IMMUNOCHEMISTRY AND MOLECULAR APPROACHES TOWARDS IDENTIFICATION OF MALAYSIAN CYPRINID HERPESVIRUS

By

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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

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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 \( \kappa \) 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 asel "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 immunohistokimia 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 didapat 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. Penjukan 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. Spesifsiti pengesanan PCR dengan kajian pecahan polimofisa DNA menunjukkan jujukan asid nukleik serupa di antara cyprinid herpesvirus dari Malaysia, Israel dan Jepun. Kaedah kuantitatifPCR berdasarkan sasaran jujukan virus PCR yang digunakan membolehkan gambaran kuantitatif terhadap tahap
jangkitan dan beban virus diselidiki, di mana keputusan awal menunjukkan bahawa
CHV memiliki expresi kinetik gen yang seakan sama dengan kumpulan
alphaherpesvirus.
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I certify that an Examination Committee met on 2\textsuperscript{nd} May 2001 to conduct the final examination of Samson Soon Min Ngen on his Doctor of Philosophy thesis entitled “Immunochemistry and Molecular Approaches Towards Identification of Malaysian Cyprinid Herpesvirus” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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9
This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy.

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Date: 12 JUL 2001
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

Date: 21st June 2001

SAMSON SOON MIN NGEN
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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

Restriction fragment profiles of CHV 433 bp PCR product amplified from three geographical regions cleared with (a) SmaI, (b) EcoNI, (c) HaeIII, (d) Fnu4HI. Lane M: 50 bp DNA size marker; Lane 1: Malaysia; Lane 2: Israel; Lane 3: Japan

A goldfish (Carassius auratus L.) showing a papilloma at the base of dorsal fin

A goldfish (Carassius auratus L.) showing an epidermal papilloma at antero-dorsal region

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

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
Digestion of the nested PCR products with SmaI restriction enzyme. M: 50 bp DNA size marker; Lane 1-4: field samples; Lane 5: distilled water.

Electron micrograph of papilloma section showing herpesvirus nucleocapsids in cell nuclei. x 50,000

Intranuclear accumulation of electron dense nucleocapsids within the nucleus of infected cells. x 50,000

Clusters of intranuclear electron dense nucleocapsids in infected cell nucleus. x 50,000

Electron micrograph of papilloma section showing herpesvirus nucleocapsids in cell nuclei. x 50,000

Electron micrograph of papilloma section showing herpesvirus nucleocapsids in cell nuclei. x 50,000

Clusters of intranuclear electron dense nucleocapsids in infected cell nucleus. x 50,000

Enveloped virions within cytoplasmic vacuoles of infected cells. x

Location of NcoNI restriction sites in the wild-type CHV 433 bp sequence

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

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

Co-amplification of 2.1 x 10³ 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 x 10⁵, 1.75 x 10⁵, 3.5 x 10⁴, 1.75 x 10⁴, 3.5 x 10³, and 1.75 x 10³ respectively; Lane 7: distilled water

Co-amplification of 2.1 x 10⁴ 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 x 10⁵, 1.75 x 10⁵, 3.5 x 10⁴, 1.75 x 10⁴, 3.5 x 10³, and 1.75 x 10³ respectively; Lane 7: distilled water

Generation of standard curve formula using log ratio WT/cCT against log number of mutant competitor molecules challenged with a) 2.1 x 10³ wild-type sequence molecules and b) 2.1 x 10⁴ wild-type sequence molecules
Quantitative PCR results of CHV infection in FHM cells over a period of eight days. SEM of three replicates
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex</td>
</tr>
<tr>
<td>ABTS</td>
<td>2,2'-Azino-di-(3-ethyl-benzthiazoline-6)</td>
</tr>
<tr>
<td>ag</td>
<td>Attogram</td>
</tr>
<tr>
<td>BB</td>
<td>Brown Bullhead</td>
</tr>
<tr>
<td>BCIP</td>
<td>Bromochloroindolyl phosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CCV</td>
<td>Channel Catfish Virus</td>
</tr>
<tr>
<td>CHV</td>
<td>Cyprinid Herpesvirus</td>
</tr>
<tr>
<td>cm²</td>
<td>Centimeters square</td>
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<tr>
<td>CPE</td>
<td>Cytopathic Effect</td>
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<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
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<td>Competitive Polymerase Chain Reaction</td>
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<td>Enzyme-linked immunosorbent assay</td>
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<td><em>Epithelioma Papulosum Cyprini</em></td>
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<td>EHV-1</td>
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<td>Equine Herpesvirus Type Two</td>
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<tr>
<td>EHV-4</td>
<td>Equine Herpesvirus Type Four</td>
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