

**MOLECULAR CHARACTERISTICS AND PATHOGENICITY OF A NOVEL
TRANSPLACENTAL RAT CYTOMEGALOVIRUS**

By

LOH HWEI SAN

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

January 2005

Dedicated with love and gratitude to:

**Father
Loh Swee Fatt**

**Mother
Chong Hoong Mooi**

**Brother and Sisters
Kian Loke, Hwei Wen and Hwei Lee**

**Fiancé
Liew Pit Kang**

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

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Chairman: Professor Mohd Azmi Mohd Lila, Ph.D.

Faculty: Veterinary Medicine

Cytomegalovirus (CMV) is a species-specific betaherpesvirus which causes acute, persistent and latent infections in both humans and animals. CMV is the most frequent congenital infection in humans. RCMV strain ALL-03 was the first CMV ever isolated from the placenta and uterus of the house rat (*Rattus rattus diardii*). As such, hypothetically, this RCMV should be a distinct strain from the existing isolates that is capable to cross placenta and infect the fetus. The objectives of the study were (i) to identify the novelty of the RCMV strain ALL-03, (ii) to characterize its immediate-early (IE) genes, and (iii) to determine its pathogenicity by developing the *in utero* transmission and neonatal infection models in rats. Overall, the present study signifies the virological and molecular detection of the RCMV antigen, DNA and mRNA in addition to the serological demonstration of the RCMV-specific immune response. Other than the traditional diagnostic methods, the study had also used advanced techniques, for examples, double antibody sandwich enzyme-linked immunosorbent

assay (DAS-ELISA), quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR. The study was commenced by characterizing the strain ALL-03. Upon infection, the virus showed delayed cytopathology, cell-association, low maximum titres, the presence of herpesviral inclusion bodies and herpesvirus related particles in infected rat embryonic fibroblast (REF) cells; specific antigen-antibody reaction with RCMV strain Maastricht; and rat-specific are all in accord with a RCMV. The genetic difference at the genome level with that of Maastricht, English, UPM/Sg and UPM/Kn strains had confirmed its novelty. The first recognized genes expressed during CMV infection, the IE genes were studied by analyzing the mRNA transcripts of infected-REF cells. The cDNA libraries were cloned into plasmids for sequencing. Each sequence was then probed towards the databanks for an identity search. Following the PCR and hybridization techniques, two distinct transcripts of unknown identities within the databanks were confirmed to be of the strain ALL-03 origin. These two IE transcripts were found considerably different to the IE genes of RCMV strains Maastricht and English. Meanwhile, a real-time RT-PCR assay was developed specifically to quantify the *in vitro* transcription levels of the two RCMV IE mRNAs. The kinetic transcription profiles and the bioinformatics analyses suggested them as exon 4 or IE1 and exon 5 or IE2. An *in utero* infection model demonstrated the clinical signs, pathological changes and anatomical virus distribution to the uterus, placenta, embryo, fetus, lung, kidney, spleen, liver and salivary gland of rats. The placenta was observed to be involved in the maternofetal RCMV infection. The maternal viremia leading to uterine infection which subsequently transmitting to the fetus through the placenta is the most likely phenomenon of congenital CMV

infection in the model. The study has established a useful rat model that mimics the neonatal CMV infection in humans especially for the virus dissemination in different organs, viremia and immune response. The kinetic quantitation of the viral antigen, DNA and antibody was assessed by DAS-ELISA, real-time PCR and ELISA respectively. This neonatal rat model demonstrated a characteristic splenomegaly and acute virus dissemination in blood, spleen, liver, lung and kidney. The salivary gland infection is suggested to augment the antibody response that may be responsible for a reduction of viremia. The study has provided important new insights of CMV disease particularly for a congenital infection in humans. The exploitation of the major IE regions has permitted greatest advances as a candidate of viral-vectored immunocontraception for rat control and generation of eukaryotic expression vectors.

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

**CIRI-CIRI MOLEKUL DAN PENGAJIAN PATOLOGI KE ATAS SEJENIS
SITOMEGALOVIRUS TIKUS RUMAH BAHARU YANG
BERUPAYA MENERUSI PLASENTA**

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Sitomegalovirus (CMV) merupakan betaherpesvirus yang menyebabkan jangkitan-jangkitan akut, berkekalan and terpendam ke atas kedua-dua manusia dan haiwan. CMV ialah jangkitan kongenital pada manusia yang paling kerap. Strain ALL-03 RCMV merupakan CMV pertama yang dipencilkan dari rahim dan plasenta tikus rumah (*Rattus rattus diardii*). Justeru itu, RCMV ini dihipotesiskan sebagai satu strain yang sepatutnya berlainan daripada pencilan-pencilan tersedia ada di mana ia berupaya menembusi plasenta untuk menjangkiti fetus. Matlamat-matlamat pengajian ini ialah (i) mengenalpastikan kebaharuan strain ALL-03 RCMV, (ii) mencirikan gen-gen 'immediate-early' (IE)nya, (iii) mengkaji bidang patologinya dengan menubuhkan model-model jangkitan *in utero* dan neonatal pada tikus-tikus. Secara keseluruhannya, pengajian ini mementingkan penemuan secara virologik dan molekular ke atas antigen, DNA dan mRNA RCMV ini di samping menunjukkan secara serologi gerakbalas keimunan yang spesifik kepada RCMV. Selain daripada kaedah-kaedah diagnostik yang

biasa, pengajian ini juga menggunakan teknik-teknik yang canggih seperti sandwich berantibodi dua-asai immunoerap terangkai enzim (DAS-ELISA), kuantitatif transkripsi balik-reaksi rangkaian polimerasi (RT-PCR) dan PCR masa-benar. Pengajian ini dimulakan dengan pencirian strain ALL-03. Semasa jangkitan, virus tersebut menunjukkan sitopatologi yang terlengah, pengkaitan-sel, paras maksima virus yang rendah, kehadiran badan-badan kandungan herpesviral dan partikel-partikel yang bersamaan herpesvirus dalam sel fibroblas lembaga tikus (REF) yang terjangkit; reaksi antigen-antibodi yang spesifik dengan strain Maastricht RCMV dan kespesifikan-tikus menyerupai satu RCMV. Ketidaksamaan genetik di paras genom dengan strain-strain Maastricht, English, UPM/Sg and UPM/Kn RCMV membuktikan kebaruannya. Sebagai gen-gen pengenalan pertama yang ternyata semasa jangkitan CMV, gen-gen IE telah dikaji dengan mengadakan analisis transkripsi mRNA-nya ke atas sel-sel REF. Perpustakaan cDNA diklonkan ke dalam plasmid-plasmid untuk diujukkan. Setiap ujukan disiasatkan ke atas bank data untuk mencari kenalannya. Justeru kegunaan teknik-teknik PCR dan penghibridasi, dua hasil transkripsi berasingan yang tiada kenalan dalam bank data telah dikenalpastikan sebagai asalan strain ALL-03. Kedua-dua hasil transkripsi IE didapati berlainan daripada gen-gen IE strain-strain Maastricht dan English RCMV. Sementara itu, satu RT-PCR masa-benar telah dikemukakan dengan spesifik untuk mengirakan paras transkripsi *in vitro* kedua-dua mRNA IE itu. Maklumat-maklumat transkripsi kinetik and analisis bioinformatik mencadangkan bahawa mereka ialah ekson 4 atau IE1 dan ekson 5 atau IE2. Satu model jangkitan *in utero* mempersembahkan kesan-kesan klinikal, perubahan patologi dan penularan virus secara anatomikal ke atas rahim, plasenta, lembaga, fetus, peparu,

ginjal, limfa, hati dan kelenjar air liur tikus-tikus. Plasenta diperhatikan bahawa terlibat dalam jangkitan maternofetal CMV. Kejadian viremia maternal yang menyebabkan jangkitan rahim seterusnya penularan kepada fetus melalui plasenta merupakan cara-cara pengjangkitan CMV secara kongenital dalam model ini. Pengajian ini juga telah mempersembahkan satu model berguna yang meyerupai jangkitan neonatal pada manusia terutamanya penularan virus dalam pelbagai organ, viremia dan gerakbalas keimunan. Pengiraan kinetik ke atas antigen virus, DNA dan antibodi dikajikan oleh DAS-ELISA, PCR masa-benar dan ELISA masing-masing. Model tikus neonatal ini menunjukkan satu sifat pembesaran limfa dan penularan akut virus dalam darah, limfa, hati, paru dan ginjal. Jangkitan dalam kelenjar air liur dijangkakan akan membantu gerakbalas antibodi yang mungkin bertanggungjawab dalam kemerosotan viremia. Pengajian ini telah menyumbangkan kepada satu kedekatan baru yang penting dalam penyakit CMV terutamanya dalam jangkitan kongenital pada manusia. Penerokaan pada bahagian IE utama merupakan satu langkah maju ke depan sebagai satu calon pencegahan hamil secara keimunan berangkutan-virus untuk kawalan tikus dan penciptaan vektor penyataan eukariot.

ACKNOWLEDGEMENTS

My utmost appreciation and gratitude are conveyed to my supervisor Prof. Dr. Mohd Azmi Mohd Lila for his invaluable guidance; constructive advice, comments and suggestions; patience and encouragement throughout the study. I would like to express my heartfelt thanks and appreciation to my co-supervisors, Prof. Dato' Dr. Hj. Sheikh-Omar Abdul Rahman and Prof. Dr. Mohd Zamri Saad for their invaluable advices, suggestions and discussions; constructive criticisms; patience and supports which were really helpful towards the completion of my study. Additionally, their efforts spent to improve the quality of the thesis are very much appreciated.

My sincere thanks and gratitude are extended to Associate Prof. Dr. Abdul Rahman Omar, Associate Prof. Dr. Mohd Hair Bejo and Prof. Abdul Rani Bahaman for granting permission to use the equipments and facilities in their laboratories and their precious supports. I am grateful to the staff members of the Biologic Laboratory and Virology Laboratory especially Madam Rodiah Husin and Mr. Mohd Kamarudin Awang Isa for their valuable technical assistances.

My greatest gratitude and thanks are dedicated to Kit Yee, May Ling, Sok Fang, Lee Shun, Tam, Chan, Kim Sing, Su Fun, Zeenat, Zuridah, Zunita, Lih Ling, Sheaw Wei, Wan, Lee Kim, Narumon, Do Yew, Elham, Hossein, John, Yatie, Suria, Elysha, Hanisa, Faizal, Kelvin, Louis, Farah and the other members in Faculty of Veterinary Medicine for their friendship, assistance and encouragement throughout the course of the study.

Last but not least, I am indebted to my beloved parents, brother, sisters, brother-in-law and Pit Kang for their endless encouragement, moral support, patience, understanding and unconditional love all the time. I thank my late pets, BoBo and Popeye for their companionships of days past and the cherished memories that they had gifted to me.

I certify that an Examination Committee met on 26th January 2005 to conduct the final examination of Loh Hwei San on her Doctor of Philosophy thesis entitled "Molecular Characteristics and Pathogenicity of a Novel Transplacental Rat Cytomegalovirus " 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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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.

LOH HWEI SAN

Date: 31/01/2005

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AP	Assembly Protein
BCIP	5-Bromo-4-Chloro-3-Indolyl-Phosphate
BHK	Baby Hamster Kidney
BMT	Bone Marrow Transplant
bp	Base Pair
BSA	Bovine Serum Albumin
C(T)	Threshold Cycle
cDNA	Complementary DNA
CDV	Cidofovir
CHPMPC	Cyclic Derivative of HPMPC
CMI	Cell-Mediated Immunity
CNS	Central Nervous System
CpA	Cytosine-Phosphate-Adenosine
CPE	Cytopathic Effect
CpG	Cytosine-Phosphate-Guanodine
CRFK	Crandal Reese Feline Kidney
CTL	Cytotoxic T Lymphocyte
DAB	3-3'-Diamino Benzidine Hydrochloride
DAS-ELISA	Double Antibody Sandwich ELISA
DEPC	Diethyl Pyrocarbonate
dH ₂ O	Distilled Water
DHPG	9-(1, 3-Dihydroxy-2-Propoxymethyl) Guanine
DMEM	Dulbecco Minimum Essential Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide Triphosphate
DTT	Dithiothreitol
E	Early
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assays
EMBL	European Molecular Biology Laboratory
FBS	Fetal Bovine Serum
FITC	Fluorescence Isothiocyanate
FOS	Pyrophosphate Analogue Fosarnet
g	Gravity
gB	Glycoprotein B
GCV	Ganciclovir (same compound with DHPG)
GPCMV	Guinea Pig Cytomegalovirus
GPCR	G-Protein-Coupled Receptor

h	Hour
H&E	Hematoxylin and Eosin
HCMV	Human Cytomegalovirus
HHV	Human Herpesvirus
HIS	Hyperimmune serum
HIV	Human Immunodeficiency Virus
HPMPC	(S)-1-(3-Dihydroxy-2-Phosphonyl Methoxypropyl) Cytosine
HSV	Herpes Simplex Virus
HVS	Herpesvirus Saimiri
i.p.	Intraperitoneal
IE	Immediate-Early
Ig	Immunoglobulin
IIF	Indirect Immunofluorescence
IIP	Indirect Immunoperoxidase
kbp	Kilo Base Pair
kDa	Kilo Dalton
L	Late
LB	Luria Bertani
M	Molar
MCMV	Mouse Cytomegalovirus
MCP	Major Capsid Protein
mCP	Minor Capsid Protein
MHC	Major Histocompatibility Complex
MIE	Major Immediate-Early
MIEP	Major Immediate-Early Promoter
min	Minute
mM	Millimolar
MOI	Multiplicity of Infection
MOPS	3- <i>N</i> -Morpholino Propanesulfonic Acid
mRNA	Messenger Ribonucleic Acid
MW	Molecular Weight
NBT	Nitro Blue Tetrazolium
NIEP	Non-infectious Enveloped Particle
NK	Natural Killer
NTC	No Template Control
OD	Optical Density
ORF	Open Reading Frame
p.i.	Post-Infection
PBS	Phosphate Buffer Saline
PBST	PBS Tween 20
PBSTx	PBS Triton X-100
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Unit
RCMV	Rat Cytomegalovirus
RE	Restriction Endonuclease
REF	Rat Embryonic Fibroblast

RK	Rabbit Kidney
RNA	Ribonucleic Acid
RNase	Ribonuclease
rpm	Revolutions per Minute
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
s	Second
s.c.	Subcutaneous
SCID	Severe Combined Immunodeficient
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEM	Standard Error of Mean
SNT	Serum Neutralization Test
SPF	Specific Pathogen Free
SPSS	Statistical Program for Social Science
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TEM	Transmission Electron Microscopy
TMB	Tetra Methyl Benzidine
TNE	Tris-NaCl-EDTA
TpG	Thymine-Phosphate-Guanodine
UL	Unique Long
UPM	Universiti Putra Malaysia
US	Unique Short
UV	Ultraviolet
V	Volt
v/v	Volume per Volume
Vero	Cell Line Derived from Green African Monkey Kidney
VP	Virion Polypeptide
vs	Versus
VZV	Varicella-Zoster Virus
w/v	Weight per Volume
w/w	Weight per Weight
X-gal	5-Bromo-4-Chloro-3-Indolyl-B-D-Galactopyranoside