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Molecular evidence of hemoplasmas in Malaysian cattle and ticks

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Abstract. Hemotrophic mycoplasma (hemoplasmas) is a cell wall-less bacterium causing infectious anemia in animals. As data on hemoplasmas infecting cattle in Malaysia is scarce, specific polymerase chain reaction assays were used for detection of hemoplasmas from blood samples of cattle and ticks in this study. Hemoplasma DNA was detected in 69 (69.0%) of 100 cattle blood samples obtained from different breeds. A total of 50.0% of the cattle in this study were infected with only *Mycoplasma wenyonii*, while 2.0% were infected with only *Candidatus* Mycoplasma haemobos and 17% were infected with both species. Based on sequence analysis of the partial or nearly full length sequences of hemoplasma 16S rRNA gene, the presence of *M. wenyonii* and *Candidatus* M. haemobos was confirmed. Hemoplasma-positive cattle of less than three years appeared to have higher infection rate compared to other age groups. *M. wenyonii* was identified for the first time in approximately 30% of cattle ticks (*Rhipicephalus microplus* and *Haemaphysalis* sp.) in this study. This study presents the first molecular evidence of hemoplasmas in Malaysian cattle and ticks.

INTRODUCTION

Hemotrophic mycoplasma (also known as hemoplasma) is an emerging bacterial pathogen to a wide variety of animals including livestock (Fujihara *et al.*, 2011; Maggi *et al.*, 2013), wildlife (Watanabe *et al.*, 2010), and companion animals (Maggi *et al.*, 2013). Infection caused by the cell wall-deficient, obligate epierythrocytic bacterium in animals is often chronic and subclinical; but may lead to more severe diseases such as hemolytic anemia when animals are under stress due to poor nutrition, pregnancy or other underlying infections (Sharifiyazdi *et al.*, 2014). Infections caused by *Mycoplasma wenyonii* and *Candidatus* Mycoplasma haemobos have been reported in cattle in countries in the Asia-Pacific region, including Japan (Tagawa *et al.*, 2008; Tagawa *et al.*, 2010; Tagawa *et al.*, 2013), China (Congli *et al.*, 2013; Song *et al.*, 2013) and Europe (Hoelzle *et al.*, 2011; Hofmann-Lehmann *et al.*, 2004). The transmission of hemoplasmas is believed to occur through various blood-feeding arthropods, including ticks (Neimark *et al.*, 2001).

Information is lacking on the presence of hemoplasmas in cattle and ticks in Malaysia. Hence, the aim of this study is to determine the occurrence of hemoplasmas using the blood samples of cattle and ticks collected from a previous study. As hemoplasmas have not yet been successfully cultivated *in vitro* (Aquino *et al.*, 2014), PCR assays targeting specific 16S rRNA gene (Jensen *et al.*, 2001) were used for detection and identification of the bacteria.

MATERIALS AND METHODS

Cattle blood and tick samples

A total of 100 cattle blood samples collected from five Malaysian farms located at Pahang (Farm A and Farm B), Kelantan (Farm C), Johore (Farm D) and Terengganu (Farm E) from a previous study (Kho *et al.*, 2015) were investigated in this study. Table 1 shows the origin and details of the cattle investigated in this study. The cattle investigated were of different breeds, i.e., Kedah Kelantan (n=37), Yellow Kedah Kelantan (n=20), Nellore (n=17), Brahman (n=3), Jersey (n=2), Drakensberger (n=1), local Indian/Zebu (n=1) and Mafriwal (n=19). The ages of the cattle ranged from 6 months to 12 years old, with majority (77.0%) less than 3 years old (Table 2). Fifty percent (50%) of the cattle were male and all were managed by rotational grazing system. Briefly, total DNA was extracted from 200 µL whole blood sample using QIAmp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. The DNA extract was stored at -20°C prior to PCR testing.

The DNA extracts from 73 ticks (29 *Rhipicephalus microplus* and 44 *Haemaphysalis bispinosa*) collected from cattle mainly from Farm B (n=37) and E (n=33) (Kho *et al.*, 2015) were included for

Table 1. Molecular detection of hemoplasma in cattle blood samples

Variable	No. (%) PCR-positive sample					Total (n=100)
	Farm A (n=20)	Farm B (n=20)	Farm C (n=20)	Farm D (n=20)	Farm E (n=20)	
Location	Jerantut, Pahang	Ulu Lepar, Pahang	Tanah Merah, Kelantan	Air Hitam, Johore	Kuala Berang, Terengganu	
Major cattle breed	Kedah Kelantan	Nellore	Kedah Kelantan	Mafriwal	Yellow Kedah Kelantan	
M. wenyonii	10 (50)	8 (40)	12 (60)	16 (80)	4 (20)	50 (50)
Candidatus M. haemobos	0 (0)	0 (0)	0 (0)	2 (10)	0 (0)	2 (2)
Dual infection	0 (0)	9 (45)	6 (30)	2 (10)	0 (0)	17 (17)
Total hemoplasma infection	10 (50)	17 (85)	18 (90)	20 (100)	4 (20)	69 (69)

Table 2. Hemoplasma infection of 100 cattle investigated in this study

Variable (no. cattle)	No. (%) PCR-positive sample			
	M. wenyonii	*Candidatus* M. haemobos	Dual infection	Total hemoplasma
Age				
<1 year (n=19)	6 (31.5)	2 (10.5)	7 (36.8)	15 (78.9)
1≤years<3 (n=58)	29 (50.0)	0 (0.0)	8 (13.7)	37 (63.7)
3≤years<5 (n=6)	5 (83.3)	0 (0.0)	0 (0.0)	5 (83.3)
≥5 years (n=17)	10 (58.8)	0 (0.0)	2 (11.7)	12 (70.5)
Sex				
Male (n=50)	24 (48.0)	0 (0.0)	10 (20.0)	34 (68.0)
Female (n=50)	26 (52.0)	2 (4.0)	7 (14.0)	35 (70.0)

investigation. The cattle ticks were obtained from the ear, eyes, flank, abdomen, tail and perineal regions. The ticks were first washed in 70% ethanol and rinsed in sterile distilled water prior to homogenization using surgical blades (Duh *et al.*, 2010). Each homogenate was then subjected to DNA extraction using QIAamp DNA mini kit (Qiagen, Hilden, Germany).

Molecular screening of hemoplasmas

The bacterial 16S rRNA gene was amplified using primers HF (5'-ACG AAA GTC TGA TGG AGC AAT A-3') and HR (5'-ACG CCC AAT AAA TCC GRA TAA T-3') as described by Jensen *et al.* (2001). A total volume of 25 µL containing 0.2 µL of each primer (25 µM), 1 U *Taq* DNA polymerase, 0.5 µL of dNTPs (100 µM), 2.5 µL of Dreamtaq™ DNA buffer and 2 µL of DNA extract were used in the PCR reaction. All PCR assays were performed in a Veriti thermal cycler (Applied Biosystems, USA). The cycling condition included denaturation at 95°C for five minutes followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 90 seconds and a final elongation step at 72°C for five minutes. PCR products (5 µL) were analysed by electrophoresis on a 1.5% agarose gel prestained with Gel Red at 100 V for 60 minutes, and the image was captured using InGenius gel documentation system (Syngene, England). A 100-*bp* DNA ladder (Solis BioDyne, Estonia) was included for molecular weight estimation. A *Mycoplasma haemofelis*-positive sample (obtained from a cat) and distilled water was used as positive and negative controls in each PCR run.

PCR-positive DNA extracts were randomly selected for amplification of the full length 16S rRNA gene using primers fD1 (5'-CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TCA G-3') and rp2 (5'-CCC GGG ATC CAA GCT TAC GGC TAC CTT GTT ACG ACT T-3') (Weisburg *et al.*, 1991). PCR was performed in a total volume of 25 µL containing 0.2 µL of each primer (25 µM), 1 U *Taq* DNA polymerase, 0.5 µL of dNTPs (100 µM), 2.5 µL of Dreamtaq™ DNA buffer and 2 µL of DNA extract. The amplification condition was denaturation at 95°C for five minutes followed by 30 cycles of 95°C for two minutes, 58°C for 30 seconds, 72°C for four minutes and a final elongation step at 72°C for 20 minutes.

Cloning of amplified fragments

Amplified fragments of the nearly full length 16S rRNA gene were cloned into pCR 4-TOPO plasmid vectors using a cloning kit (Invitrogen, USA) in accordance to the instructions of the manufacturer. In each cloning reaction, 2 µL fresh PCR product was added to the cloning reagents and incubated at room temperature (25°C) for five minutes. The ligated plasmid was then transformed to TOP 10 One Shot *Escherichia coli* strain (Invitrogen, USA) using a heat-shock method (incubation at 42°C for 30 seconds). The transformed bacterial cells were cultured on Luria Bertani agar supplemented with 50 mg/ml ampicillin. Plasmid DNA was extracted from an overnight-grown bacterial culture using GeneAll Exprep Plasmid SV Mini Kit (GeneAll Biotechnology, Seoul, Korea).

Sequence determination and analysis

Amplified fragments were randomly selected for sequence determination using forward and reverse PCR primers. Vector specific primers M13-F and M13-R (Invitrogen, USA) were used for sequence determination of bacterial plasmid. The obtained sequences were compared for similarity with sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST) program (National Center for Biotechnology Information, National Institute of Health).

Statistical analysis

Pearson's chi-square (χ^2) test (SPSS version 20) was used to determine the association between the detection of hemoplasmas with the age and sex of the cattle. A p value of <0.05 was considered as significant.

RESULTS

Figure 1 is an image of the agarose gel electrophoretic analysis of PCR products obtained from amplification of hemoplasma DNA from some representative cattle blood samples. A single amplicon of 173 bp was

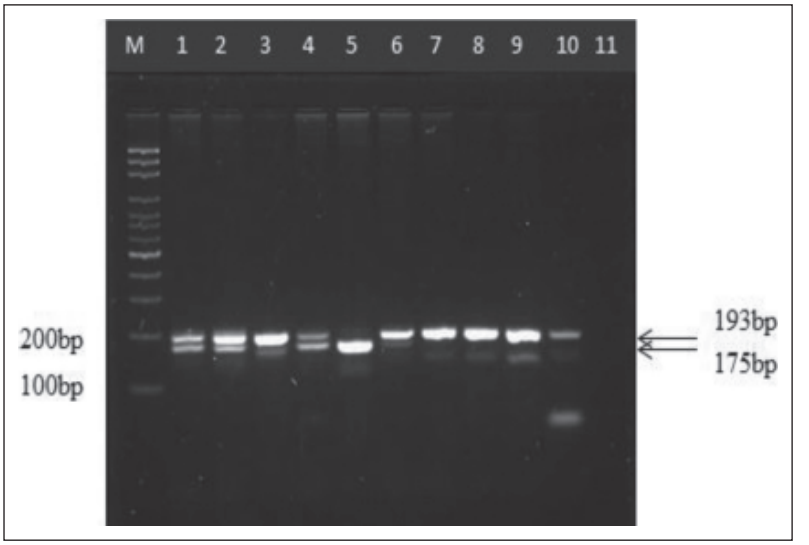

Figure 1. Agarose gel electrophoretic analysis of amplified partial 16S rRNA gene fragments (using primer HF/HR) obtained from cattle blood samples. Lane M: 100 bp DNA Ladder (Solis Biodyne, Tartu, Estonia); lane 1, 2, 4: mixed infection of *M. wenyonii* and *Candidatus* M. haemobos; lane 5: single infection of *Candidatus* M. haemobos; lane 6-9: *M. wenyonii*; lane 10: positive control (*Mycoplasma haemofelis*); lane 11: negative control (distilled water).

generated from the amplification of hemoplasma DNA from two blood samples. Identification of hemoplasma species was further confirmed based on analysis of the nearly full length 16SrRNA gene sequence (1207 bp, GenBank accession no. KT985638) which exhibited 100% sequence identity with that of *Candidatus* M. haemobos clone 311 derived from an infected cow in Switzerland (GenBank accession no. EF616468) (Meli *et al.*, 2010).

A single amplicon of approximately 195 bp was generated from the amplification of 50 cattle blood DNA samples in this study. BLAST analysis of 156 nucleotides (GenBank accession no. KT990216) amplified from all six randomly selected samples demonstrated 100% sequence similarity with that of *M. wenyonii* CH222 strain derived from an infected cow in Switzerland (GenBank accession no. GQ259756) (Hofmann-Lehmann *et al.*, 2004). Mixed infections of hemoplasmas were detected in 17 (17%) cattle when both fragments of 173 and 195 bp were amplified simultaneously from the cattle blood DNA samples in this study (Table 1).

Of the cattle investigated, the lowest detection rate (20%) of hemoplasma DNA was noted in the Yellow Kedah Kelantan breed while the Mafriwal dairy cattle (100%) had the highest detection rate (Table 1). Higher hemoplasma infection rate (67.5%) was noted in cattle less than three years old as compared to the older age group (51.5%); however the difference in the hemoplasma detection rates was not significant (Pearson Chi-square test, p=0.562). There was no difference in the hemoplasma detection rates between male and female cattle in this study (Pearson Chi-square test, p=0.829).

A total of 16 (24.2%) *Haemaphysalis* ticks and 7 (36.4%) *R. microplus* ticks were positive for *M. wenyonii* DNA, as demonstrated by the amplification of a 195 bp fragment from the tick DNA samples. Sequence analysis of amplified fragment from a positive tick (GenBank accession no. KT990217) confirms the identity of *M. wenyonii* which matched with that of *M. wenyonii* CH222 strain (GenBank accession no. GQ259756) (Hofmann-Lehmann *et al.*, 2004).

DISCUSSION

A high rate (69%) of hemoplasma infection was noted in the cattle investigated in this study. Hemoplasma detection rates ranged from 20–100% among different breeds of cattle, with the lowest rate noted in the Yellow Kedah Kelantan breed (20%). *M. wenyonii* was the predominant hemoplasma identified in this study, with the detection rates varying from as low as 20% (farm E) to as high as 100% (Farm D) (Table 1).

In this study, *Candidatus* M. haemobos was detected in a lower rate, and was mostly detected in mixed infection with *M. wenyonii*. Mixed infections of hemoplasma species have been reported in the cattle in other geographical regions. In the Asian region, Tagawa *et al.* (2008) were the first to report about hemoplasma infection in cattle. It was reported that 21.8% of Japanese cattle were infected with *M. wenyonii*, whereas 16.7% were infected with *Candidatus* M. haemobos and 5.1% were infected with both species. A higher prevalence (39.1%) of *Candidatus* M. haemobos followed by *M. wenyonii* (38.5%) was later reported by the same group of investigators in another epidemiological survey (Tagawa *et al.*, 2012). *Candidatus* M. haemobos has also been reported with a high rate (41.7%) in cattle from tropical China (Su *et al.*, 2010). Both *M. wenyonii* and *Candidatus* M. haemobos have also been implicated in an outbreak of hemotropic mycoplasmosis in cattle herds in Northern Germany (Hoelzle *et al.*, 2011). In Switzerland, 22 (37.9%) of 58 cattle blood samples were positive for *M. wenyonii* (Hofmann-Lehmann *et al.*, 2004). In another study, of the 24 cattle blood sample tested in Hungary, concurrent infection of *M. wenyonii* and *Candidatus* M. haemobos has been detected in 22 and 21 cattle, respectively (Hornok *et al.*, 2012). The difference in the hemoplasma detection rates across different geographical regions has not been thoroughly investigated, however; it may be related with various factors including the age and sex of the cattle, and animal husbandry practice.

A study in Shanghai, China reported that young cattle of 2-4 years old were most vulnerable to *M. wenyonii* infection (Congli *et al.*, 2013). Similar observation was reported from Japan whereby cattle of 1-3 years of age were more susceptible to infection with *M. wenyonii* compared to other age groups (Tagawa *et al.*, 2012). In agreement with these investigations, higher detection rate of hemoplasma DNA was noted in cattle less than three years old in this study, although the difference was not significant. It is postulated that older cattle might have obtained immunity against hemoplasma infection as compared to the younger cattle (Tagawa *et al.*, 2012). Additionally, vertical transmission has also been suggested as a possible route of neonatal infection (Fujihara *et al.*, 2011). However, hemoplasma infection was not affected by the sex of cattle in this study (Table 2).

Ticks and other arthropods play an important role in the transmission of hemoplasma infection. The presence of *M. wenyonii* through ticks was first documented in *Dermacentor andersoni* tick (Neimark *et al.*, 2001). New potential vectors i.e., the horn fly (*Haematobia irritans*), the stable fly (*Stomoxys calcitrans*) and two species of horse flies (*Tabanus bovinus*, *Tabanus bromius*) have also been documented (Hornok *et al.*, 2011). In China, 69.2% lice, 66.7% flies and 80.7% mosquitoes have been tested positive for *M. wenyonii* using loop-mediated isothermal amplification (LAMP) and PCR assays, suggesting that a variety of arthropods can serve as mechanical vectors for *M. wenyonii* (Song *et al.*, 2013).

We detected for the first time the presence of *M. wenyonii* from approximately 30% of cattle ticks (*R. microplus* and *H. bispinosa*) in this study. Previous investigation reported the detection of hemotropic mycoplasmas from 2 out of 181 *Ixodes* sp. and 1 out of 67 *Rhipicephalus* sp. ticks collected from animals (Willi *et al.*, 2007). So far, the involvement of *R. microplus* and *H. bispinosa* in the transmission cycle of hemoplasma infection has not been reported and screening of hemoplasmas in other ticks including *Haemaphysalis inermis* and *Dermacentor marginatus* have been negative (Hoffman-Lehmann *et al.*, 2004; Hornok *et al.*, 2012).

However, as bacterial DNA may also be detected from ticks after a blood meal from an infected host, the potential role of the cattle ticks such as *R. microplus* and *H. bispinosa* as vectors for transmission of hemoplasmas is yet to be established.

This study presents the first molecular evidence of hemoplasmas in Malaysian cattle and ticks. Continued surveillance and monitoring of the disease is necessary in view of the clinical impact of the bacteria to animal health and production. Further investigation on the cellular and molecular interrelationships between *R. microplus* and *H. bispinosa* ticks with *M. wenyonii* is necessary.

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