UNIVERSITI PUTRA MALAYSIA

GENETIC DIVERSITY AND ANTIGENIC VARIATION OF TRYPANOSOMA EVANSI IN MALAYSIA

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By

MAHIRA WATANABE

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

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DEDICATION

Dedicated to my parents, siblings and Nani Hazoor

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

GENETIC DIVERSITY AND ANTIGENIC VARIATION OF *TRYPANOSOMA EVANSI* IN MALAYSIA

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May 2012

Chairman: **Associate Professor Rehana Abdullah Sani, PhD**

Faculty: **Faculty of Veterinary Medicine**

To investigate the genetic diversity of *Trypanosoma evansi* field isolates in Peninsular Malaysia, 42 isolates from cattle, deer and buffalo from eight farms were analysed using five microsatellite markers and a tandemly repeated coding sequence, MORF2-REP. A total of 30 alleles and eleven multilocus genotypes (MLGs) identified from across six loci revealed genetic polymorphism among the local isolates. The high fixation index (F_{st}) and Nei's genetic distances were all indicative of genetic sub-structuring within the isolates. Nei's genetic distances showed less allelic diversity between cattle and deer isolates and greater diversity between deer and buffalo isolates. The isolates from buffalo shared the same MLG and clustered together in the

unweighted pair-group mean average (UPGMA) and neighbor-joining (NJ) trees, the deer isolates formed three sub-groups and the cattle isolates formed a number of different groups. Among the cattle isolates a number of MLGs were shared among isolates originating from different farms. Some MLGs were however unique to certain hosts or farms such as the MLGs observed in the deer isolates. The genotypes observed in the deer breeding centre were of significance as these pathogenic strains were responsible for an outbreak of trypanosomiasis which contributed to more than 27% of all deaths at the centre. The high number of repeated MLGs and high heterozygote excesses found in this study were supportive of a clonal population structure of this parasite.

In order to investigate the antigenic repertoire of *T. evansi* isolates in Malaysia in the course of a natural infection, variant surface glycoproteins (VSGs) expressed in the field strains were isolated, cloned and characterized. Out of the 41 VSGs isolated from 32 *T. evansi* field isolates, nine were identified as novel VSGs which did not have close homologues in the databases. Ten of the VSGs were identical or highly similar to each other while 31 VSGs were distinct. Some of the identical VSGs were shared between isolates originating from different host species and farms suggesting that the antigenic repertoire had no constraints placed on it by the host species or geographical location. Most of the VSGs expressed by field isolates had typical N- and C-terminal domain structures. The N-terminal domains showed high sequence diversity as compared to the more conserved C-terminal domains. As there were no conserved motifs located in the

N-terminal domain, the VSG coat remains a poor candidate for vaccine development and diagnostics.

In an attempt to elucidate the complex process of antigenic variation, the antigenic repertoire of *T. evansi* was investigated in three goats experimentally infected with a local *T. evansi* field isolate. The VSGs expressed during the course of infection were isolated, cloned and characterized. Three major parasitaemic waves were observed in all three animals from which combinations of major and minor VSG populations were isolated. A total of 190 VSGs from the three animals were isolated during the course of infection which were divided into 48 distinct groups. The order of expression of major VSGs among the infected goats followed a loose hierarchy. Some VSGs expressed during the early stages of infection were expressed again during the later stages of infection.

In conclusion, the data revealed high genetic diversity among the Peninsular Malaysia isolates of *T. evansi* which possessed a large antigenic repertoire. A loose hierarchy in the order of expression of major VSGs was observed in goats infected with a local field isolate of *T. evansi*.

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

KEPELBAGAIAN GENETIK DAN KELAINAN ANTIGEN PADA *TRYPANOSOMA EVANSI* DI MALAYSIA

Oleh

MAHIRA WATANABE

Mei 2012

Pengerusi: **Profesor Madya Rehana Abdullah Sani, PhD**

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Untuk menyiasat kepelbagaian genetik perbezaan dari pencilan *Trypanosoma evansi* di Semenanjung Malaysia, 42 pencilan daripada lembu, rusa dan kerbau dari lapan ladang yang berbeza dikaji menggunakan lima penanda mikrosatelit dan "tandemly repeated coding sequence", MORF2-REP. Sebanyak 30 alel dan 11 genotip multilocus (MLGs) yang dikenalpasti dari enam lokus menunjukkan polimorfisme genetik antara pencilan tempatan. "Fixation index" (F_{st}) yang tinggi dan jarak semua genetik Nei menunjukkan genetik sub-penstrukturan dalam pencilan. Jarak genetik Nei menunjukkan kepelbagaian alel yang kurang antara pencilan lembu dan rusa dan kepelbagaian yang

lebih antara pencilan lembu dan kerbau. Pencilan daripada kerbau berkongsi MLG yang sama dan dikumpulkan bersama, pencilan rusa membentuk tiga sub-kumpulan dan pencilan lembu membentuk beberapa kumpulan yang berbeza. Antara pencilan lembu beberapa MLGs dikongsi di kalangan pencilan yang berasal dari ladang yang berbeza. Walaubagaimanapun beberapa MLG unik kepada hos atau ladang tertentu seperti MLGs yang terdapat pada pencilan rusa. Genotip yang terdapat di ladang rusa ini adalah penting kerana strain tersebut merupakan yang virulen dan penyebab kematian beberapa rusa. Jumlah MLG yang tinggi dan heterozigot berlebihan tinggi yang ditemui dalam kajian ini menunjukkan bahawa parasit ini bermirip struktur populasi klon.

Untuk menyiasat kelainan antigen antara pencilan *T. evansi* di Malaysia dalam jangkitan semulajadi, permukaan varian glikoprotin (VSGs) yang dikeluarkan oleh pencilan dari lapangan, diasingkan, diklon dan ciri VSGs disiasat. Daripada 41 jenis permukaan varian glikoprotin yang diasingkan daripada 32 pencilan *T. evansi* dilapangan, sembilan VSGs adalah novel yang tidak mempunyai homolog rapat di pangkalan data. Sepuluh VSGs yang serupa antara satu sama lain dan 31 VSGs berbeza. Sebahagian VSG yang serupa telah dikongsi di antara pencilan yang berasal dari spesis hos dan ladang yang berbeza, ini mencadangkan repertoir antigen tidak mempunyai kekangan yang dikenakan ke atasnya oleh hos atau lokasi geografi. Kebanyakan VSG yang dikeluarkan oleh pencilan lapangan mempunyai struktur domain N-terminal dan domain C-terminal yang tipikal. Domain N-terminal menunjukkan jujukan kepelbagaian tinggi berbanding dengan domain C-terminal yang

lebih terpelihara. Oleh sebab tidak ada motif terpelihara terletak di domain N-terminal, selaput VSG bukan calon yang baik untuk pembangunan vaksin dan diagnostik.

Untuk menjelaskan proses variasi antigen yang kompleks, repertoir antigen *T. evansi* telah diperiksa dalam tiga kambing uji kaji yang dijangkiti dengan satu pencilan *T. evansi* dari lapangan. VSGs yang dikeluarkan semasa jangkitan uji kaji, telah diasingkan, diklon dan ciri disiasat. Tiga puncak parasitaema dapat diperhatikan dalam ketiga-tiga kambing dari mana campuran populasi major dan minor VSG telah diasingkan. Sebanyak 190 VSGs dari tiga haiwan tersebut telah diasingkan semasa jangkitan yang dibahagikan kepada 48 kumpulan yang berbeza. Urutan mengekspreskan major VSG dikalangan kambing yang dijangkiti mengikuti hierarki yang longgar. Beberapa VSG yang diekspreskan pada peringkat awal jangkitan mengekspreskan sekali lagi semasa peringkat akhir jangkitan.

Kesimpulannya, data menunjukkan kepelbagaian genetik di antara pencilan *T. evansi* Semenanjung Malaysia yang mempunyai repertoir antigenik yang luas. Kelainan antigen salah satu pencilan ini pada kambing yang dijangkit menunjukkan hierarki yang longgar dalam ekspresi VSG major.

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I certify that a Thesis Examination Committee has met on (Date) to conduct the final examination of Mahira Watanabe on her thesis entitled "Genetic Diversity and Antigenic Variation of *Trypanosoma evansi* in Malaysia" 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 Doctor of Philosophy.

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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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MAHIRA WATANABE

Date: 25 May 2012

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
BCT	Buffy Coat Technique
BES	Bloodstream Expression Sites
BLAST	Basic Local Alignment Search Tool
CATT	Card Agglutination Test for Trypanosomiasis
CATT/*T. evansi*	Card Agglutination Test for *Trypanosoma evansi*
cDNA	Complimentary Deoxyribonucleic acid
CRAM	Cysteine-rich Acidic Integral Membrane Protein
CS-chord	Cavalli-Sforza chord
C-terminal	Carboxyl-terminus
DNA	Deoxyribonucleic acid
DTU	Discrete Typing Units
ELISA	Enzyme Linked Immunosorbent Assay
ESAG	Expression-Site-Associated Genes
FISH	Fluorescence *in situ* Hybridization
F-statistics	Fixation Indices
GPI	Glycosyl-phosphatidylinositol
HAT	Human African Trypanosomiasis
HCT	Haematocrit Centrifugation Technique
H_e	Expected heterozygotes
H_o	Observed heterozygotes
IFA	Immunofluorescent Antibody

IPTG	Isopropyl β-D-1-thiogalactopyranoside
ISSR	Inter-Simple Sequence Repeats
ITS	Internal Transcribed Spacer
kDNA	kinetoplast -specific DNA
LAMP	Loop-Mediated Isothermal Amplification
LB	Luria-Bertani
LSU-rDNA	Single Locus Analysis of the Ribosomal RNA gene
MAECT	Miniature Anion Exchange Centrifugation Technique
MEGA	Multiplex Endonuclease Genotyping
MES	Metacyclic Expression Sites
MGE	Mobile Genetic Elements
MLEE	Multi-locus Enzyme Electrophoresis
ML	Maximum Likelihood
MLG	Multilocus Genotype
MP	Maximum Parsimony
MORF2-REP	Open Reading Frame: Tandemly Repeated Coding Sequence
NCBI	National Center for Biotechnology Information
NJ-tree	Neighbour Joining Tree
N_m	Gene Flow
N-terminal	Amino-terminus
PATT	Procyclic Agglutination Test for Trypanosomiasis
PCR	Polymerase Chain Reaction
PNA	Peptide Nucleic Acid

RAPD	Random Amplification of Polymorphic DNA
rDNA	Ribosomal Deoxyribonucleic acid
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
RoTat 1.2	Rhode Trypanozoon Antigen Type 1.2
RT-PCR	Reverse-Transcription PCR
SNP	Single Nucleotide Polymorphism
spp.	Species
SRA	Serum Resistance-associated Gene
SSR	Simple Sequence Repeat
TFM	Trypanosome Freezing Mix
UPGMA	Unweighted Pair-Group Mean Average
VAT	Variable Antigen Type
VNTR	Variable Number of Tandem Repeat
VSG	Variant Surface Glycoprotein
VSGdb	Database for Trypanosome Variant Surface Glycoproteins
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

CHAPTER 1

GENERAL INTRODUCTION

Trypanosoma evansi is a unicellular parasitic protozoa that infects the bloodstream of mammals and is the causative agent of trypanosomiasis (surra). This parasite is the most widespread pathogenic trypanosome infecting domestic and wild animals in Asia, Africa, South America and the Middle East. *T. evansi* is widely distributed due to its ability to be mechanically transmitted by biting flies such as *Tabanus* and *Stomoxys spp.* (Luckins, 1988; Stephen, 1986).

This haemoflagellate causes fever, anaemia, oedema, abortions and progressive loss of weight in infected mammals and can eventually lead to death in susceptible animals, therefore making it detrimental to the livestock industry. *T. evansi* is a cause of major constraint on livestock production and a cause of significant economic losses, therefore it is imperative to control this disease. For example, it has been reported that the estimated total cost of *T. evansi* on cattle ranchers in the Brazilian Pantanal region is US $2.4 million/ year (Seidl *et al.*, 1998). In Indonesia it was reported that morbidity and mortality caused by *T. evansi* contributed to an annual loss of US $28 million (Pearson *et al.*, 1999). In the Philippines losses incurred by *T. evansi* infection was estimated to be US $7.9 million in nine years (Manuel, 1998) during the time when outbreaks with high mortality in horses, buffaloes, cattle and goats in the Philippines were reported (Reid, 2002; Manuel, 1998).

In Malaysia there have been numerous reports of trypanosomiasis since the first report in a horse in 1903 (Fraser and Symonds, 1909). However, it was not until the early eighties that the economic impact of this disease was realized when high mortality rates were observed in buffalo and cattle farms in Malaysia (Abas-Mazni and Zainal-Abidin, 1985). *T. evansi* outbreaks have also been reported locally in horses (Ng and Vanselow, 1978), pigs (Arunasalam *et al.*, 1995) and captive Sumatran rhinoceros (Vellayan *et al.*, 2004). The most recent outbreak was reported in a deer breeding centre in Lenggong, Perak (Adrian *et al.*, 2010; Nurulaini *et al.*, 2007). One in four deaths on the farm recorded between 2006 and 2007 were due to trypanosomiasis, which indicated a significant economic loss due to *T. evansi* (Adrian *et al.*, 2010). It emphasized the economic impact of this parasite and stressed the need for effective control measures.

In order to effectively control this parasite in the country, Malaysian *T. evansi* strains must be studied extensively and meticulously. An understanding of the genetic diversity of a pathogen is essential in understanding clinical pleiotropism, its correlation with epidemics and drug resistance. This information is also crucial for diagnostic and epidemiologic research as well as evolutionary studies. Since at present there is absolutely no information regarding the genetic diversity of Malaysian *T. evansi* strains, the aim of this study was to genetically characterize the *T. evansi* field isolates in Malaysia.

Another problem associated with *T. evansi* is its long term persistence in the host due to its ability to evade the host immune modulators through a complex process of antigenic

variation. This process involves switching of the variant surface glycoprotein (VSG) homodimers that form a dense, tightly packed monolayer on the cell surface. At present there is no information available regarding the antigenic repertoire of Malaysian *T. evansi* isolates. Hence, this study was carried out to investigate the degree of antigenic variation in this parasite by characterizing the VSGs expressed by strains isolated from Malaysia and comparing these with VSGs present in the databases. This data will also provide insight into whether antigenic repertoire has any constraints placed on it by hosts or geographical location. Furthermore, the data may provide novel targets for molecular diagnostics in the form of conserved motifs.

In order to elucidate the complex mechanism of antigenic variation, this study was carried out to examine the antigenic repertoire in experimentally infected goats. The study was conducted to investigate VSG switching in experimental goats infected with a local field strain. The data obtained will provide information on predominant VSGs and the hierarchal expression of VSGs.

Specific objectives

The specific objectives of the study are:

1. To genetically characterize the field isolates of *T. evansi* in Malaysia.
2. To determine the VSG expression diversity in *T. evansi* field isolates in the course of natural infection in domestic cattle, deer and buffalo.
3. To investigate the antigenic repertoire of *T. evansi* in experimentally infected goats.

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