UNIVERSITI PUTRA MALAYSIA

EPIDEMIOLOGY OF TRYPANOSOMA EVANSI INFECTION IN HORSES IN PENINSULAR MALAYSIA AND THE VARIANT SURFACE GLYCOPROTEIN OF THE ISOLATES

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By

ELSHAFIE IBRAHIM ELSHAFIE

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

To my father's soul, Ibrahim Elshafie Hassan, my mother, Asha Mustafa Gasmi-Albary, my wife, Nazik Ahmed, my beloved kids Ibrahim and Aahd, my sisters, Tammador and Marrwa, my brothers, Isam, Yasir, Shehab, Ammar, Amir and their families for their patience, constant encouragement and support.

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

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October 2011

Chairman: **Associate Professor Rehana Abdullah Sani, Ph.D.**

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There is no current data regarding the epidemiology of *Trypanosoma evansi* infection in horses in Peninsular Malaysia. Previous publications on *T. evansi* were on reports of the disease. Therefore, a series of complementary studies were carried out on the epidemiology of *T. evansi* in horses. A cross-sectional study was conducted in eight states of Peninsular Malaysia to determine the prevalence of *T. evansi* in horses. A total of 527 blood samples was obtained and examined by Haematocrit Centrifugation Technique (HCT), Giemsa-stained thin blood smears (GSS) and Polymerase Chain Reactions (PCR). The results showed an overall parasitological prevalence of 0.57% (3/527, CI: 1.6-0.19%) with both HCT and GSS. Morphometric study revealed the mean total length of

the trypanosomes including the free flagellum was 27.94 ± 2.63 µm. PCR successfully amplified a trypanosome specific 257 bp in 1.14% of samples (6/527, CI: 2.4-0.52%) and was confirmed by nucleotide sequences. The mean packed cell volume (PCV) for the positive cases detected by HCT was lower (23% ± 7.00) compared to the cases detected by PCR alone in Terengganu (35% ± 4.73).

The overall seroprevalence detected by Card Agglutination Test for *Trypanosoma evansi* (CATT/*T. evansi*) was 13.90% (73/527, CI: 11.2-17.1%). A questionnaire was designed to collect data on risk factors associated with *T. evansi* seroprevalence. Two risk factors inferred by binary logistic regression were horse breed and gender ($p < 0.05$). The mean PCV value between the seropositive and seronegative groups was not significant ($p > 0.05$). The majority of the horse owners were not familiar with surra (85.30%). However, most of them were understandably very cautious with the health of their animals.

It is generally assumed that local ponies are resistant to the disease caused by *T. evansi*. To test this assumption, an experimental study was carried out to assess the dynamics of *T. evansi* infection in Malaysian local ponies. Four local female

ponies aged between three to seven years old were injected with a local *T. evansi* isolate, while another two ponies served as negative control. Parasitaemia was first detected on the 4th day post infection. The main clinical findings in the infected animals were fever, weight loss, successive weakness, reduction of appetite and finally led to death of one pony out of the four infected. Haematological changes in the infected group showed a significant decline in the mean total erythrocyte counts, PCV, haemoglobin, thrombocytes and neutrophils, whereas there was an increase in monocytes ($p < 0.05$). Biochemical parameters showed a significant elevation in indirect bilirubin, creatinine, urea and globulin values, while glucose, albumin, albumin/globulin ratio, aspartate aminotransferase and creatine kinase declined among the infected horses ($p < 0.05$). These haematological and biochemical changes were similar to previous reports of *T. evansi* infections in various hosts. The high morbidity and mortality observed in this study proves that Malaysian local ponies are susceptible to *T. evansi* infection.

The *T. evansi* variant surface glycoproteins (VSG) repertoire and its dynamics of expression in the infected local ponies were assessed. Variant surface glycoprotein obtained from parasitaemia peaks of *T. evansi* were analyzed by amino acid sequences. *T. evansi* isolates from each pony expressed four or five distinctive VSGs throughout the infection period of 30 days. The primary

structure of the VSGs indicated a hypervariable region in all VSG N-terminal domains, whereas C-terminal domains were relatively conserved. Certain VSGs were expressed by individual animals either at the same time or in the neighbouring parasitaemia peaks. The VSG N-terminal domain is responsible for the characteristic waves of parasitaemia in *T. evansi* infection and four to five VSGs are expressed by individual ponies in the early stage of the infection.

In conclusion, this study showed *T. evansi* infection occurred in low frequency in horses in Peninsular Malaysia, and PCR is considered as a sensitive diagnostic tool. The good management systems adopted by horse owners are probably responsible for the low *T. evansi* occurrences. The disease seropositivity is associated with gender and breed of the horse. Local ponies are highly susceptible to *T. evansi* infection which is contrary to previous assumptions and the treatment regime applied was effective to rescue the remaining surviving animals.

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

EPIDEMIOLOGI JANGKITAN *TRYPANOSOMA EVANSI* PADA KUDA DI SEMENANJUNG MALAYSIA DAN GLIKOPROTEIN PERMUKAAN VARIAN DALAM PENCILAN

Oleh

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Maklumat tiada sehingga kini berkenaan epidemiologi jangkitan *Trypanosoma evansi* pada kuda di Semenanjung Malaysia. Penerbitan yang lepas mengenai *T. evansi* semua merupakan laporan penyakitnya. Oleh itu, beberapa siri kajian dijalankan berkenaan epidemiologi jangkitan *T. evansi* pada kuda. Kajian keratan rentas dijalankan di lapan negeri di Semenanjung Malaysia untuk menentukan prevalens *T. evansi* pada kuda. Keseluruhanya 527 sampel darah diperolehi dan diperiksa dengan 'Haematocrit Centrifuge (HCT)' teknik, calitan darah nipis Giemsa (GSS) dan 'Polymerase Chain Reaction (PCR)'. Keputusan menunjukkan keseluruhan prevalen parasit adalah 0.57% (3/527, CI: 1.6-1.9%)

dengan menggunakan HCT dan GSS. Kajian morphometrik mendedahkan panjang min untuk tripanosom termasuk bahagian flagellum adalah 27.94 ± 2.63 μm. PCR berjaya menghasilkan spesifik tripanosom 257 bp di dalam 1.14% (6/527, CI: 2.4-0.52%) dan pengesahan oleh turutan nukleotida. Min untuk 'packed cell volume' (PCV) bagi kes positif adalah lebih rendah di dalam HCT (23% ± 7) jika dibandingkan dengan hanya PCR di Terengganu (35% ± 4.73).

Keseluruhan seroprevalen yang dikesan oleh 'Card Agglutination Test for *Trypanosome evansi* (CATT/*T. evansi*)' adalah 13.90% (73/527, CI: 11.2-17.1%). Soal selidik telah direka untuk mengumpul data untuk faktor yang berisiko untuk seroprevalens positif *T. evansi.* Dua faktor berisiko disimpulkan oleh perduaan logistik regresi adalah baka dan jantina kuda ($p < 0.05$). Min PCV diantara kumpulan seropositif dan seronegatif adalah tidak tererti ($p > 0.05$). Kebanyakkan pemilik kuda tidak mengenali surra (85.30%). Akan tetapi, kebanyakkan mereka mengambil berat tentang kesihatan kuda mereka.

Tanggapan secara am adalah kuda poni tempatan rintang terhadap penyakit yang dibawa oleh *T. evansi*. Untuk menguji tanggapan tersebut, satu kajian eksperimen dijalankan untuk menilai tindak balas jangkitan *T. evansi* pada kuda poni tempatan. Empat ekor kuda berusia antara tiga sehingga tujuh tahun

disuntik dengan pencilan *T. evansi* tempatan manakala dua ekor kuda poni lain bertindak sebagai kawalan negatif. Parasitaemia telah dikenalpasti pada hari ke 4 selepas infeksi. Tanda-tanda klinikal yang utama untuk haiwan yang dijangkiti termasuk demam, kehilangan berat badan, lemah beransuran, penurunan selera makan dan akhirnya kematian berlaku pada satu daripada empat ekor kuda. Perubahan hematologi di dalam kumpulan yang dijangkiti menunjukkan penurunan yang tererti di dalam min jumlah sel darah merah, PCV, hemoglobin, thrombosit dan neutrophil, sedangkan monosit bertambah ($p < 0.05$). Parameter biokimia menunjukkan kenaikan yang tererti di dalam 'indirect bilirubin', kreatinin, urea dan nilai globulin, sementara glukosa, albumin, nisbah albumin/globulin, aspartate aminotransferase dan kreatinin kinase menurun dikalangan kuda yang. Perubahan hematologi dan biokimia tersebut ada persamaan dengan laporan infeksi *T. evansi* sebelum ini di dalam pelbagai hos. Ketinggian morbiditi dan kematian yang dilihat dalam kajian ini menunjukkan kuda poni tempatan Malaysia adalah mudah dijangkiti *T. evansi*.

Himpunan Varian Permukaan Glikoprotein (VSG) *T. evansi* dan ekspresi dynamiknya di dalam kuda poni tempatan dinilai. Varian permukaan Glikoprotein *T. evansi* yang diperolehi daripada puncak parasitemia *T. evansi* dianalisa mengikut turutan asid amino. Pencilan *T. evansi* daripada setiap kuda mengekspreskan empat atau lima VSGs yang berlainan sepanjang tempoh

jangkitan iaitu 30 hari. Struktur primari VSG menunjukkan kawasan hipervariasi di kesemua domain N-terminal VSG, manakala C-terminal domain adalah terpelihara. VSG tertentu diekspresskan oleh kuda secara individual samada secara serentak atau di puncak parasitemia yang berdekatan. VSG N-terminal domain adalah berperanan dalam ciri-ciri gelombang dalam parasitemia dalam jangkitan *T. evansi* dan empat hingga lima VSG diekspreskan oleh kuda poni secara individual di dalam peringkat awal jangkitan.

Kesimpulannya, kajian ini menunjukkan *T. evansi* berlaku di dalam frekuensi yang rendah pada kuda di Semenanjung Malaysia, dan PCR dianggap sebagai alat diagnostik yang sensitif. Sistem pengurusan yang baik yang diamalkan oleh pemilik kuda kemungkinan besar memainkan peranan dalam penurunan kejadian *T. evansi*. Seropositif penyakit adalah berkaitan dengan baka dan jantina kuda. Kuda poni tempatan adalah mudah dijangkiti *T. evansi* dan ianya berbeza dengan tanggapan sebelum ini dan rejim rawatan yang digunakan adalah berkesan untuk menyelamatkan haiwan-haiwan yang masih hidup.

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I certify that a Thesis Examination Committee has met on 21st October 2011 to conduct the final examination of Elshafie Ibrahim Elshafie on his thesis entitled "Epidemiology of *Trypanosoma evansi* Infection in Horses in Peninsular Malaysia and the Variant Surface Glycoprotein of the Isolates" 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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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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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.

ELSHAFIE IBRAHIM ELSHAFIE

Date: 21 October 2011

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LIST OF ABBREVIATIONS

µg	Microgram
AFLP	Amplified Restriction Fragment Length Polymorphism
AST	Aspartate Aminotransferase
BCT	Buffy Coat Technique
bp	base pair
CATT/*T. evansi*	Card Agglutination Test for *Trypanosoma evansi*
CFT	Complement Fixation Test
CK	Creatine Kinase
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
g	Centrifugal force
GSS	Giemsa-stained blood smear
Hb	Haemoglobin
HCT	Haematocrit Centrifugation Technique
IACUC	Institutional Animal Care and Use Committee
IFAT	Immunofluorescent Antibody Test
Ig	Immunoglobulin
kDNA	kinetoplast DNA
LAT	Latex Agglutination Test`
MAECT	Miniature Anion Exchange Centrifugation Technique
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MI	Mouse Inoculation

mRNA	messenger Ribonucleic acid
NCBI	National Centre for Biotechnology Information
PBSG	Phosphate Buffer Saline Glucose
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
RAPD	Random Amplified Polymorphic DNA
RBC	Red Blood Cell
RES	Reticulo-endothelial System
RFLP	Restriction Fragment Length Polymorphism
RoTat 1.2	Rode Trypanozoon Antigen Type 1.2
TFM	Trypanosome Freezing Mix
VAT	Variable Antigen Type
VSG	Variant Surface Glycoprotein
WBC	White Blood Cell
µl	Microliter
µm	Micron
µM	Micromolar

CHAPTER 1

GENERAL INTRODUCTION

Trypanosoma evansi (*T. evansi*) is a flagellated haemoprotozoa that causes a devastating disease called surra. It is the most prevalent pathogenic trypanosome throughout the tropical and subtropical areas of the world, owing to its ability to mechanical transmission by biting flies (Hoare, 1972). *T. evansi* has a large diversity of mammalian hosts, including wild life animals. The disease is endemic in all Southeast Asian countries, including Malaysia, and causes a significant constraint to livestock development.

Among the diagnostic methods for *T. evansi* are direct methods either to detect parasite antigen and DNA by serological and molecular techniques or active parasitaemia by direct parasitological examination of blood or buffy-coat and/or inoculation of the blood into rodents (Paris *et al.*, 1982; Nantulya, 1990; Desquesnes and Dávila, 2002). The indirect methods concentrate on the detection of anti-*T. evansi* antibodies using serological techniques (Claes *et al.*, 2005b). However, the sensitivity of parasitological techniques is often limited due to low parasitaemia in the chronically infected host (Nantulya, 1990). In addition, serological techniques for antibody detection cannot differentiate between past and current infections. Polymerase chain reaction introduced

recently is considered to be the most sensitive and reliable technique in detection of *T. evansi*, both in chronic and early infections (Desquesnes and Dávila, 2002).

Among horses, *T. evansi* causes disease characterized by anaemia, body weight loss, abortion and high mortality (Losos, 1980; Silva *et al.*, 1995a). However, the severity of the disease depends on the *T. evansi* strains of particular geographical location, and there is an assumption of enzootic stability in Latin America in horses (Herrera *et al.*, 2004). According to Luckins (1988), *T. evansi* found in the Southeast Asian countries is highly virulent and cause economic losses not only in horses but also other animal hosts.

Like many other diseases, surra is a multifactorial disease, and its occurrence is exaggerated by some predisposing risk factors. In endemic trypanosome area, a major concern is the introduction or importation of naive hosts into these areas. For example, severe surra developed in buffaloes imported from Australia into Indonesia (Luckins, 1988). Within endemic areas, certain factors may influence the prevalence in animals at risk such as breed, sex, environmental factors, stress and the abundance of fly vectors.

Trypanosomes have the capacity of antigenic variation, which is the basis of their ability to escape and evade the host immune response through variant surface glycoprotein (VSG); a mechanism which hinders work in vaccine production (Hutchinson *et al.*, 2007). However, most of the published work on VSGs described the African trypanosomes such as *T. brucei*, whereas *T. evansi* had received little attention in this aspect. The VSGs repertoire and dynamics expression are not fully understood in *T. evansi* populations.

The first case of *T. evansi* in Peninsular Malaysia was detected in an Australian mare in 1903 (Fraser and Symonds, 1909). Episodes of the infection and outbreak occur sporadically and affected many hosts namely, horses, cattle, buffaloes, pigs and deer (Ng and Vanselow, 1978; Abas Mazni and Zainal-Abidin, 1985; Sani *et al.*, 1995; Arunasalam *et al.*, 1995; Adrian *et al.*, 2010), whereas Malaysia is free from other forms of trypanosomiasis. Naturally infected ponies were used to study the dynamics of *T. evansi* infection (Ng and Vanselow, 1978; Ikede *et al.*, 1983). Since then, there have been no other studies on trypanosomiasis in horses in Malaysia. Anecdotal evidence suggests that local ponies in Malaysia are resistant to *T. evansi*. However, this remains to be proven.

With the increasing interest in equestrian activities, many horses were imported from non-endemic trypanosome countries such as Australia, New Zealand, United Kingdom and America (Bashir, 1993). Therefore, it is vital that the local situation on surra in horses in Malaysia is described. In addition, the risk factors associated with the disease prevalence have not been elucidated and the dynamics of *T. evansi* in local ponies, which is a potential source for cross breeding with exogenous horses, have not been examined. The current study was conducted to generate more information to address these gaps in knowledge for potential use in disease management programs.

1.1 Aims of the Research

The aims of this study are; first, to determine the prevalence of *T. evansi* and its associated risk factors in horses in Peninsular Malaysia using different diagnostic techniques, and second, to study the dynamics of *T. evansi* infection in Malaysian local pony with emphasis on VSGs repertoire and its trend of expression.

1.2 Research Hypothesis

The hypotheses of the current research are:

1. *T. evansi* prevalence in horses is low and PCR technique serves as a sensitive tool to detect the infection.
2. A number of risk factors are important in determining the occurrence of the pathogen in horses.
3. Malaysian local ponies can control parasitaemia and anaemia induced by *T. evansi* infection because they are resistant to the parasite.
4. The VSGs repertoire of *T. evansi* is small in a biological system such as local ponies and particular VSGs are expressed in early infections.

1.3 Specific Objectives

The specific objectives of the study are to:

1. determine the prevalence of *T. evansi* in horses in Peninsular Malaysia using parasitological and molecular techniques.
2. estimate the seroprevalence of *T. evansi* infection in horses and determine its association with some risk factors.
3. determine dynamics of *T. evansi* infection in the Malaysian local pony.

4. assess the VSGs repertoire in *T. evansi* isolated and its dynamics expression in infected local ponies.

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