. Immunochemistry approaches employing hybridoma technology established a hybridoma clone (DG3-1) producing specific IgM κ light chain monoclonal antibody (MAb) against Malaysian cyprinid herpesvirus. The MAb was cross-reactive against Japanese cyprinid herpesvirus type 1 (CHV) antigens but not against Channel catfish herpesvirus (CCV) and Salmonid herpesvirus (SHV-2) in immunodot-blot assay. The cyprinid herpesvirus type-specific epitope recognised by the MAb was located on two viral polypeptides having the molecular weight of 58,000 and 67,000 daltons in Malaysian cyprinid herpesvirus and CHV through Western blot analysis. As the MAb showed no neutralization activity against virus infection in cell culture and glycosylation inhibitors did not affect the presence and migration of the antigens under polyacrylamide gel electrophoresis, evidences as such suggest the antigens are nonglycosylated components of the viral structure.



Immunohistochemical analysis on goldfish papilloma tissue sections with MAb using labeled avidin binding (LAB) method demonstrated specific staining of cyprinid herpesvirus antigens within the nucleus of infected cells. Specific localization of these viral antigens in the cell nuclei were consistent with reports of nonglycosylated herpesvirus antigens involving viral capsid components or DNA-binding proteins. Employing molecular techniques, cyprinid herpesvirus nucleic acid sequences were later confirmed to be present in the immunohistochemical positive papilloma sections through *in situ* hybridization assay using a 1,161 bp CHV nucleic acid probe.

Molecular identification by polymerase chain reaction (PCR) using CHV specific primers was extremely sensitive, specific, rapid and practical. The technique successfully amplified a 433 bp DNA fragment from frozen archival goldfish papilloma tissues and recent papillomas obtained from goldfish and carp hybrids. Nucleic acid sequencing of the DNA fragment revealed identical sequence homology with CHV, thus confirming conclusively that Malaysian cyprinid herpesvirus and CHV are members of the same group of virus. Detection sensitivity level as assessed with first step PCR, was capable of detecting viral nucleic acids from 1 fg or 200 copies of actual viral target sequences and from as low as 1-10 virus infected cells. Sensitivity level was increased 100-1000-fold when nested PCR strategy was employed. Specificity of detection evaluated by DNA fragment polymorphism demonstrated homologous DNA sequences among cyprinid herpesvirus representatives from Malaysia, Israel and Japan. A quantitative competitive PCR assay based on the current viral target sequence also provided quantitative description of infection and viral burden with preliminary results indicative of CHV possessing an alphaherpesvirus gene-like expression kinetics.

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

**PENDEKATAN IMUNOKIMIA DAN MOLEKUL DALAM
PENGENALPASTIAN HERPESVIRUS CYPRINID MALAYSIA**

Oleh

SAMSON SOON MIN NGEN

Mei 2001

Pengerusi: Dr. Hassan Hj. Md. Daud, Ph.D

Fakulti : Perubatan Veterinar

Kaedah imunokimia dan molekul telah digunakan dalam pengenalpastian herpesvirus cyprinid Malaysia, yang bertanggungjawab ke atas kejadian papiloma di dalam ikan koi (*Cyprinus carpio* L.) dan ikan emas (*Carassius auratus* L.) di Malaysia. Kaedah imunologi menggunakan teknologi hibridoma telah menghasilkan klon hibridoma (DG3-1) yang mengeluarkan antibodi monoklon (MAb) IgM dengan rantai κ yang spesifik terhadap cyprinid herpesvirus Malaysia. Antibodi monoklon menunjukkan reaksi saling terhadap antigen cyprinid herpesvirus type 1 (CHV) Jepun tetapi tidak terhadap Channel catfish herpesvirus (CCV) and Salmonid herpesvirus (SHV-2) dalam asei "immunodot-blot". Analisis "Western blot" mendedahkan bahawa epitope spesifik cyprinid herpesvirus yang dikenalpasti oleh MAb terletak pada dua polipeptida virus dengan berat molekul 58,000 and 67,000 dalton pada cyprinid herpesvirus Malaysia dan CHV. Oleh kerana MAb tidak menunjukkan neutralisasi terhadap jangkitan virus di dalam kultur tisu dan rawatan penyekat glikosilasi tidak mempengaruhi kehadiran dan migrasi antigen-antigen dalam elektrophoresis gel polyacrylamide, menunjukkan bahawa

antigen-antigen ini adalah komponen struktur teras virus yang terdiri dari polipeptida tidak berglikosilasi.

Analisis imunohistokimia ke atas keratan tisu papiloma ikan emas menggunakan teknik "Labeled Avidin Binding" (LAB) menunjukkan pewarnaan spesifik antigen cyprinid herpesvirus di dalam nukleus sel yang dijangkiti. Pengesanan antigen-antigen ini di dalam nukleus sel adalah selari dengan laporan mengenai antigen tidak berglikosilasi herpesvirus yang terdapat pada komponen kapsid virus dan protin pengikat DNA. Pendekatan teknik molekul terhadap keratan imunohistokimia papiloma yang positif menggunakan prob asid nukleik CHV bersaiz 1,161 bp dengan kaedah hibridisasi "*in situ*" turut menunjukkan kehadiran asid nukleik CHV.

Pengenalpastian melalui reaksi polimeras berantai (PCR) dengan primer spesifik CHV juga didapati sangat sensitive, spesifik, cepat dan praktikal. Kaedah ini berjaya menghasilkan fragmen DNA bersaiz 433 bp dari tisu papiloma yang dibekukan dan yang baru dari ikan emas dan kap hibrid. Penjujukan asid nukleik menunjukkan homologi yang sama dengan CHV justeru mengesahkan bahawa MCHV dan CHV adalah virus yang sama. Sensitiviti pengesanan dengan PCR dengan teknik PCR tahap satu mampu mengesan asid nukleik CHV dari 1 fg atau 200 salinan sasaran jujukan asal virus dan dari 1-10 sel terjangkit. Sensitiviti pengesanan ini dapat dipertingkatkan 100-1000 kali ganda dengan kaedah PCR bersarang. Spesifisiti pengesanan PCR dengan kajian pecahan polimofisa DNA menunjukkan jujukan asid nukleik serupa di antara cyprinid herpesvirus dari Malaysia, Israel dan Jepun. Kaedah kuantitatif PCR berdasarkan sasaran jujukan virus PCR yang digunakan membolehkan gambaran kuantitatif terhadap tahap

jangkitan dan beban virus diselidiki, di mana keputusan awal menunjukkan bahwa CHV memiliki ekspresi kinetik gen yang seakan sama dengan kumpulan alphaherpesvirus.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my committee chairman, Dr. Hassan Haji Mohd Daud for his suggestions and support throughout the completion of this program. To Professor Dr. Mohamed Shariff Mohamed Din, thank you for the constant encouragement and guidance. I'm also indebted, as you have provided the vital link with the Tokyo University of Fisheries that allowed this project to be completed. I would also like to extend my heartfelt appreciation to Associate Professor Dr. Abdul Manaf Ali for his valuable suggestions, advice and hands-on commitment in the establishment of the hybridoma clones in this research project. My gratitude also goes to Professor Dr. Hideo Fukuda from the Tokyo University of Fisheries for the CHV samples and his important assistance on CHV molecular biology. My sincere appreciation as well to Professor Dr. Ilan Paperna of the Hebrew University for providing papilloma samples from Israel used in the current work. I am likewise grateful to Associate Professor Dr. Khatijah Yusoff for her valuable discussions on molecular methodologies in the present research.

Special thanks are accorded to my colleagues, Dr. Tan Lee Tung and Dr. Lee Kok Leong for their excellent technical assistance during the course of my work. It has been a great honor and pleasure to work with the both of you. Let's continue this dynamic partnership and anticipate what the future will hold for us. My sincere thanks also to Mr. Wang Yin Geng for his excellent viewpoints on scientific matters pertaining to aquatic animal health. With all my heart, I thank you and Chen Xia for the moral and technical supports both of you have given me all these years. To Mr. T.N. Devaraj, Dr. Najiah Musa and Ms. Abeer Al-Sahtout, I will forever cherish your friendships.



To my family, thank you for your undivided love and support throughout these years. As I strived to excel in giving the best I could in my work and on other academic projects, your acceptance of me has always been for who I am and not for what I have accomplished. To my parents, Joseph and Lucy, I love you both dearly as I know I have been away from home far too long. Thank you for your patience. To my brother, Dr. Jeffrey Soon, your constant inspiration and strength will forever remain in my heart as it has seen me through some very difficult times. To my sister-in-law, Pauline, thank you for being there when the going was rough. To God I give all Praise and Glory. Thank You for the second chance. Loving you Joanne, with all my heart.



TABLE OF CONTENTS

	Page
ABSTRACT	2
ABSTRAK	4
ACKNOWLEDGEMENTS	7
APPROVAL SHEETS	9
DECLARATION FORM	11
TABLE OF CONTENTS	12
LIST OF TABLES	15
LIST OF FIGURES	16
LIST OF ABBREVIATIONS	24
CHAPTER	
I GENERAL INTRODUCTION	28
II LITERATURE REVIEW	33
Common Properties of Herpesviruses	34
Herpesvirus Classification	35
Fish Herpesviruses	36
Carp Pox	38
General Properties of <i>Herpesvirus cyprini</i>	39
Biophysical and Biochemical Properties	39
Pathogenicity and Oncogenicity	41
Cyprinid Herpesvirus Latency	44
General properties of a Cyprinid Herpesvirus Isolated in Malaysia	45
Immunochemical Approaches in the Identification of Fish Viruses	46
Application of Monoclonal Antibodies in Fish Herpesvirus	
Identification and Antigenic Characterization	51
Molecular Approaches in the Identification of Fish Viruses	53
Detection of Viral Genetic Sequences with Nucleic Acid	
Probes	54
Amplification and Characterization of Virus Nucleic Acids	
using Polymerase Chain Reaction (PCR)	56
Research Constraints	62
III DEVELOPMENT OF MONOCLONAL ANTIBODIES AGAINST	
 MALAYSIAN CYPRINID HERPESVIRUS	65
Material and Methods	69
Virus Production and Quantification in Cell Culture	69
Recovery and Purification of Virus from Infected Cell Cultures	70
Immunization of Donor Animals	71
Generation of Murine Hybridoma cells	72
Cell Fusion	74
Hybridoma Selection and Cloning	75
Antibody Screening for Malaysian Cyprinid Herpesvirus	75



	Single Cell Cloning through Limiting Dilution	76
	Freeze storage of Hybridomas	77
	Results	78
	Production, Isolation and Purification of Virus	78
	Antiserum Titer of Immunized Animals	80
	Generation of Murine Hybridoma cells	81
	Identification of Hybridoma Colonies with Reactive Antibodies against Malaysian Cyprinid Herpesvirus	81
	Discussion	88
IV	CHARACTERIZATION OF A MONOCLONAL ANTIBODY AGAINST CYPRINID HERPESVIRUS ANTIGENS	95
	Material and Methods	98
	Determination of Antibody Isotype and Neutralization of Infection	98
	Hybridoma Growth and Antibody Production Properties	99
	Cross-Reactive Immunodot Assay on Fish Herpesviruses using MAb DG3-1	101
	Virus Protein Analysis using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	102
	Identification of Viral Immunogenic Proteins with Western Blot Assay	104
	Results	107
	Isotype Determination and Neutralization Results of MAb DG3-1	107
	Hybridoma Growth and MAb Production	108
	Monoclonal Antibody DG3-1 is Specific Against Cyprinid Herpesvirus Antigens	112
	Viral Protein analyses	113
	Cyprinid Herpesvirus Antigens Recognized by MAb	116
	Discussion	120
V	DETECTION OF CYPRINID HERPESVIRUS IN GOLDFISH (<i>Carassius auratus</i> L.) PAPILOMA VIA IMMUNO- HISTOCHEMISTRY AND <i>IN SITU</i> HYBRIDIZATION	132
	Material and Methods	136
	Preparation of Goldfish Carp Pox-like lesions for Immunohistochemical Analyses	136
	Immunohistochemical Detection of Cyprinid Herpesviral Antigens with MAb DG3-1 via Labeled Avidin Binding (LAB) Method	138
	CHV Nucleic Acid Probe Preparation	139
	Synthesis of Biotin-labeled Nucleic Acid Probe by Random Priming Technique	140
	<i>In situ</i> Hybridization Procedure and Detection	142

Results	144
Immunohistochemical Detection of Cyprinid Herpesviral Antigens in Goldfish Papilloma Lesions	144
CHV Nucleic Acid Probe Generation	146
<i>In situ</i> Hybridization and Detection of CHV Nucleic Acid Sequences in Goldfish Papilloma	149
Discussion	153
VI MOLECULAR DETECTION AND QUANTITATION OF CYPRINID HERPESVIRUS NUCLEIC ACIDS WITH THE POLYMERASE CHAIN REACTION	165
Material and Method	170
Preparation of Nucleic Acid Templates for PCR Amplification	170
Amplification of Viral DNA by the PCR process	172
Analysis of PCR Products by Agarose Gel Electrophoresis	175
Molecular Cloning and Nucleotide Sequencing of PCR Amplified 433 bp DNA Fragment from Goldfish Papilloma	175
Sensitivity of PCR Amplification	178
Specificity of PCR Amplification	179
Quantitative Analysis of Amplified CHV Target Sequence by Competitive PCR	180
Development and Construction of Competitive Template Competitive Quantitative PCR of CHV Infection in FHM Cell Culture	181
Results	184
Detection of Cyprinid Herpesviral DNA by PCR Amplification	186
Molecular Cloning and Nucleotide Sequencing of Amplified PCR product	188
Sensitivity of PCR Amplification	195
Specificity of PCR Amplification	207
Establishment of Quantitative PCR	216
Quantitative PCR Analysis of CHV Infection in FHM Cells	222
Discussion	225
VII GENERAL CONCLUSIONS	250
BIBLIOGRAPHY	261
BIODATA OF THE AUTHOR	282



LIST OF TABLES

Tables		Page
1	Hybridoma seeded wells containing antibodies against Malaysian cyprinid herpesvirus, 12 days after cell fusion	82
2	The ELISA reactivity results of two hybridoma clones after first limiting dilution	86
3	Isotype determination of MAb DG3-1	107
4	Summary of viral polypeptide molecular weights as determined by SDS-PAGE involving three polyacrylamide gel concentrations	115
5	The PCR primers used in the present study	174



LIST OF FIGURES

Figures		Page
1	Purine biosynthesis salvage pathway	67
2	The general scheme in monoclonal antibodies production	68
3	A cluster of Malaysian cyprinid herpesvirus naked viral particles. Bar = 100 nm	79
4	Icosahedral Malaysian cyprinid herpesvirus particles. Bar = 100 nm	80
5	Antibody titer levels in mice eight weeks after initial challenge	80
6	One day old single hybridoma cell after cloning by limiting dilution	84
7	Division of a hybridoma cell after two days in culture	84
8	Hybridoma cells after five days of culture	85
9	High density growth of hybridoma cells after 14 days of culture	85
10	Percentage of wells screened having optical density readings above control baseline level following second limiting dilution	87
11	Cell viability of hybridoma clone DG3-1 under stir batch culture condition. SEM of three replicates	109
12	Cell viability of hybridoma clone DG3-1 under static culture condition. SEM of three replicates	110
13	Cell viability percentages of both static and stir batch culture over a period of seven days	110
14	Growth curve and antibody production level of hybridoma clone DG3-1 under stir batch culture condition	111
15	IgM production level of hybridoma clone DG3-1 under stir batch culture condition over a period of seven days. SEM of three replicates	111

16	Immunodot assay results of infected and non-infected cell culture medium. Lane 1-2: CHV infected cell culture supernatant; A1: 20 μ L; A2: 40 μ L; B1: 60 μ L; B2: 80 μ L. Lane 3: CCV infected cell culture supernatant; A3: 20 μ L; B3: 60 μ L. Lane 4: SHV-2 infected cell culture supernatant; A4: 20 μ L; B4: 60 μ L. Lane 5: FHM non-infected cell culture medium; A5: 20 μ L; B5: 60 μ L. Lane 6: BB non-infected cell culture medium; A6: 20 μ L; B6: 60 μ L. Lane 7: Immunodot blot positive control; MAb DG3-1; A7, B7: 60 μ L. Lane 8: Immunodot blot negative control; TBS buffer; A8, B8: 60 μ L	112
17	SDS-PAGE analysis of purified Malaysian cyprinid herpesvirus using a 12% polyacrylamide gel stained with silver. Lane 1: molecular weight markers electrophoresis; Lane 2: electrophoresis of viral polypeptides with Vp nomenclature on the right	114
18	Western blot analysis of MAb DG3-1 on purified Malaysian cyprinid herpesvirus. Lane 1: Molecular weight markers; Lane 2: Detection of Malaysian cyprinid herpesvirus antigens by MAb DG3-1	117
19	SDS-PAGE of CHV infected cell extracts. Lane 1: Protein ladder; Lane 2: Non-infected cell extracts; Lane 3: CHV infected cell lysates; Lane 4: Infected medium (clarified)	117
20	Western blot analysis of MAb DG3-1 on infected FHM cell extracts. Lane 1: Molecular weight markers; Lane 2: Detection of CHV antigens by MAb DG3-1	118
21	The unaffected relative mobility of the antigens after tunicamycin and monensin treatments	119
22a	A goldfish (<i>Carassius auratus</i> L.) showing several papillomatous lesions on the body	137
22b	A goldfish showing a papilloma near the dorsal fin	137
23	Immunohistochemical detection of cyprinid herpesviral antigens in fixed, paraffin embedded goldfish papilloma section. Section was probed with MAb DG3-1 without counterstaining. Note the localization of the red dots within infected nuclei . x 350	145
24	Immunohistochemical assay control involving sequential layer omission of primary or secondary antibody, counterstained with contrast BLUE. Note the enlarged nuclei of infected cells in the papilloma . x 350	145

25	Immunohistochemical detection of CHV antigens with MAb DG3-1, counterstained in contrast BLUE. Note the specific reaction of the antibody within infected cell nuclei . x 350	146
26	Nucleic acid region of CHV fragment 3 employed for nucleic acid probe development. Primer probes for DNA probe generation are underlined	147
27	Development of PCR amplified CHV DNA probe. Lane 1: 1000 bp PCR amplicon size standard; Lane 2: PCR amplified CHV DNA fragment; Lane M: 100 bp DNA size marker with orientation band at 600 bp. Size of DNA given in base pairs (bp)	148
28	Presence of weak <i>in situ</i> hybridization signal under short proteolytic digestion duration in papilloma section probed with biotin-labeled CHV DNA probe (100 ng/mL). x 350	150
29	Detection by <i>in situ</i> hybridization of CHV DNA in infected nuclei of papilloma cells using 50 ng/mL biotin-labeled DNA probe. x 700	150
30	<i>In situ</i> hybridization of CHV DNA in infected nuclei of papilloma cells using 100 ng/mL biotin-labeled DNA probe. x 350	151
31	Detection by <i>in situ</i> hybridization of CHV DNA in infected nuclei with 250 ng/mL biotin-labeled CHV probe. Note the higher hybridization signal intensity. x 350	151
32	Absence of <i>in situ</i> hybridization signal following the omission of CHV nucleic acid acid probe from hybridization solution. x 175	152
33	The PAP complex is comprised of horseradish peroxidase bound to an anti-peroxidase antibody generated in the same species as the primary antibody, which recognized the antigen of interest. The primary antibody and the PAP complex are linked via a secondary antibody generated in a second animal species against immunoglobulin of the primary animal species (Bratthauer, 1994)	156
34	In the ABC procedure, the primary antibody against the antigen of interest is linked to the avidin-biotinylated peroxidase complex via a biotinylated secondary antibody raised against immunoglobulin of the animal species used to generate the primary antibody (Bratthauer, 1994)	156



35	The LAB procedure. Horseradish peroxidase or alkaline phosphatase is covalently linked to avidin. The primary antibody against the antigen is linked to the enzyme-labeled avidin complex (LAB) via a biotinylated secondary antibody raised against immunoglobulin of the animal species used to generate the primary antibody. CCC, long carbon extension arm (Bratthauer, 1994)	156
36	Schematic diagram on the construction of the mutant competitor standard for quantitative PCR	183
37	Agarose gel electrophoresis of PCR fragments amplified from archival goldfish papilloma tissue using primer set CHV1/CHV2. M: 50 bp DNA size markers with orientation band at 350 bp; Lane 1: Distilled water; Lane 2: CHV infected cell lysate; Lane 3: Goldfish papilloma tissue. Arrow indicates the target fragment with the expected size. Note also the presence of nonspecific amplification products	187
38	Result of PCR re-amplification of excised target fragment. M: 50 bp DNA size marker with orientation band at 350 bp; Lane 1-2: re-amplified gel purified target fragment	187
39	The promoter and multiple cloning sequence of pGEM-T Easy vector	189
40	Rapid colony PCR screening of 10 transformed white colonies. M: 50 bp DNA size marker; Lane 1-10: plasmid clones carrying cloned PCR products; Lane 11: plasmid clone from a blue colony	189
41	Estimation of plasmid amount and quality after miniprep isolation. M: DNA size markers; Lane 1-5: isolated plasmid. Size of DNA markers are indicated in kilobase pairs (bp)	191
42	Digestion of plasmid with <i>NotI</i> restriction enzyme to verify presence of insert. M: DNA size markers; Lane 1-5: restriction enzyme digested plasmid clones. Note the release of cloned target	191
43	Nucleic acid sequencing results of MCHV 433 bp fragment cloned in pGEM-T Easy plasmid using SP6 sequencing primers (clone 1)	192
44	Nucleic acid sequencing results of MCHV 433 bp fragment cloned in pGEM-T Easy plasmid using T7 sequencing primers (clone 2)	193
45	Nucleic acid sequencing results of MCHV 433 bp fragment cloned in pGEM-T Easy plasmid using SP6 sequencing primers (clone 2)	194

46	Location of first step and nested primers within target region of CHV fragment No.1 (Yamamoto et al., pers. comm.)	196
47	PCR construction of the 800 bp templates for densitometric quantification with identical size known standards. M: 50 bp DNA size marker; Lane 1: PCR constructed 800 bp template containing wild-type sequence; Lane 2: PCR constructed 800 bp template containing mutant competitor sequence	197
48	Densitometric quantification of PCR constructed templates with standards series of known amounts. Note that both standards and target templates are identical in size. Lane 1:32 ng; 2: 30 ng; 3: 25ng; 4: 20ng; 5: 15 ng; 6: wild-type sequence template; 7: mutant competitor sequence template	197
49	Sensitivity of PCR amplification of CHV 433 bp target sequence at 55°C annealing temperature under various Mg ²⁺ concentration; (a) 1.5 mM, (b) 2.0 mM, (c) 2.5 mM. M: 50 bp DNA size marker; Lane 1-5: quantified actual CHV target sequence; 1: 1 pg; 2: 100 fg; 3: 10fg; 4: 1 fg; 5: 100 ag. Note the presence of nonspecific amplification at low target sequence amount	199
50	Sensitivity of PCR amplification of CHV 433 bp target sequence at 60°C annealing temperature under various Mg ²⁺ concentration; (a) 1.5 mM, (b) 2.0 mM, (c) 2.5 mM. M: 50 bp DNA size marker; (b) Lane 1-5: quantified actual CHV target target sequence; 1: 1 pg; 2: 100 fg; 3: 10fg; 4: 1 fg; 5: 100 ag. Note the strong PCR amplification signal at Mg ²⁺ 2.0 mM	200
51	First step PCR amplification of CHV 433 bp target sequence. M: 50 bp DNA size marker; Lane 1-9: quantified CHV target sequence; 1: 100 pg; 2: 10 pg; 3: 1 pg; 4: 100 fg; 5: 10 fg; 6: 1 fg; 7: 100 ag; 8: 10 ag; 9: 1 ag; 10: distilled water	202
52	Nested step PCR amplification of a CHV 310 bp target sequence. M: 50 bp DNA size marker; Lane 1-9: quantified CHV target sequence; 1: 100 pg; 2: 10 pg; 3: 1 pg; 4: 100 fg; 5: 10 fg; 6: 1 fg; 7: 100 ag; 8: 10 ag; 9: 1 ag; 10: distilled water. Note the appearance of first step PCR amplicons due to excessive product carry-over	202
53	PCR amplification of CHV 433 bp target sequence using 0.1 μM first step primers. Lane 1-4: quantified CHV target sequence; 1: 1 fg; 2: 100 ag; 3: 10 ag; 4: 1 ag; Lane 5: distilled water	203

54	Nested PCR amplification of a CHV 310 bp nested sequence with first step PCR products. Lane 1 -4: quantified CHV target sequence; 1: 1 fg; 2: 100 ag; 3: 10 ag; 4: 1 ag; Lane 5: distilled water	203
55	First step PCR assay using crude lysate of virus-infected cells as targets. Lane 1-7: virus-infected cell lysates; 1: 10 ⁴ cells; 2: 10 ³ cells; 3: 10 ² cells; 4: 10 cells; 5: 1 cell; 6: 0.1 cell; 7: 0.01 cell	205
56	Re-amplification of first step products by nested step PCR assay of crude virus-infected cell lysates. Lane 1-7: virus-infected cell lysates; 1: 10 ⁴ cells; 2: 10 ³ cells; 3: 10 ² cells; 4: 10 cell; 5: 1 cell; 6: 0.1 cell; 7: 0.01 cell	205
57	Detection of CHV DNA from goldfish paraffin embedded papilloma section using first step and nested PCR assay. M: 50 bp size marker; Lane 1: distilled water; Lane 2-3: first step PCR results; Lane 4: distilled water; Lane 5-6: detection of CHV 310 bp nested fragment	206
58	Detection of CHV by PCR amplification in papilloma samples from three countries. Lane M: 50 bp DNA size marker; Lane 1: Malaysia; Lane 2: Israel; Lane 3: Japan; Lane 4: distilled water	208
59	Restriction fragment profiles of CHV 433 bp PCR product amplified from three geographical regions cleared with (a) <i>Sma</i> I, (b) <i>Eco</i> NI, (c) <i>Hae</i> III, (d) <i>Fnu</i> 4HI. Lane M: 50 bp DNA size marker; Lane 1: Malaysia; Lane 2: Israel; Lane 3: Japan	208
60	A goldfish (<i>Carassius auratus</i> L.) showing a papilloma at the base of dorsal fin	210
61	A goldfish (<i>Carassius auratus</i> L.) showing an epidermal papilloma at antero-dorsal region	210
62	A goldfish-carp hybrid-"comet" showing several papilloma nodules of various sizes on the skin (a) Side view of the specimen showing five papilloma growth on the body; (b) A close up view of the same specimen. Note the large papilloma near the dorsal region and along the lateral line of the fish	212
63	Detection of CHV DNA by nested PCR assay in papilloma tissues from four field samples (two goldfish and two hybrids) by nested PCR assay. M: 50 bp DNA size marker; Lane 1-4: field samples; Lane 5: distilled water	213

64	Digestion of the nested PCR products with <i>SmaI</i> restriction enzyme. M: 50 bp DNA size marker; Lane 1-4: field samples; Lane 5: distilled water	213
65	Electron micrograph of papilloma section showing herpesvirus nucleocapsids in cell nuclei. x 50,000	214
66	Intranuclear accumulation of electron dense nucleocapsids within the nucleus of infected cells. x 50,000	214
67	Clusters of intranuclear electron dense nucleocapsids in infected cell nucleus. x 50,000	215
68	Enveloped virions within cytoplasmic vacuoles of infected cells [A]. Electron dense naked capsids inside cell nuclei [B]. x 50,000	215
69	Location of <i>Nco</i> NI restriction sites in the wild-type CHV 433 bp sequence	217
70	Construction of competitor template for quantitative PCR. M: 50 bp DNA size marker; Lane 1: CHV infected cell lysate; Lane 2: wild-type sequence plasmid; Lane 3: mutant competitor plasmid with an internal 66 bp wild- type sequence deletion	217
71	Visualization of heteroduplex molecules migrating slower than the 433 bp fragment after co-amplification of a constant number of wild-type template with decreasing amount of mutant competitor copy number	219
72	Co-amplification of 2.1×10^3 copies of CHV wild-type sequence template with decreasing copy number of CHV mutant competitor template. M: 50 bp DNA size marker; Lane 1-6: 3.5×10^5 , 1.75×10^5 , 3.5×10^4 , 1.75×10^4 , 3.5×10^3 , and 1.75×10^3 respectively; Lane 7: distilled water	220
73	Co-amplification of 2.1×10^4 copies of CHV wild-type sequence template with decreasing copy number of CHV mutant competitor template. M: 50 bp DNA size marker; Lane 1-6: 3.5×10^5 , 1.75×10^5 , 3.5×10^4 , 1.75×10^4 , 3.5×10^3 , and 1.75×10^3 respectively; Lane 7: distilled water	220
74	Generation of standard curve formula using log ratio WT/cCT against log number of mutant competitor molecules challenged with a) 2.1×10^3 wild-type sequence molecules and b) 2.1×10^4 wild-type sequence molecules	221

75 Quantitative PCR results of CHV infection in FHM cells over a period of eight days. SEM of three replicates

223



LIST OF ABBREVIATIONS

ABC	-	Avidin-biotin complex
ABTS	-	2,2'-Azino-di-(3-ethyl-benzthiazoline-6)
ag	-	Attogram
BB	-	Brown Bullhead
BCIP	-	Bromochloroindolyl phosphate
bp	-	Base pairs
BSA	-	Bovine serum albumin
CCV	-	Channel Catfish Virus
CHV	-	Cyprinid Herpesvirus
cm ²	-	Centimeters square
CPE	-	Cytopathic Effect
cDNA	-	Complementary Deoxyribonucleic Acid
CPCR	-	Competitive Polymerase Chain Reaction
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic Acid
ELISA	-	Enzyme-linked immunosorbent assay
EPC	-	<i>Epithelioma Papulosum Cyprini</i>
EHV-1	-	Equine Herpesvirus Type One
EHV-2	-	Equine Herpesvirus Type Two
EHV-4	-	Equine Herpesvirus Type Four

