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COMPARISON BETWEEN MICROSCOPIC EXAMINATION AND COMPETITIVE ELISA FOR DIAGNOSIS OF EQUINE PIROPLASMOSIS IN KELANTAN, MALAYSIA

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ABSTRACT. The objectives of the present study were to determine the infection rate of equine piroplasmosis (EP) in horses and ponies in Kelantan, Malaysia and compare the microscopic examination with competitive enzyme-linked immunosorbent assay (cELISA) test as methods for diagnosis of EP. 306 blood samples were randomly collected from equids including 148 horses and 158 ponies in various districts of Kelantan, from September 2013 to March 2014. Based on microscopic examination of the staining blood smears, the infection rates of *Theileria equi, Babesia caballi* and of both infections in horses were 19.59%, 25% and 8.78% respectively, whereas in ponies the infection rates were 14.55%, 19.62%, and 5.69% respectively. Based on cELISA test, the infection rates of *T. equi, B. caballi* and of both infections in horses were 50.67%, 62.16% and 33.10% respectively, whereas in ponies, the infection rates were 51.89%, 63.92% and 35.44% respectively. No significant difference were observed between equids species associated with a seroprevalence of *T. equi*, *B. caballi* and of both infections ($P \leq 0.05$). According to the Kappa value there was no compatibility between microscopic examination and cELISA on the diagnosis of *T. equi, B. caballi* and of both infections which were 0.235, 0.013 and 0.080 respectively. In conclusion, the current results for this research work indicate that equine piroplasmosis is widespread in Kelantan, Malaysia and cELISA test is more efficient than microscopic examination for diagnosis of EP.

Keywords: equine piroplasmosis, microscopic examination, cELISA, horse, ponies, Kelantan.

INTRODUCTION

Equine piroplasmosis is a tick-borne protozoal disease of equids, caused by *Babesia caballi* and *Theileria equi* (previously *Babesia equi*) (Mehlhorn and Schein, 1998). The disease has a worldwide importance because the protozoa can be transmitted by carrier equids or infected ticks to originally piroplasmosis-free countries thus threatening the horse industry (Friedhoff *et al.,* 1990). The disease is a major problem to the international movement of equines and the causative agents of the disease are endemic in many tropical and subtropical areas of the world, as well as in temperate climatic zones (de Waal, 1992; Brüning, 1996; Hailat *et al.,* 1997). Ixodidae ticks of the genera Rhipicephalus, Hyalomma, Dermacentor and Boophilus are acting as vectors for *B. caballi*, *T. equi* and both parasites (USDA-APHIS, 2008; de Waal and van Heerden, 2004). The clinical manifestations of the disease is characterised by fever, depression, reduced appetite, icterus, anemia, hemoglobinemia, bilirubinuria and occasionally, death (Knowles, 1996).

EP was listed among the diseases of the world organisation for animal health OIE and reported as disease affecting horse industry. In Malaysia, the disease is still notifiable by the Department of Veterinary Services and the Veterinary Research Institute (VRI). There were no registered report in OIE documents for the disease (OIE, 2014). Only one article and one short communication, were published in Malaysia which concluded that the prevalence rate of EP is low in the east coast of Peninsular Malaysia (Chandarwathani *et al.,* 1998; Zawida *et al.,* 2010). The animal populations susceptible to infection are diversified in Malaysia as well as potential tick vectors (Mariana *et al.,* 2005). Furthermore, ponies, mules and donkeys are acting as natural reservoirs for transmission of infections to the horses (Radostitis *et al*., 2008).

The disease could be diagnosed by different methods including microscopic examination of Giemsa-stained blood smears, enzyme-linked immunosorbent assay (ELISAs) and polymerase chain reaction (PCR) (Friedhoff and Soule, 1996; Moretti *et al.,* 2010; Alsaad *et al.,* 2012). Studies of EP in Kelantan, Malaysia are scarce and little information had been provided, therefore, the aims of this work were to determine the infection rate of EP in horses and ponies and comparison between microscopic examination and cELISA test for diagnosis of EP in Kelantan, Malaysia.

MATERIAL AND METHODS

This study was conducted on 306 blood samples from equids including 148 horses and 158 ponies, which were randomly collected from different districts in Kelantan, using the Win Episcope 2.0 sampling program (Dohoo *et al*., 2010). The equids were sampled between September 2013 to March 2014. A whole blood sample has been used for microscopic examination and cELISA test were obtained via jugular

venipuncture placed in sterile vacutainer® tubes with and without anticoagulant ethylenediamine tetraacetic acid (EDTA). Thin and thick blood smears were prepared from the tube containing EDTA blood for each animal upon arrival at the laboratory, then fixed with absolute methanol for 3-5 minutes. After drying and staining with Giemsa's solution 5% (Azur-eosin-methylene blue solution, Merck Sdn. Bhd., Germany) for 30-35 minutes, they were examined microscopically (100×) to determine the presence of *T. equi* and *B. caballi* (Hendrix and Robinson, 2006). The serum were separated from tube anticoagulant-free blood by using centrifuge at 2500 rpm for 15 minutes and stored at -20 °C until used for cELISA test (Kouam *et al.,* 2010). Commercial c-ELISA kits (VMRD, Inc. Pullman, and WA99163 USA) were used for detection of *T. equi* and *B. caballi* antibodies in serum samples according to the manufacturer's instruction.

STATISTICAL ANALYSIS

Data analysis were done using IBM SPSS statistics 19 (SPSS Inc.) to compare microscopic examination and cELISA in diagnosing EP in Kelantan, based on Kappa values. Kappa ≥1 means high compatibility between the two tests. Whereas, Kappa ≤ 0 means no compatibility between the two tests. Two-sided Chi-square tests were used to analyse the difference between equid species associated with the infection rate. P values ≤ 0.05 was considered significant.

RESULTS

The results indicated that the infection rates of *T. equi, B. caballi* and of both infections in horses were 19.59%, 25% and 8.78% respectively. In ponies, the infection rates were 14.55%, 19.62% and 5.69% respectively based on microscopic examination (Table 1). The infection rate of *T. equi, B. caballi* and of both infections in horses were 50.67%, 62.16% and 33.10% respectively. In ponies, the infection rates were 51.89%, 63.92% and 35.44% respectively (Table 2). No

Table 1. The infection rate of EP (*T. equi, B. caballi* and both infections) in horses and ponies by microscopic examination of blood smears.

Equids species	Samples No.	*T. equi*			*B. caballi.*			Both infection		
		No. /P	No./N	P%	No. /P	No./N	P%	No. /P	No./N	P%
Horse	148	29	119	19.59	37	111	25	13	135	8.78
Pony	158	23	135	14.55	31	127	19.62	9	149	5.69
Total	306	52	254	16.99	68	278	22.22	22	284	7.18

No.= Samples Number N= Negative samples P= Positive samples

Table 2. The infection rates of EP (*T. equi, B. caballi* and of both infections) in horses and ponies by cELISA.

Equids species	Samples No.	*T. equi*			*B. caballi.*			Both infection		
		No. /P	No./N	P%	No. /P	No./N	P%	No. /P	No./N	P%
Horse	148	75	73	50.67	92	64	62.16	49	99	33.10
Pony	158	82	76	51.89	101	49	63.92	56	102	35.44
Total	306	157	149	51.30	193	113	63.07	105	201	34.31

No.= Samples Number N= Negative samples P= Positive samples

Table 3. Comparison between microscopic examination and cELISA based on kappa values for diagnosis of *B. caballi.*

		Microscopic examination		
		infected	Uninfected	Total No.
cELISA	Infected	44	**149***	193
	Uninfected	**24****	89	113
		68	238	306

* Mean false negative ** Mean false positive

Table 4. Comparison between microscopic examination and cELISA based on kappa values for diagnosis of *T.equi.*

		Microscopic examination		
		Infected	Uninfected	Total No.
cELISA	Infected	45	**112***	157
	Uninfected	**7****	142	149
		52	254	306

*Mean false negative ** Mean false positive

Table 5. Comparison between microscopic examination and cELISA based on kappa value for diagnosis of both infections.

		Microscopic examination		
		Infected	uninfected	Total No.
cELISA	Infected	12	**93***	105
	Uninfected	**10****	191	201
		22	284	306

* Mean false negative ** Mean false positive

significant difference between horses and ponies were associated with infection rates of *T. equi*, *B. caballi* and of both infections, based on cELISA test (Table 2). There was no compatibility between microscopic examination and cELISA based on overall infection rates of *T. equi*, *B. caballi* and of both infections in equids where Kappa values were 0.235, 0.013 and 0.080 respectively. This means that cELISA is highly efficient for diagnosis of *T. equi, B. caballi* and of both infections in equids (Tables 3, 4 and 5).

DISCUSSION

Piroplasmosis in equids is a very important disease because of its effect in international movements of horses in equine sports competitions, horse meat markets and for countries restricting the entrance of horses serologically positive for piroplasma species. For these reasons, specific and sensitive tests to detect EP infections are needed (Osman *et al.,* 2009). The results of the current study observed higher infection rate of causative agents of EP in the horses and ponies based on microscopic examination and cELISA test. These results disagree with Chandrawathani *et al.* (1998) which reported that the infection rates of *T. equi* and *B. caballi* in horses and ponies were 0% in Kelantan based on microscopic examination of only 91 samples. Furthermore, in Zawida *et al.* (2010), the infection rate of *T. equi* and *B. caballi* in horses were 20% and 1% respectively in 12 states of Malaysia based on cELISA tests on 180 serum samples. In other studies, in Greece, Kouam *et al.,* (2010) mentioned that the infection rates of EP in horses and ponies using cELISA test for *T. equi* were 9.2% and 28.6% respectively and 1.1% and 14.3% respectively for *B. caballi,* and 0.8% and 14.3% respectively for both infections; whereas using microscopic examination of blood smears, it was 0% for *T. equi* and *B. caballi*. In Spain, cELISA test was 50.3% for *T. equi*, 11.4% for *B. caballi* and 10.8% for both infections in the horses (Garcia-Bocanegra *et al.,* 2013). Furthermore, in Venezuela, the infection rates of *T. equi*, *B. caballi* and of both infections in the horses were 14.0%, 23.2% and 13.0% respectively by using the same test (Rosales *et al.,* 2013). The differences in the infection rates of EP in the different countries may be related to management practices, differences in the prevalence of tick vectors and climatic factors such as temperature, humidity and rainfall which influences the habitat of the ticks (Oncel *et al.,* 2007). With regards to equids. There was no significant difference between horses and ponies with respect to the seroprevalence of *T. equi* and *B. caballi* as well as both infections. This could be due to the fact that horses and ponies were reared together and exposed to the same environmental and management conditions. This is in agreement to studies by Kouam *et al.*, (2010). However, it was also noted by Kouam *et al.*, (2010) that due to the insignificant difference a larger sample can be tested to further prove this effect. Our study showed a low positive

infection rate for *T. equi*, *B. caballi* and of both infections using microscopic examination compared to cELISA test. According to Kappa values, cELISA test is more efficient than microscopic examination for diagnosis of EP, which is in agreement with Servinc *et al.,* (2008) and Moretti *et al.,* (2010). A visual detection of piroplasma in the erythrocytes of stained blood smears by microscopic examination is possible during an acute form of the disease, whereas during the subclinical latent stage, positive detection of parasitemia is very low (Schein, 1988; Zweygarth *et al.,* 2002). The c-ELISA test has been shown to be highly specific for each of the two species of piroplasmosis agents (OIE, 2010; Abdullah *et al.,* 2012). Researchers like Salim *et al.* (2008) and Servinc *et al.* (2008) proposed that the c-ELISA test be used as an alternative method of detection of acute and latent infections by piroplasms. In the current study, it was observed that EP was a widespread disease in Kelantan, which may be due to the high population size of equids in Kelantan, some of them imported from different countries where the disease was endemic such as Thailand (Adams, 2005). The immense distribution of ticks in Peninsular Malaysia play another important role in alleviating the infection rate of EP (Mariana *et al*., 2005, 2008).

CONCLUSIONS

The research work indicates that equine piroplasmosis is of high rate of spread in Kelantan, Malaysia. According to Kappa values, the cELISA test is more efficient for diagnosis of EP compared to the microscopic examination method. Although the microscopic method has a low sensitivity, its use is simple, easy and cheap compared to other techniques. However in the case of regular screening, it should be supplemented by other more accurate and sensitive tests like serological tests and molecular techniques.

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