Bulgarian Journal of Veterinary Medicine, 2021, **24**, No 3, 395–408
ISSN 1311-1477; DOI: 10.15547/bjvm.2019-0141

Original article

MOLECULAR EVIDENCE AND EPIDEMIOLOGY OF "*CANDIDATUS MYCOPLASMA HAEMOBOS*" AMONG CATTLE IN PENINSULAR MALAYSIA USING THE *16S rRNA* GENE

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Summary

Ola-Fadunsin, S. D., D. A. Abdullah, F. I. Gimba, F. J. F. Abdullah & R. A. Sani, 2021. Molecular evidence and epidemiology of "*Candidatus Mycoplasma haemobos*" among cattle in Peninsular Malaysia using the *16S rRNA* gene. *Bulg. J. Vet. Med.*, **24**, No 3, 395–408.

Haemotropic *Mycoplasma* infection among cattle has not been seriously looked into, despite the fact it is associated with poor weight gain and depression. One thousand and forty-five blood samples from apparently healthy cattle from forty-three farms within the entire Peninsular Malaysia were examined over a one-year period. Using polymerase chain reaction to amplify the *16S rRNA* gene, the presence of "*Candidatus Mycoplasma haemobos*" as one of the haemotropic *Mycoplasma* species that affects cattle in the country was detected. Forty-seven percent of the sampled population was positive for "*C. M. haemobos*", with the antigen present in all herds sampled. Breed of cattle, gender, age, production type, physiological status, herd size, management system, farm size, farm age, prophylactic treatment against blood parasites, presence of ticks, frequency of de-ticking, zone, closeness to forest, closeness to waste area, closeness to human settlement and closeness to body of water were all significantly associated ($P<0.05$) with the detection of "*C. M. haemobos*". The findings from this study will be pivotal in government policy on this blood *Mycoplasma* infection, as the study happens to be the first comprehensive molecular survey of the microorganism in the country.

Key words: *16S rRNA* gene, "*Candidatus Mycoplasma haemobos",* cattle, Peninsular Malaysia, polymerase chain reaction (PCR)

INTRODUCTION

The genus *Mycoplasma* was first described in 1934, and was formerly known as *Eperythrozoon* (Neimark & Kocan, 1997). The species was recently reclassified within the group of haemotropic *Mycoplasma* species based on analysis of the

16S rRNA gene (Neimark & Kocan, 1997; Neimark *et al*., 2002). Mycoplasmas are Gram-negative, wall-less epierythrocytic bacteria that parasitise mammalian erythrocytes (Neimark *et al*., 2001; Messick, 2004; Bauer *et al*., 2008). The organism is pleomorphic and may be observed as rings, rods, or cocci during cytological examination of blood smears (Genova *et al*., 2011). These haemotrophic mycoplasmas are the causative agents of an infectious anaemia (Hoelzle *et al*., 2010), with a sporadic, febrile and haemolytic clinical disease (Sudan *et al*., 2012). Mycoplasmas are common and widespread among animals (Willi *et al*., 2007; Bauer *et al*., 2008), and may also infect immunocompromised humans (Dos Santos *et al*., 2008).

At present the nomenclature of bovine mycoplasmas is not definite and provisional designations are commonly used (Tagawa *et al*., 2010). Based on analysis of the 16S rRNA, "*Candidatus Mycoplasma haemobos*" was proposed to infect cattle in Switzerland and Japan (Hofmann-Lehmann *et al*., 2004; Tagawa *et al*., 2008). *Mycoplasma wenyonii* is another species of cattle that is found attached to red blood cells or free in the plasma of infected cattle (Messick, 2004). It is an uncultivable, cell wall-less organism although it can be found in ring, rod, or cocci forms during cytologic examination of blood smears stained with Romanowski staining methods (Neimark & Kocan, 1997). Both species have a cosmopolitan distribution and are reported to elicit anaemia, oedema, reproductive problems and various other clinical signs (Tagawa *et al*., 2008; Hoelzle *et al*., 2011). Of the two, "*C. M. haemobos*" appears to be more pathogenic (Tagawa *et al*., 2010).

The most important vector for bovine haemotrophic *Mycoplasma* species are the sucking lice, where infection is through trans-stadial transmission (Morel, 1989). *Hyalomma anatolicum* is believed to be responsible for the transmission of *Mycoplasma wenyoni* (Morel, 1989; Kaufmann, 1996). Transplacental infection and vector-borne (*Haematobia irritans*, *Stomoxys calcitrans*, *Tabanus bovinus* and *Tabanus bromius*) transmission may contribute to the spread of both bovine haemoplasmas (*M. wenyonii* and "*C. M. haemobos*") (Hornok *et al*., 2011).

The 16S ribosomal RNA (*16S rRNA*) gene is believed to be the part of the DNA that is now most commonly used for taxonomic purposes for microorganisms (Tortoli, 2003; Harmsen & Karch, 2004). *16S rRNA* is a conserved gene of the DNA and it is used to mark evolutionary distance and relatedness of organisms (Harmsen & Karch, 2004). The *16S rRNA* gene sequence has been determined for a large number of strains (Clarridge, 2004). GenBank, the largest databank of nucleotide sequences, has over 20 million deposited sequences, of which over 90,000 are of *16S rRNA* gene (Clarridge, 2004). The diverse properties of *16S rRNA*, such as its essential function, ubiquity, and evolutionary properties, have allowed it to become the most commonly used molecular marker in microbial ecology (Case *et al*., 2007). Su *et al*. (2010), Girotto *et al*. (2012) and Tagawa *et al*. (2013) have documented reports on the efficacy of *16S rRNA* gene in the detection of "*C. M. haemobos*" in cattle.

Bovine mycoplasmiosis has only recently been reported to occur in Peninsular Malaysia with a prevalence of 17.0% (Chin, 2007); earlier work done by Lee & Whitten (1982) indicated the absence of the microorganism among local cattle. Subsequently, Nur Mahiza (2010) using the conventional parasitological method found this bacteria to be one of the pre-

dominant blood organism infecting cattle in Malaysia. The aim of the study was to determine the presence of "*Candidatus Mycoplasma haemobos*" among cattle in Peninsular Malaysia and to identify the main risk factors associated with the prevalence of this *Mycoplasma* species. This study appears to be the first to elucidate on the epidemiology of "*C. M. haemobos*" of cattle in Peninsular Malaysia.

MATERIALS AND METHODS

Study area, design and sample collection

Peninsular Malaysia occupies a geographic coordinate of 4° 0' 0″ N, 102° 30'0″ E and it encompasses a total land mass of 131,598 km^2. It is located at the southern-most tip of the Asian mainland, bordered by Thailand in the north, and separated from Singapore by the straits of Johor in the south.

A total of 1,045 blood samples were obtained from 43 cattle farms from the entire Peninsular Malaysia. The study was conducted from August 2015 to October 2016 comprising 379 dairy and 666 beef cattle. Sampling was carried out following a cross sectional study design based on convenience and willingness of the cattle owners. The minimum sample size required in this study was calculated as suggested by Thrusfield (2005) for the estimation of prevalence in a large population. For sampling purposes, the country was arbitrarily divided into six zones (North, Northwest, Northeast, Southwest, Southeast and South).

All sampled cattle were apparently healthy. About 5 milliliters of blood was collected from each animal via jugular or coccygeal venipuncture depending on the age of the cattle. The blood was collected into a labelled ethylenediaminetetraacetic acid tube and put in an ice box for immediate transportation to the Parasitology laboratory of the Faculty of Veterinary Medicine Universiti Putra Malaysia.

PCR detection of "Candidatus Mycoplasma haemobos"

Deoxyribonucleic acid (DNA) was extracted from each blood sample using the Qiagen DNeasy® Blood and Tissue kit according to the manufacturers' protocol.

The PCR amplification was performed on the extracted genomic DNA using the My Cycler^{TM} (Bio-Rad Laboratories, USA) thermal cycler as described by Ola-Fadunsin *et al*. (2018), with oligonucleotide primers specific for "*C. M. haemobos*". A set of nucleotide sequence of the primer Forward – 5' GAGTTAGTT ATTAAAGCTTTAT 3', Reverse – 5' ATTCATGAGGTACTATCAGTTG 3' was used to amplify a 279 base pair of the *16S rRNA* gene of "*C. M. haemobos*" (Su *et al*., 2010) with the following programme: initial denaturation at 94 °C (5 min), 40 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (30 s), extension at 72 °C (30 s) with final extension at 72 °C for 7 min. The PCR amplicons were electrophoresed on a 2% agarose gel (Vivantis, USA) at 90V with TAE (Tris-acetic acid-EDTA) buffer, stained with ethidium bromide, and viewed under a UV transilluminator (GeneDoc^{TM}, Bio-Rad Laboratories) with 100 bp DNA ladders as standard size markers (Vivantis, USA). Images were captured using a digital camera and computer software (GeneSnap ^{TM}, 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.

Positive amplicons of 279 bp were excised from the agarose gel with a sterile scalpel and purified using the QIAquick Gel Extraction Kit (Qiagen, Germany) according to the manufacturers' protocol. The obtained DNA was subsequently sequenced using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA). Confirmation of the obtained sequences of *16S rRNA* gene fragments of "*C. M. haemobos*" was done using the National Centre for Biotechnology Information (NCBI) GenBank using the Basic Local Alignment Search Tool (BLAST) as described by Abdullah *et al.* (2019).

Questionnaire and statistical analysis

To determine the risk factors associated with the prevalence of "*C. M. haemobos*", a well-structured, interviewer-administered questionnaire containing open-ended and closed-ended (dichotomous or multiple choice) questions was designed to obtain information on host, demography, environmental and management factors and bio-security. The questionnaires were administered on farms where samples were obtained. A respondent was someone who was actively involved in the daily activities of the farm and was not necessarily the farm owner.

The molecular prevalence of "*C. M. haemobos*" was calculated based on the number of PCR "*C. M. haemobos*" positive cattle divided by the total number of cattle sampled. The odd ratio and statistical significance between positive and negative samples were determined using Chi Square (univariable model) for discrete variables at 95% confidence interval. Multivariable unconditional logistic regression was used to determine the factors for "*C. M. haemobos*" controlling for other covariate at $P<0.2$ and biologically plausible variables. SPSS v. 22.0. was used for the statistical analysis and $P<0.05$ was taken as statistically significant.

RESULTS

A 279-base pair of the *16S rRNA* gene of "*C. M. haemobos*" was obtained (Fig. 1). The overall prevalence was 47.0% (491/1045, 95 % CI: 44.0 – 50.0). "*Can-*

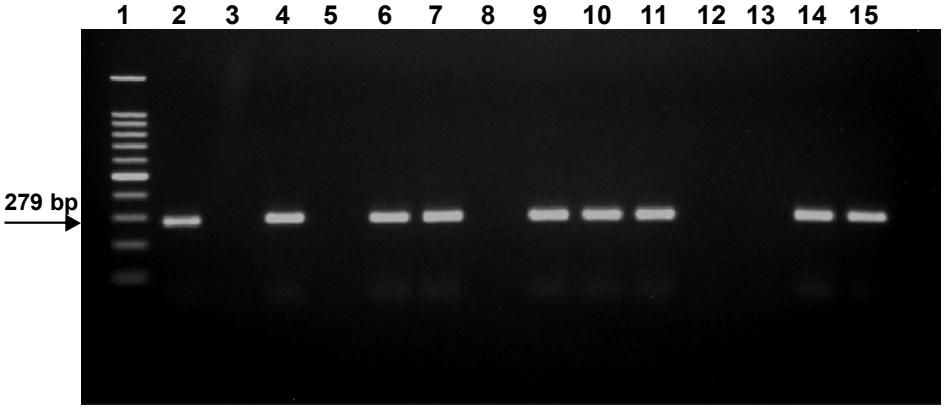

Fig. 1. Agarose gel electrophoresis showing PCR amplicons of the *"Candidatus Mycoplasma haemobos" 16S rRNA* gene fragment (279 bp) among cattle in Peninsular Malaysia. Lane 1: 100 bp size markers, lane 2: positive control, lane 3: negative control, lanes 4, 6, 7, 9–11, and 14–15: positive samples, lanes 5, 8, 12 and 13: negative samples.

didatus Mycoplasma haemobos" was detected in the entire sampled herd with a prevalence ranging from 16.2% to 90.0%. The prevalence among the different zones was statistically significant (P<0.05), with the southwest zone having the highest prevalence of 59.7% (111/186, 95% CI: 52.5–66.6) and the south zone having the lowest prevalence of 32.6% (56/172, 95% CI: 25.9–39.8) (Table 1).

Cattle breed, gender, age, production type, physiological status, herd size, management system, farm size, farm age, frequency of prophylactic treatment against blood organisms (prophylaxis), presence of ticks, frequency of de-ticking, zone, distance to forest, distance to waste area, distance to human settlement and distance to body of water were all significantly associated (P<0.05) with the detection of "*C. M. haemobos*" (Tables 2–4).

The multivariate model (Table 5) revealed breed, gender, age and presence of ticks as the statistically significant risk factors for "*C. M. haemobos*" infection, while others failed to reject the null hypothesis. The Brahman × LID breed was 2.0 times more likely to be PCR positive compared to the local KK breed. However, the latter was 3.0, 3.1 and 4.3 times more likely to be PCR positive compared to the KK × Friesian, KK × Charolais and Friesian × Charolais breeds, respectively. Male cattle were 1.4 times at higher risk of the infection compared to females.

Age groups were also associated to the PCR positivity; cattle that are $\leq$ 1 year and >1 – $\leq$ 2 year old were 1.4 and 2.9 times, respectively, more likely to be positive compared to the cattle that are above five years old. Cattle with tick infestation had a lower odd (0.54) of been PCR positive compared to cattle that were not infested.

DISCUSSION

The obtained 279-base pair of the *16S rRNA* gene of "*C. M. haemobos*" confirms that this haemotropic *Mycoplasma* is one of the *Mycoplasma* species affecting cattle in Peninsular Malaysia. The presence of "*C. M. haemobos*" in all the sampled herds suggests that it is endemic among cattle in Peninsular Malaysia.

The overall molecular prevalence of "*C. M. haemobos*" in Peninsular Malaysia was 47.0%, the prevalence frequency obtained in this study is higher than the reported 39.1% molecular prevalence in cattle in Eastern Hokkaido, Japan (Tagawa *et al*., 2012). In addition, it appears lower compared to that of Fujihara *et al.* (2011) and Girotto *et al*. (2012) who reported the prevalence of "*C. M. haemobos*" in cattle as 42.0%–3.0% and 61.0% in the Western Part of Japan and Southern Brazil, respectively. The disparities in the prevalence of "*C. M. haemobos*" in these countries suggest that factors in geographical latitude may affect infections as well as the differences in the arthropod vectors activities and distribution.

Interestingly, our reported prevalence was higher than the documented 11.2% prevalence detected in cattle in Peninsular Malaysia using conventional parasitological techniques (Nur Mahiza, 2010). This disparity however, could be due to the difference in the diagnostic techniques employed. The use of thin blood smears for diagnosis of blood organisms is usually difficult and is highly dependent on the expertise of the investigator to correctly identify the organisms in their various developmental stages (Bell-Sakyi *et al*., 2004; Ola-Fadunsin *et al*., 2017). Molecular detection using PCR is more sensitive and specific than examination of blood smears, particularly in cases of low

Table 1. Molecular prevalence (%) of *"C. M. haemobos"* infecting cattle in Peninsular Malaysia categorised according to the various sampling zones

Zones	Number	Number positive (prevalence)	95% confidence interval
North	105	53 (50.5) [a]	41.0 – 60.0
Northwest	220	116 (52.7) [a]	46.1 – 59.3
Northeast	176	85 (48.3) [a]	41.0 – 55.7
Southwest	186	111 (59.7) [b]	52.5 – 66.6
Southeast	186	70 (37.6) [c]	30.9 – 44.8
South	172	56 (32.6) [c]	25.9 – 39.8

CI = Confidence Interval. Different alphabet superscripts (a,b,c,d) indicate significant differences ($P<0.05$) between rows.

Table 2. Univariate association between host epidemiological factors and *"Candidatus Mycoplasma haemobos"* infection among cattle in Peninsular Malaysia.

Factors	Positive (%)	Negative (%)	OR (95% CI)	P
Breeds				
KK [a]	61 (41.5)	86 (58.5)	1.00	
KK × Brahman	112 (45.9)	132 (54.1)	1.20 (0.79, 1.81)	0.40
KK × Friesian	8 (32.0)	17 (68.0)	0.67 (0.26, 1.63)	0.38
KK × LID	41 (54.7)	34 (45.3)	1.70 (0.97, 2.99)	0.07
KK × Yellow China	6 (24.0)	19 (76.0)	0.45 (0.16, 1.16)	0.10
KK × Charolais	5 (17.2)	24 (82.8)	0.30 (0.10, 0.78)	0.01*
Brahman	21 (43.8)	27 (56.2)	1.10 (0.57, 2.12)	0.78
Brahman × LID	45 (60.0)	30 (40.0)	2.11 (1.20, 3.74)	0.01*
Friesian × Jersey	42 (47.2)	47 (52.8)	1.26 (0.74, 2.14)	0.40
Friesian × Sahiwal	146 (53.5)	127 (46.5)	1.62 (1.08, 2.44)	0.02*
Friesian × Charolais	4 (26.7)	11 (73.3)	0.52 (0.14, 1.65)	0.28
Gender				
Male	121 (54.8)	100 (45.2)	1.48 (1.10, 2.00)	0.01*
Female [a]	370 (44.9)	454 (55.1)	1.00	
Age (years)				
≤ 1	105 (48.2)	113 (51.8)	1.77 (1.17, 2.70)	0.01*
$> 1 - \leq 2$	144 (64.0)	81 (36.0)	3.39 (2.23, 5.18)	<0.01*
$> 2 - \leq 5$	185 (42.4)	251 (57.6)	1.41 (0.97, 2.05)	0.07
> 5 [a]	57 (34.3)	109 (65.7)	1.00	
Physiological status				
Immature	237 (54.9)	195 (45.1)	2.12 (1.48, 3.06)	<0.01*
Mating stock	28 (49.1)	29 (50.9)	1.68 (0.91, 3.10)	0.09
Pregnant	57 (39.0)	89 (61.0)	1.12 (0.71, 1.76)	0.63
Lactating	106 (44.7)	131 (55.3)	1.41 (0.95, 2.12)	0.09
Dry [a]	63 (36.4)	110 (63.6)	1.00	

[a] Reference category, * significant, OR = odds ratio, CI = confidence interval, KK = Kedah Kelantan, LID = Local Indian Dairy.

Table 3. Univariate association between management epidemiological factors and *"Candidatus Mycoplasma haemobos"* infection among cattle in Peninsular Malaysia.

Factors	Positive (%)	Negative (%)	OR (95% CI)	P
Production type				
Dairy	194 (51.2)	185 (48.8)	1.30 (1.01, 1.68)	0.04*
Beef [a]	297 (44.6)	369 (55.4)	1.00	
Herd size				
Small (≤ 100)	266 (48.7)	280 (51.3)	1.47 (1.03, 2.07)	0.03*
Medium (> 100 – 300)	157 (48.2)	169 (51.8)	1.43 (0.99, 2.09)	0.06
Large (> 300) [a]	68 (39.3)	105 (60.7)	1.00	
Management system				
Semi-intensive	346 (44.3)	435 (55.7)	0.51 (0.26, 0.97)	0.04*
Extensive	120 (53.8)	103 (46.2)	0.75 (0.37, 1.47)	0.40
Intensive [a]	25 (61.0)	16 (39.0)	1.00	
Farm size (acres)				
≤ 10	204 (50.2)	202 (49.8)	2.35 (1.60, 3.48)	<0.01*
> 10 – 20	90 (52.9)	80 (47.1)	2.61 (1.67, 4.12)	<0.01*
> 20 – 50	87 (48.3)	93 (51.7)	2.17 (1.39, 3.40)	<0.01*
> 50 – 100	61 (48.4)	65 (51.6)	2.18 (1.34, 3.55)	<0.01*
> 100 [a]	49 (30.1)	114 (69.9)	1.00	
Farm age (years)				
≤ 10	213 (55.8)	169 (44.2)	1.96 (1.15, 3.01)	0.01*
> 10 – 20	125 (38.3)	201 (61.7)	0.97 (0.56, 1.70)	0.91
> 20 – 30	101 (47.6)	111 (52.4)	1.42 (0.80, 2.53)	0.23
> 30 – 40	27 (44.3)	34 (55.7)	1.24 (0.60, 2.54)	0.56
> 40 [a]	25 (39.1)	39 (60.9)	1.00	
Prophylaxis				
Every 2 months	15 (37.5)	25 (62.5)	0.61 (0.31, 1.17)	0.14
Every 3 months	5 (12.5)	35 (87.5)	0.15 (0.05, 0.35)	<0.01*
Every 6 months	56 (43.4)	73 (56.6)	0.78 (0.53, 1.13)	0.19
No treatment [a]	415 (49.6)	421 (50.4)	1.00	
Presence of ticks				
Yes	358 (43.5)	465 (56.5)	0.52 (0.38, 0.70)	<0.01*
No [a]	133 (59.9)	89 (40.1)	1.00	
Frequency of de-ticking				
Monthly	28 (68.3)	13 (31.7)	1.87 (0.94, 3.88)	0.08
Every 2 months	25 (41.7)	35 (58.3)	0.62 (0.35, 1.10)	0.10
Every 3 months	14 (23.3)	46 (76.7)	0.27 (0.14, 0.50)	<0.01*
Every 4 months	51 (41.5)	72 (58.5)	0.62 (0.40, 0.95)	0.03*
Every 6 months	214 (46.0)	251 (54.0)	0.74 (0.55, 1.00)	0.05
Once a year	12 (57.1)	9 (42.9)	1.16 (0.47, 2.95)	0.75
Not practiced [a]	147 (53.5)	128 (46.5)	1.00	

[a] Reference category, * significant, OR = odds ratio, CI = confidence interval.

Table 4. Univariate association between environmental epidemiological variables and *"Candidatus Mycoplasma haemobos"* infection among cattle in Peninsular Malaysia

Factors	Positive (%)	Negative (%)	OR (95% CI)	P
Distance to forest area				
≤ 200 m	265 (54.3)	223 (45.7)	2.80 (1.88, 4.21)	<0.01*
> 200–500 m	63 (55.3)	51 (44.7)	2.90 (1.73, 4.89)	<0.01*
> 500–1000 m	32 (28.3)	81 (71.7)	0.93 (0.54, 1.61)	0.80
> 1000–1500 m	89 (47.1)	100 (52.9)	2.09 (1.32, 3.34)	<0.01*
> 1500 m [a]	42 (29.8)	99 (70.2)	1.00	
Distance to waste area				
≤ 200 m	71 (50.7)	69 (49.3)	1.28 (0.87, 1.86)	0.21
> 200–500 m	110 (53.4)	96 (46.6)	1.42 (1.02, 1.97)	0.04*
> 500–1000 m	38 (28.8)	94 (71.2)	0.50 (0.33, 0.76)	<0.01*
> 1000–1500 m	59 (65.6)	31 (34.4)	2.36 (1.48, 3.81)	<0.01*
> 1500 m [a]	213 (44.7)	264 (55.3)	1.00	
Distance to human settlement				
≤ 200 m	35 (36.1)	62 (63.9)	0.57 (0.36, 0.89)	0.01*
> 200–500 m	77 (57.0)	58 (43.0)	1.34 (0.92, 1.97)	0.13
> 500–1000 m	56 (48.7)	59 (51.3)	0.96 (0.64, 1.44)	0.85
> 1000–1500 m	66 (36.5)	115 (63.5)	0.58 (0.41, 0.82)	<0.01*
> 1500 m [a]	257 (49.7)	260 (50.3)	1.00	
Distance to water body				
≤ 200 m	200 (53.2)	176 (46.8)	1.50 (1.07, 2.11)	0.02*
> 200–500 m	75 (44.4)	94 (55.6)	1.06 (0.70, 1.59)	0.80
> 500–1000 m	83 (40.3)	123 (59.7)	0.89 (0.61, 1.32)	0.57
> 1000–1500 m	40 (51.3)	38 (48.7)	1.39 (0.83, 2.35)	0.22
> 1500 m [a]	93 (43.1)	123 (56.9)	1.00	

[a] Reference category, * significant, OR = odds ratio, CI = confidence interval.

parasitaemia (Carelli *et al*., 2007; Shahnawaz *et al*., 2011). As such, PCR assays are considered to be the "gold standard" for the detection of infection in cattle (De Eschaide *et al*., 2005). 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 *"C. M. haemobos"* over the duration of time. The multiple modes of transmission (horizontal, vertical, iatrogenic) of this haemotropic *Mycoplasma* species (Morel, 2000; Hornok *et al*., 2011) make difficult the control especially in areas where there are subclinical infections and reservoir animals among the herd.

The differences in the prevalences seen in the different geographic zones in our study, confirms the study by Fujihara *et al.* (2011) who reported differences in the prevalence of haemoplasma in two different zones in Western Japan. This present study has shown that there was no particular trend in the level of "*C. M. haemobos*" harboured by the indigenous breed (KK) and the exogenous breeds and its crosses as some crosses showed lower prevalence and some higher prevalence compared to the indigenous breed. This is

Table 5. Multivariate association between epidemiological factors and *"Candidatus Mycoplasma haemobos"* infection among cattle in Peninsular Malaysia

Factors	β	SE	P	OR	95% CI	
					Lower	Upper
Breed						
KK[a]				1.000		
KK × Brahman	0.363	0.245	0.14	1.438	0.889	2.325
KK × Friesian	–1.106	0.563	0.04*	0.331	0.110	0.996
KK × LID	0.373	0.317	0.24	1.452	0.780	2.702
KK × Yellow China	–0.583	0.573	0.31	0.558	0.182	1.717
KK × Charolais	–1.130	0.568	0.04*	0.323	0.106	0.984
Brahman	–0.146	0.362	0.69	0.865	0.425	1.758
Brahman × LID	0.712	0.345	0.04*	2.038	1.036	4.009
Friesian × Jersey	0.060	0.317	0.85	1.062	0.570	1.977
Friesian × Sahiwal	0.417	0.235	0.08	1.518	0.957	2.408
Friesian × Charolais	–1.455	0.680	0.03*	0.234	0.062	0.885
Gender						
Male	0.346	0.179	0.04*	1.413	0.996	2.006
Female [a]				1.00		
Age (years)						
≤ 1	0.310	0.243	0.02*	1.364	0.847	2.196
> 1 – ≤ 2	1.060	0.240	<0.01*	2.888	1.803	4.626
> 2 – ≤ 5	0.197	0.208	0.34	1.218	0.810	1.832
> 5 [a]				1.00		
Presence of ticks						
Yes	–0.608	0.201	<0.01*	0.544	0.367	0.808
No [a]				1.00		

[a] Reference category, * Significant, OR = odds ratio, CI = confidence interval.

not in agreement with earlier reports which indicated that indigenous cattle were more resistant to haemo-organisms compared to the imported breeds (Muhanguzi *et al*., 2010; Atif *et al*., 2013). It is well established that nutritional and physiological status, environmental conditions, type of management practice and season of sampling can affect the prevalence of haemo-organisms (Sehgal *et al*., 2005; Akinpelu, 2008). In the multivariate analysis, the indigenous KK breed had a significantly lower prevalence compared to the Brahman × LID breed, but were more susceptible compared to KK × Friesian, KK × Charolais and Friesian × Charolais breeds. While this result is not readily explainable, it may be possible that the Charolais and Friesian cattle may be genetically more resistant to the *Mycoplasma* species. In addition, these breeds have long been introduced into the country and as such must have acclimatised to the local environment, making them more resistant to "*C. M. haemobos*" and its vectors.

Male cattle have a higher risk of being positive than their female counterpart in this study. It is difficult to explain the effect of gender without conducting an ex-

perimental infection of the *Mycoplasma* species in both sexes. It is possible that gender is a confounding factor that is linked to certain biological characteristics of the different sex (Elshafie *et al*., 2013). Stress of work among male animals may result in a higher susceptibility to diseases (Rowlands *et al*., 1993). Also, the practice of keeping cows longer for the purpose of milking and reproduction, allowing them to develop immunity to the organism may support our findings.

Age played a significant role in the epidemiology of "*C. M. haemobos*" in Peninsular Malaysia. Our study shows that younger cattle were at a higher risk of been infected compared to older cattle. Higher prevalence of haemoplasma infections has been reported in younger cattle than older cattle in Japan (Fujihara *et al*., 2011; Tagawa *et al*., 2012), this may likely be due to the softer skin of young cattle predisposing them to more attack from the arthropod vectors.

Fascinatingly, the presence of tick vectors was a significant risk factor as cattle infected with ticks were about twice less likely to be infected with *"C. M. haemobos*" than cattle that are not infected with ticks. This may not be quick to explain as sucking lice are believed to be the most important vector for bovine haemotrophic *Mycoplasma* species (Morel, 1989) although *Hyalomma anatolicum*, *Stomoxys calcitrans Aedes aegypt*, *Tabanus bovinus* and *Tabanus bromius* have been reported to transmit *Mycoplasma* species to pigs and ruminants (Prullage *et al*., 1993; Kaufmann, 1996; Hornok *et al*., 2011). Considering the species of tick vectors affecting cattle in Peninsular Malaysia, such as *Rhipicephalus* (*Boophilus*) *microplus* and *Haemaphysalis bispinosa* (as observed in a different study carried out by us), these species could be involved in the transmission of this pathogen; however, this observation needs in-depth investigation since ticks have been suspected as a vector for feline haemoplasma infection (Willi *et al*., 2007).

Dairy cattle were of higher risk of the disease compared to beef cattle. A number of factors affects the prevalence of blood organisms as it relates with the production type of cattle. The physiological and nutritional status of the animal as well as the density of vectors on the farm contribute to the acquisition and spread of infection. Also, farms with small herd size (less than 300 cattle) had the highest prevalence of *"C. M. haemobos*" compared to the medium and large herd farms. This may be associated with the fact that the smaller the number of cattle in a farm the higher the vector density per cattle. During sampling, it was observed that cattle in small herds were often kept in a restricted space, affording closer contact between the individual animals, thus allowing the infection to spread at a higher rate.

Other factors that had a significant univariate statistical association with the detection of *"C. M. haemobos*" were management system, farm size (acres), farm age (years), prophylaxis treatment against blood parasites, frequency of de-ticking, distance to forest area, distance to waste area, distance to human settlement and distance to water body. It is postulated that poor management, lack of tick control practices and inadequate economic sustainability of poor resource small holder farmers for the implementation of proper management and animal health practices contributed to the higher prevalence of vector borne diseases in cattle (Swai *et al*., 2005).

CONCLUSION

Our study revealed the presence of *"C. M. haemobos*" among cattle in Peninsular Malaysia. The high prevalence and the wide spread of the *Mycoplasma* species recorded suggests that the organism is of great nuisance to the improvement of cattle production in the country. It also provides a more accurate representation of the prevalence due to the sensitive PCR detection method used as opposed to conventional microscopy used previously. Based on the model of multivariate logistic regression, breed, gender, age and presence of ticks were determined as the potential risk factors associated with the molecular prevalence of *"C. M. haemobos*" in cattle in Peninsular Malaysia.

REFERENCES

Abdullah, D. A., S. D. Ola-Fadunsin, K. Ruviniyia, F. I. Gimba, P. Chandrawathani, Y. A. L. Lim, F. F. A. Jesse & R. S. K. Sharma, 2019. Molecular detection and epidemiological risk factors associated with *Cryptosporidium* infection among cattle in Peninsular Malaysia. *Food and Waterborne Parasitology*, https://doi.org/10.1016/j.fawpar.2019.e00035.

Akinpelu, A. I., 2008. Prevalence and intensity of blood parasites in wild pigeons and doves (Family: Columbidae) from Shasha Forest Reserve, Ile-Ife, Nigeria. *Asian Journal of Animal & Veterinary Advances*, **3**, 109–114.

Atif, F. A., M. S. Khan, F. Muhammad & B. Ahmad, 2013. Sero-epidemiological study of Anaplasma marginale among cattle. *Journal of Animal & Plant Sciences*, **23**, 740–744.

Bauer, N., H. J. Balzer, S. Thüre & A. Moritz, 2008. Prevalence of feline haemotropic mycoplasmas in convenience samples of cats in Germany. *Journal of* Feline *Medicine & Surgery*, **10**, 252–258.

Bell-Sakyi, L., E. B. M. Koney, O. Dogbey & A. R. Walker, 2004. Incidence and prevalence of tick-borne haemoparasites in domestic ruminants in Ghana. *Veterinary Parasitology*, **124**, 25–42.

Carelli, G., N. Decaro, A. Lorusso, G. Elia, E. Lorusso, V. Mari, L. Ceci & C. Buonavoglia, 2007. Detection and quantification of *Anaplasma marginale* DNA in blood samples of cattle by real-time PCR. *Veterinary Microbiology*, **124**, 107–114.

Case, R. J., Y. Boucher, I. Dahllo, C. Holmström, W. F. Doolittle & S. Kjelleberg, 2007. Use of *16S rRNA* and *rpoB* genes as molecular markers for microbial ecology studies. *Journal of Applied Environmental Microbiology*, **73**, 278–288.

Chin, P. L., 2007. Prevalence of blood parasites in UPM cattle. DVM Thesis, Universiti Putra Malaysia, pp. 55–58.

Clarridge, J. E., 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. *Clinical Microbiology Reviews*, **17**, 840–862.

De Eschaide, S. T., M. F. Bono, C. Lugaresi, N. Aguirre, A. Mangold, R. Moretta, M. Farber & C. Mondillo, 2005. Detection of antibodies against *Anaplasma marginale* in milk using a recombinant MSP5 indirect ELISA. *Veterinary Microbiology*, **106**, 287–292.

Dos Santos, A. P., R. P. Dos Santos, A. W. Biondo, J. M. Dora, L. Z. Goldani, S. T. De Oliveira, A. M. de Sá Guimarães, J. J. Timenetsky, H. A. De Morais, F. H. González & J. B. Messick, 2008. Hemoplasma infection in HIV-positive patient, Brazil. *Emerging Infectious Diseases*, **14**, 1922–1924.

Elshafie, E. I., R. A. Sani, L. Hassan, R. Sharma, A. Bashir & I. A. Abubakar, 2013. Seroprevalence and risk factors of *Trypanosoma evansi* infection in horses in Peninsular Malaysia. *Research in* Veterinary Science, **94**, 285–289.

Fujihara, Y., F. Sasaoka, J. Suzuki, Y. Watanabe, M. Fujihara, K. Ooshita, H. Ano &

R. Harasawa, 2011. Prevalence of hemoplasma infection among cattle in the western part of Japan. *Journal of Veterinary Medical Science*, **73**, 1653–1655.

Genova, S. G., R. N. Streeter, K. E. Velguth, T. A. Snider, K. M. Kocan & K. M. Simpson, 2011. Severe anemia associated with *Mycoplasma wenyonii* infection in a mature cow. *Canadian Veterinary Journal*, **52**, 1018–1021.

Girotto, A., A. F. Zangirólamo, A. L. G. Bogado, A. S. L. Souza, G. C. Ferreira da Silva, J. L. Garcia, L. A. Vilas Boas, A. W. Biondo & O. Vidotto, 2012. Molecular detection and occurrence of '*Candidatus Mycoplasma haemobos'* in dairy cattle of Southern Brazil. *Brazilian Journal of Veterinary Parasitology*, **21**, 342–344.

Harmsen, D. & H. Karch, 2004. 16S rDNA for diagnosing pathogens: A living tree. *American Society for Microbiology News*, **70**, 19–24.

Hoelzle, K., R. Hofmann-Lehmann & L. E. Hoelzle, 2010. '*Candidatus Mycoplasma* haemob*os*', a new bovine haemotrophic *Mycoplasma* species? *Veterinary Microbiology*, **144**, 525–526.

Hoelzle, K., M. Winkler, M. M. Kramer, M. M. Wittenbrink, S. M. Dieckmann & L. E. Hoelzle, 2011. Detection of '*Candidatus Mycoplasma haemobos'* in cattle with anaemia. *The Veterinary Journal*, **187**, 408–410.

Hofmann-Lehmann, R., M. L. Meli, U. M. Dreher, E. Gönczi, P. Deplazes, U. Braun, M. Engels, J. Schüpbach, K. Jörger, R. Thoma, C. Griot, K. D. Stärk, B. Willi, J. Schmidt, K. M. Kocan & H. Lutz, 2004. Concurrent infections with vector borne pathogens associated with fatal hemolytic anemia in a cattle herd in Switzerland. *Journal of Clinical Microbiology*, **42**, 3775–3780.

Hornok, S., A. Micsutka, M. L. Meli, H. Lutz & R. Hofmann-Lehmann, 2011. Molecular Investigation of transplacental and vector-borne transmission of bovine haemoplasmas. *Veterinary Microbiology*, **152**, 411–414.

Kaufmann, J., 1996. Parasitic Infections of Domestic Animals: A Diagnostic Manual, Birkhäuser Basel.

Lee, C. C. & L. K. Whitten, 1982. Parasites of domestic animals encountered in routine diagnostic procedures in the Veterinary faculty, University of Agriculture. Malaysia. Malaysia. *Journal of Medical and Health Laboratory Technology, Malaysia*, **8**, 36–39.

Messick, J. B., 2004. Hemotropic mycoplasmas (hemoplasmas) A review and new insights into pathogenic potential. *Veterinary Clinical Pathology*, **33**, 2–13.

Morel, P., 1989. Tick borne diseases, Aberystwyth, UK: Cambrian Printers, United Kingdom.

Morel, P. C., 2000. Maladies à tiques du bétail en Afrique. In: *Précis de parasitology vétérinaire tropicale*, eds C. Chartier, J. Itard, P. C. Morel & P. M. Troncy, Editions Médicales internationales, Cachan, Editions TEC et DOC III, pp. 452–761.

Muhanguzi, D., E. Matovu & C. Waiswa, 2010. Prevalence and characterization of *Theileria* and *Babesia* species in cattle under different husbandry systems in Western Uganda. *International Journal of Animal & Veterinary Advances*, **2**, 51–58.

Neimark, H. & K. M. Kocan, 1997. The cell wall-less rickettsia *Eperythrozoon wenyonii* is a *Mycoplasma*. *FEMS Microbiology Letters*, **156**, 287–291.

Neimark, H., K. E. Johansson, Y. Rikihisa & J. G. Tully, 2001. Proposal to transfer some members of the genera *Haemobartonella* and *Eperythrozoon* to the genus *Mycoplasma* with descriptions of '*Candidatus Mycoplasma haemofelis*', '*Candidatus Mycoplasma haemomuris', "Candidatus Mycoplasma haemosuis*" and *Candidatus Mycoplasma wenyonii'. International Journal of Systematic & Evolutionary Microbiology*, **51**, 891–899.

Neimark, H., K. E. Johansson, Y. Rikihisa & J. G. Tully, 2002. Revision of haemotro-

phic *Mycoplasma* species names. *International Journal of Systematic and Evolutionary Microbiology*, **52**, 683–688.

Nur Mahiza, M. I., 2010. Prevalence of bovine haemoparasites and risk factors associated with *Trypanosoma evansi* infection in Malaysia. MSc Thesis, Universiti Putra Malaysia, pp. 112–124.

Ola-Fadunsin, S. D., A. M. Maizatul, A. R. Ibrahim, A. Amlizawathy, P. Chandrawathani, F. F. A. Jesse, R. A. Sani & R. S. K. Sharma, 2017. Molecular prevalence and species co-infection of bovine haemoparasites in Peninsular Malaysia. *Malaysian Journal of Veterinary Research*, **8**, 13–22.

Ola-Fadunsin, S. D., F. I. Gimba, D. A. Abdullah, R. S. K. Sharma, F. F. A. Jesse & R. A. Sani, 2018. Epidemiology and risk factors associated with *Anaplasma marginale* infection of cattle in Peninsular Malaysia. *Parasitology International*, **67**, 659–665.

Prullage, J. B., R. E. Williams & S. M. Gaafa, 1993. On the transmissibility of *Eperythrozoon suis* by Stomoxys *calcitrans* and *Aedes aegypti*. *Veterinary Parasitology*, **50**, 125–135.

Rowlands, G. J., W. Mulatu, E. Authie, G. D. M. D'leteren, S. G. A. Leak, S. M. Nagda & A. S. Peregrine, 1993. Epidemiology of bovine trypanosomiasis in the Ghibe valley, Southwest Ethiopia. 2. Factors associated with variations in trypanosome prevalence, incidence of new infections and prevalence of recurrent infections. *Acta Tropica*, **53**, 135–150.

Sehgal, R. N. M., H. I. Jones & T. B. Smith, 2005. Blood parasites of some West African rainforest birds. *Journal of Veterinary Medical Science*, **67**, 295–301.

Shahnawaz, S., M. Ali, M. A. Aslam, R. Fatima, Z. I. Chaudhry, M. U. Hassan, M. Ali & F. Iqbal, 2011. A study on the prevalence of a tick transmitted pathogen, *Theileria annulata*, and hematological profile in cattle from Southern Punjab (Pakistan). *Parasitology Research*, **109**, 1155–1160.

Su, Q. L., H. Q. Song, R. Q. Lin, Z. G. Yuan, J. F. Yang, G. H. Zhao, W. Y. Huang & X. Q. Zhu, 2010. The detection of "*Candidatus Mycoplasma haemobos*" in cattle and buffalo in China. *Tropical Animal* Health *& Production*, **42**, 1805–1808.

Sudan, V., R. L. Sharma, S. R. Gupta, M. K. Borah & R. Mishra, 2012. An occurrence of clinical eperythrozoonosis in a German Shepherd dog and its therapeutic management. *Journal of Parasitic Diseases*, **36**, 181–183.

Swai, E. S., E. D. Karimuribo, N. H. Ogden, N. P. French, J. L. Fitzpatrick, M. J. Bryant & D. M. Kambarage, 2005. Seroprevalence estimation and risk factors for *A. marginale* on smallholder dairy farms in Tanzania. *Tropical Animal* Health *and Production*, **37**, 599–610.

Tagawa, M., K. Matsumoto & H. Inokuma, 2008. Molecular detection of *Mycoplasma wenyonii* and '*Candidatus Mycoplasma haemobos'* in cattle in Hokkaido, Japan. *Veterinary Microbiology*, **132**, 177–180.

Tagawa, M., K. Matsumoto, N. Yokoyama & H. Inokuma, 2010. Comparison of the effect of two hemoplasma species on hematological parameters in cattle. *Journal of Veterinary* Medical Science, **72**, 113–115.

Tagawa, M., K. Yamakawa, T. Aoki, K. Matsumoto, m. ishii & h. inokuma, 2013. Effect of chronic hemoplasma infection on cattle productivity. *Journal of Veterinary Medical Science*, **75**, 1271–1275.

Tagawa, M., A. P. Ybanez, K. Matsumoto, N. Yokoyama & H. Inokuma, 2012. Prevalence and risk factor analysis of bovine hemoplasma infection by direct PCR in Eastern Hokkaido, Japan. *Journal of Veterinary Medical Science*, **74**, 1171–1176.

Thrusfield, M., 2005. Veterinary Epidemiology, 3rd edn, Blackwell Science, Oxford, England.

Tortoli, E., 2003. Impact of genotypic studies on mycobacterial taxonomy: The new mycobacteria of the 1990s. *Clinical Microbiology Reviews*, **16**, 319–354.

Willi, B., C. Filoni, J. L. Catão-Dias, V. Cattori, M. L. Meli, A. Vargas, F. Martínez, M. E. Roelke, M. P. Ryser-Degiorgis, C. M. Leutenegger, H. Lutz & R. Hofmann-Lehmann, 2007. Worldwide occurrence of feline hemoplasma infections in wild felid species, *Journal of Clinical Microbiology*, **45**, 1159–1166.

Paper received 24.10.2019; accepted for publication 11.01.2020

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