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Seroprevalence of *Babesia caballi* and *Theileria equi* in horses and mules from Northern Thailand

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ABSTRACT

In the present study the seroprevalence of *Babesia caballi* and *Theileria equi*, the causative agents for equine piroplasmosis was investigated in Northern Thailand through a cross sectional survey carried out in December 2011. Two hundred forty equids sera from a complex of farms located in Chiang Mai Province were examined by enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and polymerase chain reaction (PCR). Overall seroprevalence of *T. equi* by ELISA and IFAT was 5.42% (13/240) and 8.75% (21/240) while that of *B. caballi* was 2.50% (6/240) and 5.00% (12/240), respectively. Molecular assay found *T. equi* infection in 3 mules (1.25%) while none of *B. caballi* was detected. The IFAT results showed that prevalence of *T. equi* was significantly higher in mules (10.73%) than in horses (3.17%), whereas prevalence of *B. caballi* was higher in horses (11.11%) than in mules (2.82%). Risk factor analysis based on IFAT results showed that occupation related to the host breed was significantly associated to equine piroplasmosis seropositivity ($p < 0.05$). Present results provide important information regarding the prevalence of equine piroplasmosis in horses and mules in the Northern region of Thailand, which should be beneficial for better prevention and control of this disease.

Keywords: *Babesia caballi*; ELISA; IFAT; PCR; *Theileria equi*

INTRODUCTION

Equine piroplasmosis (EP) is a tick-borne protozoal disease affects all members of the *Equus* genus (horses, donkeys, zebras and their cross breeds) (OIE, 2008). EP is found in most tropical, subtropical as well as temperate areas affecting many parts of Europe, Africa, Arabia and Asia (except Japan) (Brüning, 1996). The causative agents are two distinct apicomplexa protozoan parasites *Babesia caballi* and *Theileria* (previously designated as *Babesia*) *equi*. *Babesia caballi* and *T. equi* are transmitted by ticks of the genera *Dermacentor*, *Hyalomma* and *Rhipicephalus* (Ueti *et al*., 2008). The typical clinical features of EP characterized by fever, pale mucous membranes and icterus are generally seen in horses, yet some have nonspecific mild symptoms (Zobba *et al*., 2008). However, the active infections coincide with anemia and result in acute cases with death reports all of which makes EP an important disease, with a significant impact on international movement of horses (Knowles *et al*., 1996).

EP can be monitored by clinical diagnosis, serological tests and molecular diagnosis. Clinical diagnosis is based on observation of clinical signs and the microscopic examination, but clinical signs may be confused due to the variety of other conditions and microscopic examination obtained low sensitivity. The serological tests including complement fixation test (CFT), indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA), are more reliable and capable of detecting the antibodies against the parasites (Rothschild, 2013). IFAT is more sensitive than CFT but is time consuming and required large amount of antigens. Recently, ELISA has been widely used for surveillance and export certification (OIE, 2008). Despite, serological tests provide evidence of antibodies against the parasites but these tests do not distinguish between the past and current infection. Thus, molecular detection based on polymerase chain reaction (PCR) technique provide high sensitivity and specificity in detection of parasite DNA have been developed (Fahrimal *et al*., 1992; Figueroa *et al*., 1993) and become a useful tool for determination of current infection. Therefore, combination of molecular and serological detection for the infection provides powerful tools for accurate diagnosis as well as for epidemiological investigation (Terkawi *et al*., 2012).

Currently, approximately 10,000 equids are habited in Thailand (DLD, 2012), majority of them being racing horses and the others are donkeys and mules. However, few EP epidemiological studies have been conducted. Therefore, this study aimed to measure the seroprevalence of EP in horses and mules from the largest farm in the northern region of Thailand based on ELISA and IFAT, the current infection was monitored using PCR. Risk factors associated to EP have also been evaluated.

MATERIALS AND METHODS

A total of 240 blood samples were collected during December 2011 from 63 horses and 177 mules in complex farm of Chiang Mai province, the largest equids farm in northern region of Thailand. The packed cell volume (PCV), sex, age, breed and occupation of sampled animals were also recorded. Sexes of animals consist of stallion and mare. Age was categorized as followed; 1-5 years (young), 6-12 years (adult) and >12 years (old). Breeds of animals contained mule and horse. Occupations of animal consist of riding animals and draft animals. PCV were grouped as followed; PCV < 30% (low), 31% < PCV < 48% (normal) and PCV > 48% (high) (http://www.merckmanuals.com). Giemsa's stained blood smear was prepared and screened by microscopic examination. Serum samples from horses experimentally infected with *B. caballi* or *T. equi* and non-infected were used as positive and negative control in serological tests. All serum samples were tested in duplicate by ELISA along with positive and negative controls. The C-terminal antigen derived from *B. caballi* 48-kDa rhoptry protein (BC48/CT) and the *T. equi* truncated merozoite antigen 30-kDa (EMA-2t) expressed as glutathione S-transferase (GST) fusion recombinant proteins were used as antigen. They were produced according to the methods described by Terkawi *et al*., 2012 and Kumar *et al*., 2004, respectively. ELISA was conducted as previously described (Terkawi *et al*., 2012). In order to justify the results, Relative Percentage Positivity (RPP) was calculated as previously described (Desquesnes *et al*., 2009). Cut-off value (COV) of *B. caballi* and *T. equi* were calculated by mean RPP + 2SD (standard deviation) on the basis of 100 presumably negative samples. The COV were 26% and 27% for *B. caballi* and *T. equi*, respectively. IFAT slides were prepared from cultured *B. caballi* and *T. equi*, and used to test all serum samples as previously described (Avarzed *et al*., 1997). Genomic DNA was extracted from blood samples using Qiagen DNA extraction kit (Qiagen, USA). The PCR for

detection of *Theileria* and *Babesia* was conducted using primers described previously with some modifications (Gubbels *et al*., 1999). The PCR product (460 bp and 520 bp for *Theileria* and *Babesia*, respectively) was purified and subsequently sequenced using Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA). DNA sequences were determined using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). Obtained sequences were analyzed using GenBank BLASTn and subsequently submitted in the GenBank (www.ncbi.nlm.nit.gov).

The proportion of agreement was calculated (Thrusfield, 2005) based on the number of agreement observed from ELISA and IFAT to evaluate their concordance. The overall prevalence by IFAT, ELISA and PCR were calculated using Epi Info Version 7.1 (Center for diseases control and prevention, USA). The chi-square test (χ^2) was used to evaluate differences ($p < 0.05$) of seroprevalences among animals on the basis of breed, sex, age groups, occupation and PCV groups.

RESULTS

The overall seroprevalences by ELISA were 2.50% (6/240) and 5.42% (13/240) for *B. caballi* and *T. equi*, respectively. The overall seroprevalences by IFAT were 5.00% (12/240) and 8.75% (21/240) for *B. caballi* and *T. equi*, respectively (Table 1). No mixed infection was detected. The overall seroprevalence of *T. equi* and *B. caballi* detected by IFAT (8.75% and 5.00%) were higher than ELISA results (5.42% and 2.50%) (Table 1). Observation of parasite under light microscope, one mule was positive (Data not shown). Detection of parasites DNA by PCR targeting 18S rRNA for piroplasm showed that, 3 mules out of 240 equids were positive for *T. equi* (GenBank: KF732846, KF732847), but none of *B. caballi* were detected (Table 1). The combination of ELISA/IFAT showed that 25 (10.5%) and 18 (7.5%) animals carried *T. equi* and *B. caballi* IgG antibodies. The proportion of agreement between IFAT and ELISA were calculated based on the frequencies of positive and negative sample of both tests and showed by 89.58% (215/240) and 92.50% (222/240) for *T. equi* and *B. caballi*, respectively (Table 2). In fact, the whole parasites were used as antigen for IFAT which may produce nonspecific reaction and consequently showed higher seropositive than ELISA. Meanwhile, recombinant antigen based ELISA used in this study provided higher specificity compared to IFAT. For these reasons, disagreement of two tests has been observed.

The risk factors analysis showed statistically significant difference of *B. caballi* seroprevalence on the basis of animal breeds and occupation. There was significantly higher seroprevalence for *B. caballi* in riding horses than draft mules ($p < 0.05$), conversely there was higher seroprevalence for *T. equi* in draft mules than riding horses ($p > 0.05$) (Table 3). Older equids depicted a *T. equi* seroprevalence higher than young and adult equids ($p < 0.05$) (Table 3). No statistically significant differences of seroprevalence for *T. equi* and *B. caballi* among stallion/ mare and PCV groups ($p > 0.05$).

Table 1. Prevalence of *Theileria equi* and *Babesia caballi* in horses and mules from Northern Thailand by ELISA, IFAT and PCR.

Breeds	N	*Theileria equi*			*Babesia caballi*		
		ELISA	IFAT	PCR	ELISA	IFAT	PCR
		n (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)	*n* (%)
Horses	63	0 (0)	2 (3.17)	0 (0)	1 (1.58)	7 (11.11)	0 (0)
Mules	177	13 (7.34)	19 (10.73)	3 (1.69)	6 (3.39)	5 (2.82)	0 (0)
Overall	240	13 (5.42)	21 (8.75)	3 (1.25)	6 (2.50)	12 (5.00)	0 (0)

N; Number of examined

n; Number of positive

Table 2. Detection of *T. equi* and *B. caballi* infections by ELISA and IFAT in equids.

	ELISA[a]		IFAT[b]		ELISA/IFAT[c]	
			(+)	(-)		
Theileria equi	(+)	13	9	4	(+)	25 (10.5%)
	(-)	227	12	215	(-)	215 (89.5%)
	Total	240	21	219	Total	240
Babesia caballi	(+)	6	0	6	(+)	18 (7.5%)
	(-)	234	12	222	(-)	222 (92.5%)
	Total	240	12	228	Total	240

[a] The frequencies of positive and negative samples as results of ELISA.

[b] The frequencies of positive and negative samples as results of IFAT cross-tabulated with ELISA results.

[c] The frequencies of positive and negative samples of combined ELISA/IFAT results.

A sample is positive for the ELISA/IFAT when it is positive for either or both of the tests and is negative when it is negative for the two tests.

Table 3. Analysis of risk factors associated for equine piroplasmosis in northern Thailand.

Risk factors	N	*Theileria equi*			*Babesia caballi*		
		n (%)	χ^2, df	*p* value	*n* (%)	χ^2, df	*p* value
Breed			3.325, 1	0.0682		6.716, 1	0.0095*
Horses	63	2 (3.17)			7 (11.11)		
Mules	177	19 (10.73)			5 (2.82)		
Occupation			3.326, 1	0.0682		6.716, 1	0.0096*
Draft	177	19 (10.73)			5 (2.82)		
Riding	63	2 (3.17)			7 (11.11)		
Age groups			38.982, 2	0.0000*		0.630, 2	0.7292
1-5 years (young)	79	2 (2.53)			2 (2.53)		
6-12 years (adult)	97	4 (4.12)			3 (3.09)		
>12 years (old)	64	15 (23.44)			7 (10.84)		
Sex			0.071, 1	0.7892		0.4393, 1	0.5074
Stallion	142	13 (9.15)			6 (5.26)		
Mare	98	8 (8.16)			6 (6.13)		
PCV groups			-	-		-	-
Low	114	9 (7.89)			6 (5.26)		
Normal	125	12 (9.60)			6 (4.80)		
High	1	0 (0)			0 (0)		

χ^2; chi square test

df; degree of freedom

p; Probability value

*; Statistical significant differences ($P < 0.05$)

DISCUSSION

In Thailand, few studies have been carried out on EP diagnosis even though it is an important disease affecting horse trading and movement. Here we first reported the combination of ELISA, IFAT and PCR detection and analyzed risk factors of EP in Thailand. Even though, seroprevalence from our study is slightly lower than previously reported in Thailand (Tantaswadi *et al*., 1998; Chungvipat and Viseshakul, 2005). This can be due to different diagnostic method, sample size and the geographical area investigated. Thus, continually monitoring and control of EP in this region might sooner help to eliminate this disease. Our results also showed that seroprevalence with IFAT were higher than with ELISA for both parasites. This may be due to higher sensitivity of IFAT while ELISA using recombinant antigen bring a higher specificity (OIE, 2008; Salim *et al*., 2009). On the other hand, the predominant *T. equi* versus *B. caballi* seropositivity was found both with ELISA and IFAT. This is in agreement with previous studies (Frerichs *et al*., 1969; Abdoon, 1984; Boldbaatar *et al*., 2005; Salim *et al*., 2008; Kouam *et al*., 2010). Indeed, EP caused by *T. equi* is more pathogenic (Zaugg and Lane, 1992), establishes a persistent infection (Kouam *et al*., 2010) and is usually predominant in equid populations. Therefore, the treatment with diminazene aceturate does not completely cure infected animals (Brüning 1996; de Waal and van Heerden, 1994). For these reasons, seroprevalence of *T. equi* was higher than *B. caballi*.

In fact, the farmer tended to use mule as draft animals while horses were used for riding. Therefore, breed is a confounding factor associated with EP in this study. Risk factors analysis indicated that occupation related to the host breed showed to be associated for *B. caballi* seropositivity ($p < 0.05$). This observation may due to the facts that *B. caballi* always showed low parasitemia and is difficult to detect by microscopic examination (the common diagnostic method used in this farm) as also confirmed in this study. Thus, misdiagnose may result in the absence of treatment of animals which become apparently healthy carriers. Conversely, our result showed higher seroprevalence for *T. equi* in draft mules than riding horses. The draft animals were generally working in the border area with Myanmar for 2-3 years before coming back to the farm. This probably increased the opportunity for tick infestation resulting in higher seroprevalence of *T. equi* (Boldbaatar *et al*., 2005) in draft animals than riding animals. Since *T. equi* infection appears to be associated with cattle, the principal host of *Boophilus microplus* (Heuchert *et al*., 1999), which is widespread in Thailand (Tanskul *et al*., 1983; Ahantarig *et al*., 2008). The higher seroprevalence of *T. equi* in mules than horses might result from the close contact of mules with cattle (Barbosa *et al*., 1995) while they were used as draft in border area, this may increase the risk of tick infestation. However, the study on tick infestation in equids of Thailand is required. Moreover, old animals showed higher seroprevalence than young animals which depicted that old animals were more exposed to both *B. caballi* and *T. equi* infection than younger animals. This result was concordant with previous studies (Kouam *et al*., 2010). Infection with equine piroplasms has been reported as the important pathogen responsible for red urine among equids, with various symptoms from acute to subclinical signs especially in horses newly introduced into endemic area. Our results showed that equids exposed *T. equi* and *B. caballi* in this area do not show much clinical signs. Moreover, low prevalence has been reported but EP still exists in the region. Thus, the possibility of acute infection in newly equid imported to this area may occur. This study provided the most recent situation of EP in northern Thailand and suggests that introducing of the new equid to this area might be at risk of active infection. However, studies on molecular epidemiology, geographical distribution and risk factor of EP need to be investigated for the whole country, to allow better control of EP in Thailand.

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