MOLECULAR PREVALENCE AND SPECIES CO-INFECTION OF BOVINE HAEMOPARASITES IN PENINSULAR MALAYSIA

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ABSTRACT. Bovine haemoparasites are cosmopolitan in distribution and are known to cause substantial losses to the cattle industry. In spite of their economic importance, there remains a dearth of information on their molecular epidemiology in many parts of the world including Malaysia. To ascertain the molecular prevalence and species co-infection of bovine haemoparasites in the country, blood samples were collected from 1,045 heads of beef and dairy cattle on 43 farms from six geographical zones throughout Peninsular Malaysia. Samples subjected to PCR amplification of parasite species-specific genetic fragments revealed that *Anaplasma marginale* was the most prevalent haemoparasite (72.6%), followed by *Theileria orientalis* (49.8%), *Candidatus Mycoplasma haemobos* (47.0%), *Babesia bovis* (32.5%), *Babesia bigemina* (30.5%) and *Trypanosoma evansi* (17.9%). A high percentage (92.1%) of cattle was infected with either one or more haemoparasites. Triple haemoparasite species co-infection was the most prevalent (25.6%), followed closely by double species co-infection (25.1%). The most common (8.8%) and significantly corellated ($r_s = 0.250$; $p<0.01$) combination was *A. marginale* + *T. orientalis*. The present study constitutes the first attempt in the country to document the molecular prevalence and species co-infection of bovine haemoparasites over a wide spatial distribution. The data obtained will facilitate treatment, control and prevention measures to improve the local cattle industry.

Keywords: bovine haemoparasites, Peninsular Malaysia, *Anaplasma marginale, Theileria orientalis, Candidatus Mycoplasma haemobos, Babesia bovis, Babesia bigemina*, *Trypanosoma evansi*

INTRODUCTION

The domestication and farming of livestock present many challenges including prevention and control of diseases that could affect productivity. Haemoparasites are among the pathogens of concern for successful livestock farming throughout the world (Jongejan and Uilenberg, 2004; Sivakumar *et al*., 2012). In Malaysia, various genera of haemopathogens namely *Anaplasma, Babesia, Mycoplasma, Theileria* and *Trypanosoma* have been documented to exert negative impacts on cattle (Lee and Whitten, 1982; Chin, 2007; Nur Mahiza 2010; Rahman *et al*., 2012). Infection with these haemoparasites are often sub-clinical,

leading to a large pool of asymptomatic carriers that act as a source of infection for ticks and other blood sucking insect vectors (Bell-Sakyi *et al*., 2004). The hot and humid tropical climate of Malaysia encourages the breeding and survival of various arthropod vectors that are incriminated in the transmission of these haemoparasites (Saharee and Fatimah, 1993). In the attempt to boost the cattle industry and to attain self-sufficiency in beef and dairy products, various breeds of cattle from Europe, Australia and South America have been imported into the country since the 1970s. This has potentially led to the importation and expansion of parasites since studies have shown that haemoparasites are more prevalent in imported breeds of cattle compared to the local Kedah-Kelantan breed and their crosses (Fadzil and Ragavan, 1986; Chin, 2007; Nur Mahiza, 2010; Rahman *et al*., 2012; Haron *et al*., 2015).

In the past numerous studies have been carried out on the microscopy and serological detection of bovine haemoparasites in Malaysia (Rajamanickam, 1970; Lee and Whiten, 1982; Salleh, 1984; Fadzil and Ragavan, 1986; Kamio *et al*., 1990; Lee *et al*., 1991; Chandrawathani *et al*., 1993, 1994, 1998, 2014; Sani *et al*., 1995; Sharifah, 2001; Chin, 2007; Nur Mahiza, 2010; Rahman *et al*., 2010, 2012; Pong and Nik Him, 2012; Nik Him *et al*., 2013; Haron *et al*., 2015). However, apart from a single study on the molecular detection of *Anaplasma* (Tay *et al*., 2014), there remains a paucity of information on the molecular prevalence, species diversity and epidemiology of these pathogens in the country. With the increasing consumer demands and the need to attain self-sufficiency in beef and dairy products locally, it is vital that all aspects of cattle farming and production should be optimized. This includes the reduction and control of haemoparasitic diseases. The present investigation was therefore undertaken to provide current information on the occurrence of cattle haemoparasites in Peninsular Malaysia, utilising molecular detection methods to determine the species diversity, prevalence and co-infection on beef and dairy cattle farms throughout the country.

MATERIALS AND METHODS

The study was conducted on 43 cattle farms over 33 locations throughout Peninsular Malaysia (Table 1), comprising 23 beef farms, 16 dairy farms and four mixed beef-dairy farms. A total of 1045 cattle were sampled including 379 dairy and 666 beef cattle. Sampling was carried out following a cross sectional study design and animals were recruited from each farm by convenience sampling. Approximately 5ml of blood was collected from each animal *via* jugular or coccygeal venipuncture and placed in ethylenediaminetetraacetic acid (EDTA) coated blood collection tubes. Samples were transported on ice to the Parasitology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia for storage at -80 °C and subsequent molecular detection. The farm coordinates were loaded into a geo-database (ArcGIS 9.1TM, ESRI, Redlands, CA, USA) for mapping and spatial analyses.

Deoxyribonucleic acid (DNA) was extracted from frozen (-80 °C) whole blood using the Qiagen DNeasy® Blood and Tissue

Table 1. Distribution of cattle farms in Peninsular Malaysia sampled for the prevalence of bovine haemoparasites. Beef farms (b), dairy farms (d), mixed beef-dairy farms (bd).

Zones and sampling locations	No. farms	No. cattle (%)
North Bukit Mertajam, Pulau Pinang, Perlis, Kuala Ketil, Sik	6 (3b, 3d)	105 (10.0)
Northwest Kinta, Kampar, Ulu Bernam, Gopeng, Air Papan, Raub, Cameron Highlands	8 (5b, 3d)	220 (21.1)
Northeast Gua Musang, Kuala Krai, Pasir Puteh, Permaisuri, Hulu Terengganu	6 (3b, 2d, 1bd)	176 (16.8)
Southwest Serdang, Dengkil, Alor Gajah, Giadek, Jelebu, Temerloh	9 (4b, 3d, 2bd)	186 (17.8)
Southeast Jerantut, Rompin, Pekan, Dungun, Kemaman	5 3b, 1d, 1bd)	186 (17.8)
South Muar, Labis, Mersing, Pontian, Kota Tinggi	9 (5b, 4d)	172 (16.5)
Total	43 (23b, 16d, 4bd)	1045 (100.0)

kit according to the manufacturers' protocol. PCR amplification was carried out to amplify nuclear and ribosomal gene fragments of the various haemoparasites using species-specific primer sets and thermocyclic profiles (Table 2). PCR was carried out in a 25 µl reaction comprising 100-150 ng genomic DNA, 1x reaction buffer (Green Go Tag Flexi Buffer, Promega Madison, USA), 1mM of each deoxynucleoside triphosphate (Promega), 5mM $MgCl_2$ (Promega Madison, USA), 1mM of each primer, and 0.5 units of Taq DNA polymerase (Promega Madison, USA). Negative controls (template DNA substituted with sterile Type-1^+ purified water) and positive controls (positive blood samples confirmed by microscopy and sequencing of the PCR amplicons) were incorporated into each PCR run. Amplification was done using the MyCycler™ (Bio-Rad Laboratories, USA) thermal cycler. Amplicons were electrophoresed on a 2% agarose gel (Vivantis, USA) at 90 V with TAE (Tris-acetic acid-EDTA) buffer, stained with ethidium bromide, and viewed under a UV transilluminator (GeneDoc™, Bio-Rad Laboratories). Images were captured using a digital camera and computer software (GeneSnap™, Bio-Rad Laboratories). In order to prevent cross-contamination, work areas were designated solely for DNA extraction, PCR reagent preparation, and PCR amplification. In addition, reagent preparation was done in a dedicated biosafety cabinet which was UV illuminated at the end of each session to avoid DNA crossover and contamination. Representative amplicons from each parasite species were gel-extracted and purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturers' protocol. The amplified products were

Table 2. Species-specific primers, thermocyclic profiles and expected amplicon size of the various gene fragments used for the detection and PCR amplification of bovine haemoparasites in Peninsular Malaysia. Thermocyclic profiles include initial denaturing (ID), denaturing (D), annealing (A), extension (E), and final extension (FE).

Parasite	Primer sequence (5'-3') Forward (F) and Reverse (R)	Gene	Thermocyclic profile	Amplicon Size (bp)	Reference
Anaplasma marginale	F- CATCTCCCATGAGTCACGAAGTGGC R- GCTGAACAGGAATCTTGCTCCAAG	MSP4	ID: 95°C /5mins D: 95°C /1min A: 65°C /2mins E: 72°C /1min No of cycles: 40 FE: 72°C /10mins	761	Shkap *et al.* (2008)
Babesia bigemina	F- TACTGTGACGAGGACGGATC R- CCTCAAAAGCAGATTCGAGT	AMA-1	ID: 95°C /5mins D: 95°C /30secs A: 59°C /1min E: 72°C /1min No of cycles: 40 FE: 72°C /10mins	211	Sivakumar *et al.* (2012)
Babesia bovis	F- CAAGCATACAACCAGGTGG R- ACCCCAGGCACATCCAGCTA	VESA 1α	ID: 95°C /4mins D: 94°C /50secs A: 62°C /40secs E: 72°C /1min No of cycles: 40 FE: 72°C /10mins	166	Bilgic *et al.* (2013)
"Candidatus Mycoplasma haemobos"	GAGTTAGTTATTAAAGCTTTAT ATTCATGAGGTACTATCAGTTG	16SrRNA	ID: 94°C /5mins D: 94°C /30secs A: 55°C /30secs E: 72°C /30secs No of cycles: 40 FE: 72°C /7mins	279	Su *et al.* (2010)
Theileria orientalis	CTTTGCCTAGGATACTTCCT ACGGCAAGTGGTGAGAACT	MPSP	ID: 94°C /4mins D: 94°C /1min A: 63°C /1min E: 72°C /1min No of cycles: 40 FE: 72°C /7mins	776	Ota *et al.* (2009)
Trypanosoma evansi	GCGGGGTGTTTAAAGCAATA ATTAGTGCTGCGTGTGTTCG	RoTat 1.2	ID: 94°C /4mins D: 94°C /1min A: 59°C /1min E: 72°C /1min No of cycles: 40 FE: 72°C /5mins	205	Urakawa *et al.* (2001)

Table 3. Spearman's correlation coefficient (r_s) for the co-occurrence of haemoparasites infecting cattle in Peninsular Malaysia.

	Ama	*Tor*	*CMh*	*Bbg*	*Bbo*	*Tev*
Anaplasma marginale (*Ama*)	1.000					
Theileria orientalis (*Tor*)	0.250**	1.000				
C. Mycoplasma haemobos (*CMh*)	0.178**	0.083**	1.000			
Babesia bigemina (*Bbg*)	0.053	0.022	0.171**	1.000		
Babesia bovis (*Bbo*)	0.133**	0.065*	0.193**	0.125**	1.000	
Trypanosoma evansi (*Tev*)	0.063*	0.065*	0.021	0.021	0.011	1.000

** Correlation is significant at the 0.01 level (2-tailed). * Correlation is significant at the 0.05 level (2-tailed).

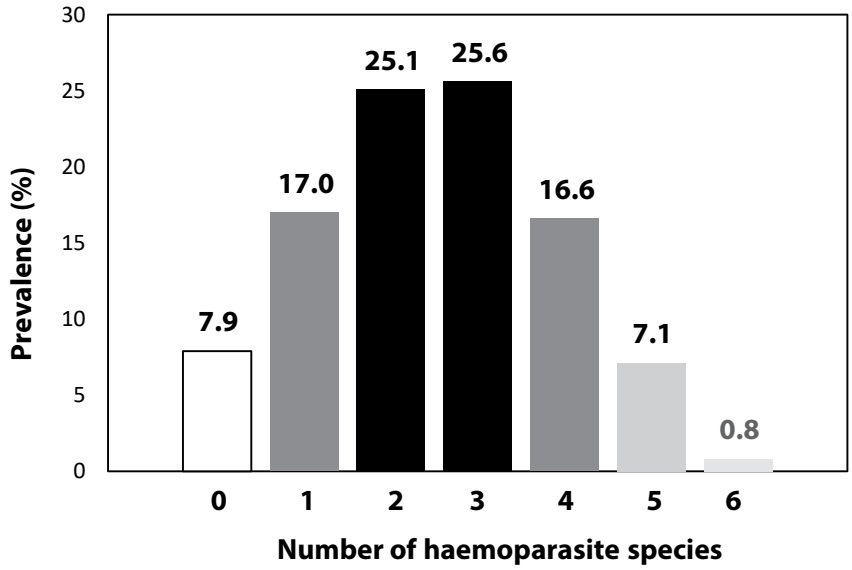

Figure 1. Prevalence (%) of haemoparasites co-infection among cattle in Peninsular Malaysia. Triple species co-infection recorded the highest prevalence, followed closely by double species co-infection.

sequenced using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). In order to facilitate identification of the haemoparasites, representative sequences of each parasite was then compared with known parasite gene fragments curated by the National Center for Biotechnology Information (NCBI) GenBank using the Basic Local Alignment Search Tool (BLAST).

Data were analysed using the Statistical Package for Social Sciences version 22.0 (SPSS Inc. Chicago, Illinois). The prevalence of each of parasite species and the occurrence of co-infection were statistically analyzed using the Chi Square test (univariable model) for discrete variables at a 95% confidence interval at $P<0.05$. Spearman's correlation was used to measure the strength of association for species co-infection.

RESULTS

A total of six species of haemoparasites were detected by PCR from the 1,045 cattle examined. These include *Anaplasma marginale, Babesia bigemina, Babesia bovis, Candidatus Mycoplasma haemobos, Theileria orientalis* and *Trypanosoma evansi*. *Anaplasma marginale* was the most prevalent haemoparasite, detected in 72.6% of the cattle. High infection rates were also apparent for *T. orientalis* (49.8%), "*C. M. haemobos*" (47.0%), *B. bovis* (32.5%) and *B. bigemina* (30.5%). The least prevalent haemoparasite was *T. evansi,* where 17.9% of the cattle were infected. All farms harbored cattle that were infected with these haemoparasite species with the exception of a beef farm in Kota Tinggi which was free from *T. orientalis* and *T. evansi*. *Anaplasma marginale* was the predominant parasite infecting cattle in a majority of the farms, while infection with *T. evansi* was consistently low across the sampling sites.

Analysis of haemoparasite species single and co-infections among cattle in Peninsular Malaysia revealed that *A. marginale* was the most prevalent single species infection (7.5%), while sole infections with *T. evansi* was the least common (1.1%). *Anaplasma marginale* + *T. orientalis* was the most prevalent form of double species infection (8.8%), whereas *B. bovis* + *T. evansi* was the least encountered (0.1%). The most prevalent form of triple haemoparasites infections was the *A. marginale* + *T. orientalis* + *C. M. haemobos* combination (6.6%). The *B. bigemina* + *B. bovis* + *T. evansi* was the least prevalent combination in its category (0.1%). In the four parasites co-infection grouping, *A. marginale* + *T. orientalis* + *C. M. haemobos* + *B. bovis* combination was the most prevalent (4.4%). In the five parasites co-infection category, *A. marginale* + *T. orientalis* + *C. M. haemobos* + *B. bigemina* + *B. bovis* combination was the most prevalent (4.0%). The spread of haemoparasite co-infection assumed as normal distribution curve (Figure 1). Eight (0.8%) animals were concurrently infected with all six haemoparasites species, while 83 (7.9%) were free from infection. The dominant co-infection categories were triple (25.6%) and double (25.1%) species co-infections. The Spearman's correlation coefficient for haemoparasite species co-occurrence (Table 3) revealed a significant association between a number of species. High correlations ($p<0.01$) were observed

between *A. marginale* and *T. orientalis* (r_s = 0.250), *C. M. haemobos* and *B. bovis* (r_s = 0.193), *A. marginale* and *C. M. haemobos* (r_s = 0.178), and *C. M. haemobos* and *B. bigemina* (r_s = 0.171).

CONCLUSION

Haemoparasitic diseases are of major constraint to livestock production in tropical and subtropical regions of the world (Jongejan and Uilenberg, 2004; Sivakumar *et al*., 2012). However, information on the prevalence, distribution, abundance and risk factors that affect these haemoparasites using molecular diagnostic methods is either inadequate or entirely lacking in most of these countries. In Malaysia, studies carried out on the prevalence of cattle haemoparasites had been done using traditional microscopy (Rajamanickam, 1970; Lee and Whiten, 1982; Salleh, 1984; Kamio *et al*., 1990; Lee *et al*., 1991; Sharifah, 2001; Chin, 2007; Nur Mahiza, 2010; Chandrawathani *et al*., 2014; Haron *et al*., 2015) and serological diagnostic methods (Chandrawathani *et al*., 1993, 1994, 1998; Sani *et al*., 1995; Nur Mahiza, 2010; Rahman *et al*., 2010; Pong and Nik Him, 2012; Rahman *et al*., 2012; Nik Him *et al*., 2013). The present study provides a more comprehensive insight on the species diversity, occurrence of co-infection and spatial distribution pattern of bovine haemoparasites in Peninsular Malaysia using species-specific PCR amplification of targeted gene fragments. It is also the first attempt to sample a large number of cattle over a wide distribution throughout the country using these molecular tools. In addition, bovine *Theileria*, *Babesia* and *Mycoplasma* are detected for the first time among beef and dairy cattle in the country using these molecular techniques.

The overall prevalence (92.1%) of cattle infected with haemoparasites in this study was much higher compared to the most recent report (Nur Mahiza, 2010) in Malaysia where only 27.1% of the 1562 cattle sampled were haemoparasite positive. A study done in neighbouring Thailand revealed that approximately half (53.0%) of the 162 cattle sampled were infected (Kaewthamasorn and Wongsamee, 2006). This disparity however, could be due to the difference in the diagnostic techniques employed. The use of thin blood smears for diagnosis of haemoparasites is usually difficult and is highly dependent on the expertise of the investigator to correctly identify the parasites in their various developmental stages (Bell-Sakyi *et al*., 2004). Molecular detection using PCR is more sensitive and specific than examination of blood smears, particularly in cases of low parasitaemia (Makler *et al*., 1998; Molad *et al*., 2006; Carelli *et al*., 2007; Bhoora *et al*., 2009; Shahnawaz *et al*., 2011).

The most common haemoparasite infecting cattle in Peninsular Malaysia was *A. marginale*, with a prevalence rate of 72.6%. This is comparable with the prevalence observed by previous researchers in the country. Rahman *et al*. (2012) reported a seroprevalence of 77.6% from five states in Peninsular Malaysia, while a larger serological survey throughout the country by Pong and Nik Him (2012) and Nik Him *et al*. (2013) reported prevalence rates of 79.4% and 77.6%, respectively. This is interesting because the PCR technique used in the present investigation detects

active infection while serological methods provide information on exposure and seroconversion. A recent molecular screen by Tay *et al*. (2014) revealed the 84.4% of the cattle screened on a farm in Selangor harboured *Anaplasma* spp. The present body of evidence strongly suggest that anaplasmosis is endemic in the country with high infection rates among farmed cattle. However, it is interesting that clinical anaplasmosis is not common, suggesting that these infected animals remain as effective reservoirs for the parasite. The overall molecular prevalence of *B. bigemina* (30.5%) and *B. bovis* (32.5%) is higher than that recorded by Chandrawathani (2000) and Rahman *et al*. (2010), which ranged between 16%-17% for both parasites. An earlier country-wide serological screen by Chandrawathani *et al*. (1993) detected antibodies in 72.0% of the cattle examined. This indicates that the local cattle are being constantly exposed with these piroplasmids, but with individuals having the ability to clear the parasites or harbour low levels of parasitaemia.

The high prevalence (47.0%) of *C. M. haemobos* among the cattle examined was greater than that reported by Nur Mahiza (2010), who documented a prevalence of 11.2%. Apart from the more sensitive diagnostic technique employed here, it cannot be ruled out that the four-fold increase may possibly indicate a change in the epidemiology of this haemopathogen over the duration of time. The multiple modes of transmission (horizontal, vertical, iatrogenic) of *C. M. haemobos* (Hornok *et al*., 2011) makes it difficult to control especially in areas where there are subclinical infections and reservoir animals among the herd. The prevalence of *T. orientalis* (49.8%) detected here is well within the range (16%-100%) reported in Malaysia from 1990 to 2014 (Kamio *et al*., 1990; Chin, 2007; Nur Mahiza, 2010; Haron *et al*., 2014). The molecular prevalence of *T. evansi* (17.9%) is much higher than that reported by Nur Mahiza (2010), whereby examination of thin blood films and haematocrit centrifugation (HCT) revealed prevalence levels of 0.6% and 2.1%, respectively. The present data, however, is comparable to the seroprevalence rates (18.0% and 14.7%) reported by Sani *et al*. (1995) and Rahman *et al*., (2012), respectively. Interestingly, the molecular prevalence of *T. evansi* is much lower than the levels of seroconversion detected by Chandrawathani *et al*. (1998) (58.0%) and Nur Mahiza (2010) (52.1%). Higher seroprevalence for this parasite is expected as antibodies tend to linger on even after treatment or following spontaneous recovery, and it does not necessarily indicate active infection (Luckins *et al*., 1979; Nantulya *et al*., 1986).

The spatial distribution pattern of haemoparasites in the country was fairly consistent albeit a higher prevalence of *T. orientalis, B. bovis* and *C.M haemobos* in certain farms. The reason for this may be related to breed or age susceptibility among the herds, or the presence of specific arthropod vectors. However, such assumptions can only be qualified once a comprehensive epidemiological study is conducted to determine the specific risk factors influencing the infection with these haemoparasites. Similarly, the most common and highly correlated co-infection combination of *A. marginale* + *T. orientalis*

cannot be explained with certainty given the present body of data. With the former being a rickettsial proteobacteria and the latter an apicomplexan parasite, this opens new ground for investigation into the co-infection synergism that may be occurring between these haemopathogens in both the ruminant host and the arthropod vector.

As the country is moving towards self-sufficiency in livestock production for meat and related products, it is vital that all aspects of cattle health and husbandry be addressed to ensure optimal productivity. It is envisaged that the data obtained in this study will contribute to a clearer understanding of the epidemiology of bovine haemoparasitic diseases in Peninsular Malaysia, which is an important facet for the formulation of effective treatment, control and prevention measures to assist in improving the local cattle industry.

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