UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION AND PARTIAL ENVELOPE GENE SEQUENCE OF AN ENDOGENOUS BOID RETROVIRUS

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

CHARACTERIZATION AND PARTIAL ENVELOPE GENE SEQUENCE OF AN ENDOGENOUS BOID RETROVIRUS

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January 2013

Chairperson : Professor Noordin Mohamed Mustapha, PhD

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Inclusion body disease (IBD) is a worldwidely prevalent and highly fatal disease of boids (*Boinae* and *Phytoninae*) believed to be caused by a retrovirus. The possibility of horizontal transmission within species has occurred likely to be mediated by the envelope (env) gene of the virus. In Malaysia, most cases of boid IBD were diagnosed based on histology and no documentation was made on the characteristics of the retrovirus. Thus, this forms the basis of this study to characterize and partially sequence the endogenous retrovirus isolated from cases of IBD in Malaysia.

The isolated endogenous boid retrovirus was propagated in Vero cells systems, purified, amplified and envelope gene was sequenced to characterize the isolate. Attempt to propagate the virus in Vero systems was successful with the formation of cytopathic effect (CPE). The pool propagated supernatant was purified using continuous sucrose gradient (20-50% density gradient) technique. Following ultracentrifugation, two thick bands at sucrose density of 25% and 35% sucrose density were procured. A faint band of 45% sucrose density was also visible. The purified virus was further analyzed by electron micrographs examination, buoyant density, SDS-PAGE and western blotting. Retrovirus characteristic has been classified according to the viral tropism formation, buoyant

density of 1.15g/ml-1.17g/ml, and protein pattern analysis. Electrophoresis analysis of the purified virus by SDS-PAGE showed significant differences of protein migration pattern compared from filtered ground tissues and filtered Vero supernatant sample. However, through western blotting analysis there was consistent presence of main protein band of 68 kDa in all samples which was believed that this main protein was associated with IBD.

Further characteristics of the env gene encoding the SU and TM protein was amplified, purified and sequenced. The envelope sequence was compared among a series of envelope gene retrovirus using multiple sequence alignment program. The virus phylogenetic relationship classified the boid snakes in their own group. However, sequence analysis of env gene revealed 30%-40% homology to other species. Further studies should be conducted to verify the pathogenicity of the isolated virus via Koch's postulate.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENCIRIAN DAN PENJUJUKAN SERPIHAN GEN ENVELOP DARIPADA RETROVIRUS ENDOGEN BOID

Oleh

ROSLINA BINTI HASSAN

Januari 2013

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Penyakit jasad rangkuman (IBD*)* yang prevalens seluruh dunia dan membawa maut pada boid (*Boinae dan Phytoninae*) dipercayai disebabkan oleh retrovirus. Kemungkinan perpindahan secara horizontal di antara spesis berlaku berantarakan gen envelop (env) virus. Di Malaysia, kebanyakan kes IBD didiagnosis berdasarkan histologi tanpa dokumen mengenai cirian retrovirus tersebut. Dengan itu, tujuan utama kajian ini adalah untuk separa mencirikan dan menjujuk retrovirus endogen yang diasingkan daripada kes IBD di Malaysia.

Retrovirus endogen IBD yang diasingkan, dibiak, ditulenkan, digandakan dalam sistem sel Vero dan gen env dijujukkan bagi pencirian identiti retrovirus endogen boid. Percubaan membiakkan virus dengan menggunakan sistem sel Vero telah berjaya menunjukkan kehadiran kesan sitopati (CPE). Tabungan supernatan yang diganda telah ditulenkan dengan menggunakan kaedah ketumpatan sukros (20%-50% kecerunan ketumpatan sukros). Selepas daya emparan-ultra, dua jaluran tebal masing-masing pada ketumpatan sukros 25% dan 35% terhasil. Manakala jaluran nipis pada ketumpatan sukros 45% juga kelihatan. Virus yang telah ditulenkan, dianalisis dengan lebih lanjut melalui pemeriksaan mikrograf elektron, ketumpatan pengapungan, elektroforesis

poliakrilamida gel dan penyerapan western. Berdasarkan pembentukan tropism, mikrograf, ketumpatan pengapungan pada kadar 1.15g/ml-1.17g/ml, dan pergerakan protein, virus dikelaskan sebagai cirri retrovirus. Analisis elektroforesis gel poliakrilamida menunjukkan pergerakan jaluran protein virus tulen sedikit berbeza dibandingkan dengan sampel tisu kisar bertapis dan Vero supernatant bertapis. Bagaimanapun, melalui penyerapan western kehadiran jaluran protein unik pada 68 kDa adalah konsisten di semua sampel yang mana dipercayai protein tersebut berkait rapat dengan penyakit IBD.

Dalam pencirian selanjutnya, gen env yang mengkodkan protein SU dan TM digandakan, ditulenkan dan serpihan gen dijujukkan. Turutan jujukan diperolehi daripada penulenan produk tindakbalas polimerase berantai. Jujukan serpihan gen envelop dibandingkan diantara spesis dengan menggunakan program penjajaran jujukan berbilang. Perbandingan hubungan filogenetik bagi jujukan gen env mengklasifikasikan ular boid berada di kumpulan tersendiri. Bagaimanapun, analisis jujukan gen env menujukkan terdapat 30%-40% persamaan homologi dengan spesis yang lain. Kajian lanjut harus dijalankan bagi mengesahkan kepatogenan virus yang diasingkan melalui postulat Koch.

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I certify that a Thesis Examination Committee has met on 18 January 2013 to conduct the final examination of Roslina Binti Hassan on her thesis entitled "Characterization and partial envelope gene sequence of an endogenous boid retrovirus" in accordance with the Universities 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 degree of Master of Science.

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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 at any other institution.

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ROSLINA BINTI HASSAN

Date:

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
BCIP	Bromo-4-chloro-3-indoyl phosphate
bp	Base pair
cDNA	Complementary DNA
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled water
ddH_2O	Deionized distilled water
dNTP	Deoxyribonucleotides
DMSO	Dimethyl sulphoxide
EDTA	Ethylenediaminetetraacetic acid
FCS	Fetal calf serum
FBS	Fetal bovine serum
hr	Hour
kDa	Kilodalton
l	Liter
MEM	Minimal essential medium
mg	milligram
min	minute
ml	milliliter
mM	milimolar
$NaHCO_3$	Natrium hydrogen bicarbonate
NaOH	Natrium hydroxida
NBT	Nitroblue tetrazolium
ng	Nanogram
PBS	Phosphate buffer saline
pmol/µl	Picomol per microliter
PSK	Penicillin, Streptomycin and Kanamycin sulfate
RT-PCR	Reverse transcriptase-polymerase chain reaction
SU	Surface

TM	Trans membrane
TNE buffer	Tris- natrium chloride-EDTA buffer
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TBE	Tris-borate-EDTA buffer
TEMED	Tetramethylethylenediamine
TPB	Triptose phosphate broth
TTBS	Tween 20 tris-natrium chloride buffer
TV	Trypsin versine
U	Unit (s)
v	Volt
v/v	Volume per volume
w	Watt
w	Weight per volume
%	Percentage
^{o}C	Degrees celcius

CHAPTER I

INTRODUCTION

Boid inclusion body disease (IBD) in snakes is a viral infection recognized since mid seventies (Knotek *et al*., 2007). In 1994, a retrovirus was incriminated as responsible for IBD (Schumacher *et al*., 1994). World widely, many cases of IBD have been reported (Carlisle-Nowak *et al*., 1998; Oros *et al*., 1998; Zwart *et al*., 2001; Vancraeynest *et al*., 2006). This is the most problematic disease in snakes since chronically infected snake shed the virus before clinical sign are noted. In addition, this virus is contagious, and euthanasia is the only option for affected animals in order to prevent its spread (Raymond *et al*., 2001; Pasman *et al*., 2008)

Currently, there is no available treatment for IBD and it is variably fatal in python (Schumacher *et al*., 1994; Chandra *et al*., 2001). Tissues of most of snakes with tumours or proliferative virus were detected with retrovirus-like particles (Chandra *et al*., 2001). However, normal tissues may also harbor the virus (Huder *et al*., 2002). Recently, a systemic PCR-based search for endogenous retrovirus related to murine leukemia virus (MLV) showed a wide distribution of such C-type sequences among terrestrial animals including amphibians, reptiles, birds and mammals (Martin *et al*., 1999). In addition, various exogenous and endogenous viruses found in varying mammals and have been isolated from reptiles and birds (Knipe *et al.*, 2007). Retrovirus consisted of two identical positive-sense single stranded RNA of approximately 7 kb that codes for at least three main genes including gag, pol, and env genes flanked by LTR. *In vivo* neutralization experiments study suggested that this viral env might lead to preventive approaches to control virus diseases required the role of the viral envelope proteins and viral protein epitopes involved in the virus infection process (Burton *et al*., 2000; Huang *et al*., 2002). In an effort to create a vaccine against retrovirus disease, analysis using viral envelope

have been studied in human immunodeficiency virus, HIV (Rodriguez *et al*., 1991), simian immunodeficiency virus, SIV (Jone *et al*., 1994) and feline immunodeficiency virus, FIV (Uhl *et al*., 2008). However, variations in these regions made it difficult for the host to provide virus-neutralizing antibodies (VNAs) with broad neutralizing activities (Uhl *et al*., 2008) and recently RNAi technology become a promising technology as future preventing method of retrovirus infection (Hu *et al*., 2004). Since there is a possibility of horizontal transmission, is essential to identify and characterize the env gene of IBD in Malaysia compared to other species and as protective purposes.

Histologically, IBD has characterized by the formation of intracytoplasmic inclusion bodies in the epithelial cells of all vital organ such as liver, kidneys, lungs, intestine, stomach, heart, spleen, and pancreas (Knotek *et al*., 2007) but mainly in the visceral epithelium and neurons (Vancraeynest *et al*., 2006). Ultrasructurally, type-C like retrovirus (Ziegel *et al*., 1969) later shown to be related to D-type retrovirus (Anderson *et al*., 1970) were seen. Presently, type A retroviral particles associated with adenocarcinoma was diagnosed in an Emerald Tree Boa (*Corallus caninus*) that had metastatic intestinal adenocarcinoma (Oros *et al*., 2004). Retroviral virion is spherical 90-120nm in diameter with a buoyant density of 1.15-1.17g/ml (Petry *et al*., 1992). Current findings suggest that the inclusion body contain an antigenically distinct 68-kDa protein against specific monoclonal antibody (Wozniak *et al*., 2000) and it is unknown whether all IBD cases in different snake species consist of exactly the same protein or is species dependent (Chang and Jacobson, 2010). All the samples have been reviewed from different countries, but no information could be found for boid species in Malaysia.

To address this problem, it is a passionate interest to identify and characterize the local viral isolates as a preliminary step in the development of an effective preventive strategy

against IBD. If they are closely related, than these viral envelope proteins could be used to snake and other animal model that have similarities or are related. The relationship of genetic information availability from this study could facilitate future diagnostic technique development against retrovirus disease. In addition, due to the lack of knowledge in the control, treatment and diagnosis of IBD so that, from such study could be able to provide the knowledge and might be a discovery of IBD in Malaysia.

Therefore, the objectives of this study are:

1. To isolate, identify and characterize the virus isolated from IBD (inclusion body disease)
2. To determine the sequence of the partial env gene of boid retrovirus

The hypotheses of this study are:

1. The CPE development of multinucleated giant cells and syncytium formations in Vero cells related with retrovirus CPE
2. The buoyant density in ranged of 1.0-1.17g/ml and morphology of 80-100nm in diameter size responsible for retrovirus infection
3. The identification of retroviral agent of BIBD associated with the presence of 68 kDa protein

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