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Active infection and morphometric study of *Trypanosoma evansi* among horses in Peninsula Malaysia

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Abstract. Apart from occasional reports of clinical disease affecting horses, there is no information about *Trypanosoma evansi* in horses in Peninsula Malaysia. Thus, a cross-sectional study was conducted in eight states in Peninsula Malaysia to determine the active presence of *T. evansi* in horses. A total of 527 blood samples were obtained and examined by haematocrit centrifugation technique (HCT), Giemsa-stained thin blood smear (GSS), morphometric measurements, polymerase chain reaction (PCR) and cloning of PCR products. 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 positive cases detected by PCR alone in the state of Terengganu (35% ± 4.73). In conclusion, this study showed *T. evansi* infection occurred in low frequency in horses in Peninsula Malaysia, and anaemia coincided with parasitaemic animals. PCR is considered as a sensitive diagnostic tool when parasitaemia is undetectable. The slight lengthier mean of parasite and anaemia may indicate a virulent strain of *T. evansi* circulating throughout the country. Thus, it's highly recommended to shed light on host-parasite relationship for better epidemiological understanding.

INTRODUCTION

Trypanosoma evansi is a haemoflagellate extracellular parasite causing a well-known disease termed surra. In Southeast Asia, *T. evansi* is the predominant pathogenic trypanosome, and its occurrence greatly influences livestock development (Luckins, 1988). The episode of *T. evansi* infection in horses in Peninsula Malaysia has been poorly reported. Even though *T. evansi* was first discovered in Malaysia in 1903, there are merely two available publications that demonstrated the parasite in horses (Ng & Vanselow, 1978; Ikede *et al.*, 1983). The parasite has been broadcasted in the country among different animal species, and the recent reports were in cattle and deer (Adrian *et al.*, 2010; Md-Isa, 2010). Therefore, it seems logical that horses would be affected by this parasite as horses are highly susceptible to the infection (Marques *et al.*, 2000). In addition, the equine industry is expanding in the country and horses are being imported from several countries known to be free from *T. evansi* (Bashir, 1993). Therefore these horses may serve as a susceptible pool of naive animals for the infection.

The diagnosis of *T. evansi* may combine two or more detection methods such as mouse inoculation (MI), HCT and GSS to increase the sensitivity in detection (Monzón *et al.*, 1990). In addition, morphometric measurements of *T. evansi* can enhance result obtained by GSS in mixed trypanosomes infections as morphological

dissimilarities between *T. evansi* strains appear to be indistinguishable, although in rare occasions may develop stumpy forms (Hoare, 1972). Meanwhile, introduction of PCR in detection of *T. evansi* both in early and chronic infections has improved epidemiological investigations of the disease (Sukhumsirichart *et al.*, 2000). Therefore, a cross-sectional study was conducted in eight out of the 11 states in Peninsula Malaysia to estimate the occurrence of *T. evansi* infection in horses using parasitological and molecular techniques.

MATERIALS AND METHODS

Target population and sampling method
A total of 527 blood samples were collected, each into an ethylenediaminetetraacetic acid (EDTA) tube, from 66 horse stables located in eight states in Peninsula Malaysia as previously mentioned by Elshafie *et al.* (2013). Positive samples were maintained by MI and parasite densities were estimated using a haematocytometer counting chamber, and then stored in liquid nitrogen.

Giemsa-stained thin blood smears
Thin blood smears were prepared from whole blood and stained with 10% Giemsa in phosphate-buffered saline (PBS), pH 7.2 for 30 min. Each stained smear was examined around the tail end of the smear under oil microscope objective (x1000).

Morphometric measurements
Biometrical measurements were conducted as described by Hoare (1972) using a light microscope (Nikon eclipse 80*i*, Japan) (x1000) connected to specific software (NIS-Elements D, version 3.0). Trypanosome measurements were made from 50 trypanosomes observed in the blood film of each positive sample. Measurements involved; PK: posterior end to kinetoplast, KN: kinetoplast to middle of nucleus, PN: posterior to middle of nucleus, NA: middle of nucleus to anterior end, FF: free flagellum, TL: total length, NL: nucleus length, NW: nucleus width, NI: nuclear index (PN/NA).

Haematocrit centrifugation technique and packed cell volume
The procedure was done as described by Woo (1970). In brief, each capillary tube was filled with approximately 70 µl of EDTA blood and centrifuged at 12,000 g for 5 min. The PCV was measured using a manual haematocrit reader. The capillary tube was then gently snapped at the junction between the lower buffy-coat and RBC layer using a diamond pen. Plasma and buffy-coat layer was evacuated onto a clean slide, covered by 18x18 mm cover slip and examined under a light microscope using x100 or x400 objective.

DNA extraction and concentration
Genomic DNA was extracted from each blood sample using commercially available QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. The concentrations and purity were estimated using DNA spectrophotometer (Bio-Rad Laboratories).

DNA amplification and visualization
The specific primer used to amplify *T. evansi* DNA, was derived from a trypanosome-specific repetitive nucleotide sequence fragment that amplified 257 bp (Sukhumsirichart *et al.*, 2000). The PCR amplification was performed using a thermal cycler (MyCycler™ thermal cycler, Bio-Rad Laboratories). Each reaction was carried out in a 25 µl volume containing 1X PCR buffer, 5 mM $MgCl_2$, 200 µM each of the four deoxynucleotides triphosphates, 0.5 µM of each primer, TR3 (5'-GCGCGGATTCTTT GCAGA CGA-3') and TR4 (5'-TGCAGACACT GGAATGTTACT-3'), 1 unit of *Taq* polymerase and 25 µg/ml of template genomic DNA. The PCR amplification was performed as follows; hotstart for 1 cycle at 90ºC for 7 min and then denatured at 90ºC for 30 sec, annealing at 60ºC for 30 sec, extension at 72ºC for 30 sec and a final extension cycle at 72ºC for 5 min. The PCR amplification was performed for 30 cycles. PCR amplicons were separated by a horizontal 1.5% agarose gel electrophoresis (120 V for 50 min), stained with ethidium bromide for 30 min and visualized under UV

transilluminator (GeneDoc™, Bio-Rad Laboratories).

Cloning of the PCR products

Cloning was conducted to confirm the results obtained by conventional PCR. All procedures were carried out using standard protocol according to the manufacturer's instructions. Briefly, the amplicons were excised from the agarose gel. The desired bands were purified using DNA purification kits (GeneAll®, Biofrontier Technology) and ligated into pJET 1.2 (CloneJET™ PCR cloning kit, Fermentas) cloning vector following the sticky-end cloning protocol at an insert/vector ratio of 3:1. The ligation mixtures were transformed into *Escherichia coli* (Top10 *E. coli*, Invitrogen) competent cells after treated with $CaCl_2$ using the heat-shock method at 42°C for 30 sec. After transformation, *E. coli* was poured onto LB agar plates contained ampicillin at a concentration of 100µg/ml and incubated at 37°C overnight. Recombinant clones were detected on bacteria colonies using TR3 and TR4 primer. Positive bacteria colonies were picked up and propagated into 10 ml LB broth contained ampicillin (100µg/ml) overnight at 37°C and 200 rpm (Zhicheng®, ZHWY-100B, China). Plasmid extraction was conducted using plasmid purification kits (GeneAll®, Biofrontier Technology) and plasmid concentration was determined by spectrophotometer (Bio-Rad Laboratories). Three plasmids for each isolate were sent for sequencing (Automated fluorescence DNA sequence, Medigene Sdn Bhd, Malaysia).

Data Analysis

The data obtained were analysed using SPSS version 18 (SPSS Inc. Chicago) at $\alpha = 0.05$ significance level. Cohen's kappa statistic was used to compare agreement between different diagnostic techniques. The mean total length of *T. evansi* was compared with a reference value obtained from the mean total length (24 µm) reported by Hoare (1972) using one-sample T test. The DNA sequences were edited using BioEdit version 7.0 (Hall, 1999) and multi-alignment was performed using ClustalW 1.83 incorporated in Geneious Pro 4.8 software (http://www.geneious.co). The homologues of the examined PCR amplicons were compared with available sequences at the NCBI database (http://www.ncbi.nlm.nih.gov/) by pairswise alignments.

RESULTS

Prevalence of *T. evansi* in Peninsula Malaysia

The overall prevalence based on parasitological methods and PCR was 0.57% (3/527, CI: 1.6-0.19%) and 1.14% (6/527, CI: 2.4-0.52%) respectively (Figure 1). The highest prevalence was recorded in Negeri Sembilan 13% (3/23) by HCT, GSS and PCR, while low prevalence was detected in Terengganu by PCR only at 2.67% (3/112). No parasite was detected in horses from the remaining states. Positive samples propagated in mice revealed about $1x10^6$ trypanosomes/ml three to four days post-infection.

The three isolates detected by HCT, GSS and PCR were from three horses in one stable in Negeri Sembilan, where the horses grazed with buffaloes and cattle and the stable is surrounded by a thick jungle. The other three isolates were detected by PCR in horses from different stables in Terengganu and these horses were not mixed with ruminants. The mean PCV values (±SD) for the three HCT positive cases in Negeri Sembilan and the three PCR positive horses from Terengganu

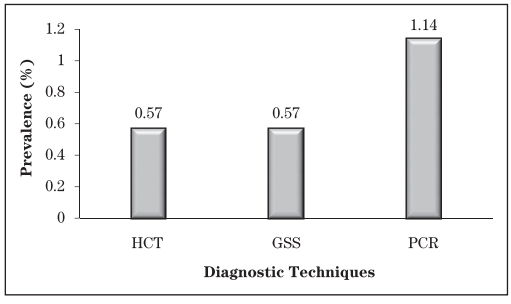

Figure 1. Detection of *T. evansi* (%) in West Malaysia using conventional parasitological and PCR techniques: HCT: Haematocrit centrifugation technique, GSS: Giemsa-stained thin blood smears, PCR: polymerase chain reactions

Table 1. Location of the *T. evansi* positive samples and its corresponding PCV and health status

Isolate ID	Location	HCT	GSS	PCR	PCV (%)	Health status
NS01	Linggi, Negeri Sembilan	√	√	√	20	Lethargic
NS02	Linggi, Negeri Sembilan	√	√	√	18	Apparently healthy
NS03	Linggi, Negeri Sembilan	√	√	√	31	Apparently healthy
TR04	Kuala Terengganu	-ve	-ve	√	34	Apparently healthy
TR05	Kuala Terengganu	-ve	-ve	√	41	Apparently healthy
TR06	Setiu, Terengganu	-ve	-ve	√	32	Apparently healthy

Table 2. *T. evansi* morphometric measurements

Measurements (µm)	Parameters								
	PK	KN	PN	NA	FF	TL	NL	NW	NI
N	150	150	150	150	150	150	150	150	150
Mean	1.46	7.66	9.50	7.30	11.28	27.94	2.55	1.24	1.32
SD	0.67	0.76	1.02	1.12	1.68	2.63	0.46	0.29	0.18

N: number of trypanosomes measured, PK: posterior end to kinetoplast, KN: kinetoplast to middle of nucleus, PN: posterior to middle of nucleus, NA: middle of nucleus to anterior end, FF: free flagellum, TL: total length, NL: nucleus length, NW: nucleus width, NI: nuclear index (PN/NA).

were 23%±7.00 and 35.67%±4.73 respectively (Table 1).

Cohen's kappa statistic showed very slight or no agreement between HCT and PCR ($\kappa = 0.011$, $p = 0.001$), whereas HCT and GSS indicated a perfect agreement ($\kappa = 1$, $p = 0.001$).

Morphometric measurements

The mean total length of *T. evansi* was significantly longer from the mean reference value of 24 µm (Hoare, 1972) ($t = 18.35$, $p = 0.001$). Only the slender form of *T. evansi* was observed with a mean nuclear index of 1.32 ± 0.18 µm (Table 2).

Identity of the PCR products with the available NCBI database

The consensus sequences which generated from the six isolates of *T. evansi* revealed a 257 bp and showed a high percentage of identity with the nucleotide fragment in *T. brucei* chromosome 1 (AL929603.2). The highest identity was 99%, whereas lowest was 96% and the average identity was 97.5%. The similarity between the six sequences using multi-alignment analysis was 97.4%.

DISCUSSION

The current study presents the first comprehensive investigation of *T. evansi* infection in horses in eight states of Peninsula Malaysia. Combining a highly sensitive detection technique (PCR) and conventional parasitological techniques (HCT and GSS), the overall active prevalence of *T. evansi* in horses was 0.57% (3/527, CI: 1.6-0.19%). PCR revealed a higher prevalence of 1.14% (6/527, CI: 2.4-0.52%) compared to HCT and GSS. Due to the limitations of different parasitological techniques in *T. evansi* detection, it is recommended to use a combination of two or more techniques to increase accuracy of diagnosis (Monzón *et al.*, 1990).

The HCT technique confirmed the trypanosome detected to be the causal agent of surra (*T. evansi*) rather than dourine (*Trypanosoma equiperdum*). The *T. equiperdum* is a tissue parasite and seldom appears in blood of infected horses (Clausen *et al.*, 2003; Claes *et al.*, 2005). In addition, MI of our isolates produced high parasitaemia whereas *T. equiperdum* lacks the ability of

propagating in mouse (Brun *et al*., 1998). *Trypanosoma brucei* was ruled out since the tsetse vector is restricted to a certain zone in sub-Saharan Africa (Hoare, 1972). Therefore, our results indicated *T. evansi* as the solely cause of trypanosomiasis in horses in Peninsula Malaysia. This result concurs with the previous findings which reported the absence of causative agents of dourine and nagana in horses in the country (Ng & Vanselow, 1978; Ikede *et al*., 1983).

The HCT prevalence obtained in horses in this study was relatively lower compared to cattle (2.1%) (Md-Isa, 2010). The reasons for the low prevalence in this study were probably due to the good management system, and the small number of horses owned in each stable, which facilitated quality management regarding feeds, husbandry and treatment as explained by Elshafie *et al*. (2013). The high prevalence that occurred in Negeri Sembilan (13%) may be attributed to the open grazing system practiced by the stable, which is located adjacent to the jungle and the horses grazed with buffaloes and cattle. The jungle environment serves as a natural habitat for the fly vectors of *T. evansi*. In southeast Asia, buffaloes and cattle are known to have subclinical infections with *T. evansi* thus serving as reservoir hosts (Luckins, 1988). Therefore, the environmental conditions accompanied with the presence of reservoir hosts in the stable in Negeri Sembilan may enhance the chance for those horses to be infected with *T. evansi*.

Hoare (1972) indicated that *T. evansi* exists as a monomorphic slender form with sporadic occasions of pleomorphism in different geographical locations and diverse mammalian hosts. He reported the morphometric total length of *T. evansi* ranged between 15 and 34 µm with an average length of 24 µm, and the nucleus positioned in the anterior part of the trypanosome with PN/NA > 1. In the present study, the range of total length including the free flagellum was 20.61 to 33.44 µm, whereas a significant increase in the mean total length was observed compared to the mean reference value (mean difference = 3.94 µm). Only the slender form of *T. evansi* was observed in this study, and the nucleus was located in the anterior half (1.32 ± 0.18 µm) of the cell. A variation in total length of *T. evansi* has been observed in rodents infected with strains originating from horses. This variation may be attributed to the adaption of the parasite from the horse to the new environment in the rodent (Dávila *et al*., 1998). In addition, increase in the mean length of *T. evansi* occurs commonly in equine strains in eastern parts of the world and is associated with high virulence (Gill, 1977). A study in India reported a significant variation in total length of *T. evansi* between buffalo and dog isolates (John *et al*., 1992). Distinct variation can be observed in the morphology of *T. evansi* found in the same animal species, therefore biometrical observations can provide only approximate diagnosis (Stephen, 1986; Tamarit *et al*., 2011). Thus, confirmation of the infection by molecular tools is necessary to overcome the diversity seen in morphological diagnosis.

The slight agreement between PCR and HCT ($\kappa = 0.011$) observed in this study is probably due to the highly sensitive nature of PCR in detection of *T. evansi*. PCR has been demonstrated to be a more sensitive technique to detect *T. evansi* infection either in early or chronic infections compared to traditional parasitological and serological techniques (Wuyts *et al*., 1995). The specific primer (Sukhumsirichart *et al*., 2000) used in this study, which can detect 1 pg of purified *T. evansi* genomic DNA, revealed six positive samples. Three of them were negative by HCT which may indicate an early or chronic infection with low parasitaemia that HCT was not able to detect. The high identity obtained by the nucleotide sequence of six *T. evansi* isolates obtained from this study and *T. brucei* obtained from the genomic database indicated that the primer target the trypanosomes DNA rather than that of other pathogens. It is not surprising that *T. evansi* sequences obtained in this study highly aligned with *T. brucei* sequence. This may be due to two factors: firstly, there are no *T. evansi* sequences available for that repetitive nucleotide sequence targeted in

this study at the NCBI database; Secondly, the full genome sequences of *T. brucei* which has been published, increased the chance of high identity with our isolates since *T. brucei* and *T. evansi* are indistinguishable from each other, and the only difference between them is the lack of maxicircle kDNA in *T. evansi* (Borst *et al.*, 1987; Berriman *et al.*, 2005).

It is noteworthy that HCT-positive horses in Negeri Sembilan had lower mean PCV value compared to infected horses detected only by PCR in Terengganu. It is likely that this occurrence is due to the destruction of red blood cells in parasitaemic horses, which indicated that the anaemia in the HCT-positive cases was due to prolonged instead of early or light infection. Our results agree with Herrera *et al.* (2004) who suggested that the absence of correlation in positive horses using serological and PCR techniques with low PCV values was due to low parasitaemia.

In conclusion, equine trypanosomiasis due to *T. evansi* is prevalent in horses in Peninsula Malaysia. In this study *T. evansi* is confirmed by both morphometric measurements and cloning of PCR products. Biometrical measurements indicated that *T. evansi* is monomorphic with a slight increase in the mean total length as compared to the mean reference value. PCR is highly sensitive compared to the conventional methods in the detection of *T. evansi* when parasitaemia is low and thus can improve the diagnosis of *T. evansi* infections. Anaemia is a common finding in *T. evansi* infection in horses with parasitaemia. In fact, the pathogenicity of *T. evansi* infection in horses in the country should be deliberated in details for better understanding of host-parasite relationship.

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