Original Article

Prevalence and risk factors of Haemotropic *Mycoplasma ovis* infection in selected smallholder sheep and goat flocks in Malaysia

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Abstract

Outbreaks of haemotropic *Mycoplasma ovis* cause haemolytic anaemia with decreased production outcomes and mortality in sheep and goats worldwide but there is a lack of published data on the epidemiology of *M. ovis* in Malaysia's small ruminant flocks. This preliminary study investigates the prevalence and risk factors of *Mycoplasma ovis* infection in selected smallholder farms in Negeri Sembilan state. A total of 341 blood samples was randomly collected from 5 farms between January and December 2019. The farmers also completed a structured questionnaire to provide farm management data and environmental information. Giemsa stained blood smears were examined microscopically to detect *M. ovis* and classify the infection as mild (1-29% infected red cells), moderate (30-59%) or severe (above 60%). The packed cell volume (PCV) was determined by microhaematocrit centrifugation technique and reported as anaemic or non-anaemic. Microscopic examination of blood smears revealed an overall prevalence of 50.7% (95%CI= 45.5-56.0), and there were higher (p<0.05) numbers of mild (34.3%) than moderate (12.6%) and severe (3.8%) infections. The mean PCV was significantly lower (p<0.05) in animals with severe (22.69%) than mild (29.11%) and moderate (30.53%) infections. *Mycoplasma ovis* infection was associated with the breeds physiological status, farm location, management system, farm ownership, frequency of vector control and deworming regime but the risk of infection was higher in Malin sheep (OR=3.97), Boer goats (OR=2.23), pregnant (OR=2.89) and lactating animals (OR= 2.23). The prevalence of *M. ovis* infection in smallholder flocks is associated with the breed and physiological status of sheep and goats and may potentially affect production.

Keywords: Anaemia, *Mycoplasma ovis*, Prevalence, Risk factors, Severity

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Introduction

Haemotropic mycoplasmas (also known as Haemoplasmas) are small wall-less bacteria that inhabit the external surface of erythrocytes in domestic and wild artiodactyl mammals (Messick, 2004; Faraj and Kamal, 2017; Martínez-Hernández *et al.,* 2019). *Mycoplasma ovis* (formerly *Eperythrozoon ovis*) and the related '*Candidatus M. haemoovis*' are the aetiological agents of hemotropic mycoplasmosis in sheep and goats worldwide (Hornok *et al.,* 2009; Stuen, 2016; Aktas and Ozubek, 2017; Machado *et al.,* 2017; Wang *et al.,* 2017). *Mycoplasma ovis* is transmitted mechanically by biting flies such as *Stomoxys calcitrans* and *Melophagus ovinus* (Hornok *et al.,* 2009), occasionally by various hard ticks (Aktas and Ozubek, 2017; Mohd Hasan *et al.,* 2017) and blood-contaminated instruments during flock immunisation, ear-tagging, marking, mulesing and wool shearing operations (Campbell *et al.,* 1971).

Generally, outbreaks of haemotropic mycoplasmosis cause severe anaemia and high mortality in kids and lambs (Hornok *et al.,* 2009; Martínez-Hernández *et al.,* 2019) but clinical disease in adults usually contrasts between an overt life-threatening haemolytic anaemia and subtle chronic anaemia, ill-thrift and infertility (Messick, 2004; Wang *et al.,* 2017). Clinically affected small ruminants show signs of fever, anorexia, pale mucous membranes, lymphadenopathy, rough wool coat, haemoglobinuria and decreased milk production (Faraj and Kamal, 2017). Specific economic loss caused by *M. ovis* infection in small ruminants is due to reduced milk production, an extended duration of fattening, abortion, mortality and the high cost of antibiotic treatment (Sutton and Jolly, 1973; Tagawa *et al.,* 2012; Urie *et al.,* 2019; Wang *et al.,* 2017). Haemotropic mycoplasmosis is endemic in sheep and goat producing areas worldwide and is currently present in Africa (Ilemobade and Blotkamp, 1978; Rjeibi *et al.,* 2015), Asia (Suzuki *et al.,* 2011; Tagawa *et al.,* 2012; Aktas and Ozubek, 2017; Wang *et al.,* 2017), Australia (Sheriff and Geering, 1969; Campbell *et al.,* 1971; Kabay *et al.,* 1991; Mason and Statham, 1991), Europe (Hornok *et al.,* 2009, 2012; Stuen, 2016), South America (Machado *et al.,* 2017; Martínez-Hernández *et al.,* 2019; Souza *et al.,* 2019) and North America (Hampel *et al.,* 2014; Urie *et al.,* 2019).

In Malaysia, clinical cases of haemotropic *Mycoplasma ovis* have been reported in small ruminants (Jesse *et al.,* 2013; Jesse *et al.,* 2015, 2017) and the related *Candidatus* M. wenyonii has also been reported in cattle (Mohd Hasan *et al.,* 2017). To date, there has been no single report on the overall prevalence of haemotropic *Mycoplasma ovis* among sheep and goat flocks under field conditions. This study was designed to investigate the overall prevalence and risk factors of haemotropic *Mycoplasma ovis* among small ruminants in selected smallholder farms focusing on the state of Negeri Sembilan.

Materials and Methods

Ethical approval and consent to participate: The sampling, data collection and laboratory protocols used in this study were approved by the Institutional Animal Use and Care Committee (IACUC), Universiti Putra Malaysia (UPM/IACUC/AUP-R041/2019) and the Department of Veterinary Services Negeri Sembilan state. Following the IACUC guidelines, we also obtained informed consent from selected smallholder farmers before enrolment in this study.

Study area: Negeri Sembilan is on the southwest coast of Peninsula Malaysia (2.7258°N, 101.9424°E) and has 63,673 (12.8% of the entire national stock) individual sheep and goats kept mostly by resource-poor individual smallholders within rural localities. This study collected samples and data from 4 individual smallholders in Lenggeng, Mendon, Senawang and Seremban, and one government farm in Jelebu. The smallholder farms practised semi-intensive management which allowed limited grazing with housing and a small amount of feed supplementation. In contrast, the more organised government farm practised an intensive management system which confines animals to pens without grazing.

Sample Size: We calculated the sample size for this study at a 95% confidence interval (Z=1.96) according to the Thrusfield (2005) formula ($n = Z^2. P (1- P) / d^2$) based on the assumptions of an expected prevalence (P) of 73% (Urie *et al.,* 2019), a 5% desired absolute precision (d) and an extensive small ruminant population in Peninsular Malaysia. Accordingly, the minimum number of samples required to estimate the prevalence of *M. ovis* was 303 but we randomly sampled 341 individual animals from the five selected farms to increase precision.

Study design and sampling: A cross-sectional survey was conducted to collect samples, essential demographic and management data simultaneously. We initially contacted the small ruminant farmers through the Department of Veterinary Services Negeri Sembilan and visited the five farms which agreed to partake in the study to collect blood samples and data from January to December 2019. We conducted a physical examination on individual animals during each farm visit to determine their gender, age, breed, reproductive status, Faffa Malan chart (FAMACHA©) and body condition scores. Animals were aged by dentition and stratified into young (less than one year) and adult (one year and above) categories. We also identified the individual gender and breed of small ruminants using physical characteristics. The colour of the ocular mucous membranes of each animal was examined once and classified according to the FAMACHA© as 1= red (non-anaemic), 2= red-pink (non-anaemic), 3= pink (mildly-anaemic), 4= pink-white (anaemic) and 5= white (severely anaemic) (Kaplan *et al.,* 2004). We determined the body condition scores (BCS) of sheep and goats by palpation to estimate the muscle and fat cover in the lumbar and sternum region and classified them as emaciated (1), thin (2), average (3), fat (4) and obese (5) (Russel, 1984). The farmers also completed a structured questionnaire to furnish data on the farm management type, husbandry, biosecurity and herd health practices for risk analysis. The site of jugular venipuncture was scrubbed with a cotton swab, and five millilitres of

blood sample was collected into labelled EDTA-vacutainer tubes and kept refrigerated in a cold box at 4°C.

Detection of Mycoplasma ovis and evaluation of infection severity: Immediately after blood collection, we prepared two thin smears from each sample on grease-free microscope glass slides (75mm x 25mm). A pinhead-sized drop of blood placed at one end of a labelled glass slide was carefully smeared and dried in air for 30 mins at room temperature. We fixed dried smears with absolute methanol in a Coplin jar for 3 mins and dried in air for 20 mins before staining in filtered 10% Giemsa solution (pH 7.2) for 30 mins (Gulland *et al.*, 1987a). After staining, we examined at least ten high powered bright fields on each blood smear under oil immersion (100x) objective of a compound microscope to detect *Mycoplasma ovis* on the erythrocytes membrane (Sutton and Jolly, 1973). We identified *M. ovis* cells as wall-less coccoid, coco-bacillary, ring, dumb-bell or horseshoe-shaped blue bodies measuring approximately 0.3-3µm, appearing either singly, in chains or as clusters on the erythrocyte membrane and /or freely in the background of the slide (Littlejohns, 1960; Hampel *et al.*, 2014). The per cent infection was calculated as the number of infected cells/800RBC x100, and the severity was reported as mild (1-29% infected cells), moderate (30-59% infected cells) and severe (above 60% infected cells) (Gulland *et al.*, 1987b).

Determination of packed cell volume: Packed cell volume (PCV) was determined by microhematocrit centrifugation technique (Grindem, 2011). Fresh anticoagulated blood in an EDTA vacutainer tube was thoroughly mixed and dispensed into microhaematocrit tubes sealed at one end with plasticine before centrifugation at 620g for 5 mins (Sigma, Osterode, Germany). The height of haematocrit was read on a micro haematocrit reader and recorded as the value of PCV and reported as anaemic (PCV<22% in goats, PCV<27% in sheep) or non-anaemic (PCV>22% in goats, PCV>27% in sheep) (Jackson and Cockcroft, 2007).

Statistical analysis: The prevalence of haemotropic *M. ovis* and corresponding 95% confidence intervals (CI) was calculated using EpiTools® statistical calculators. Field and laboratory data were first summarised in Microsoft Excel Spreadsheet version 2016 and imported into the IBM Statistical Package for Social Sciences Software (SPSS) Version 22.0 (SPSS Inc. Chicago, Illinois) for further analysis. We initially screened for associations between the prevalence of *M. ovis* and assumed risk factors at the individual animal level in a case-control design, where *M. ovis* positive and negative categories were associated with exposure factors by univariable analysis using the Chi-square test at a 5% level of significance. All significant variables (p<0.05) in the univariable analysis were further analysed in a backwards-stepwise (conditional) multivariable logistic regression model and Hosmer-Lemeshow's goodness-of-fit test while controlling for other covariates at p>0.10.

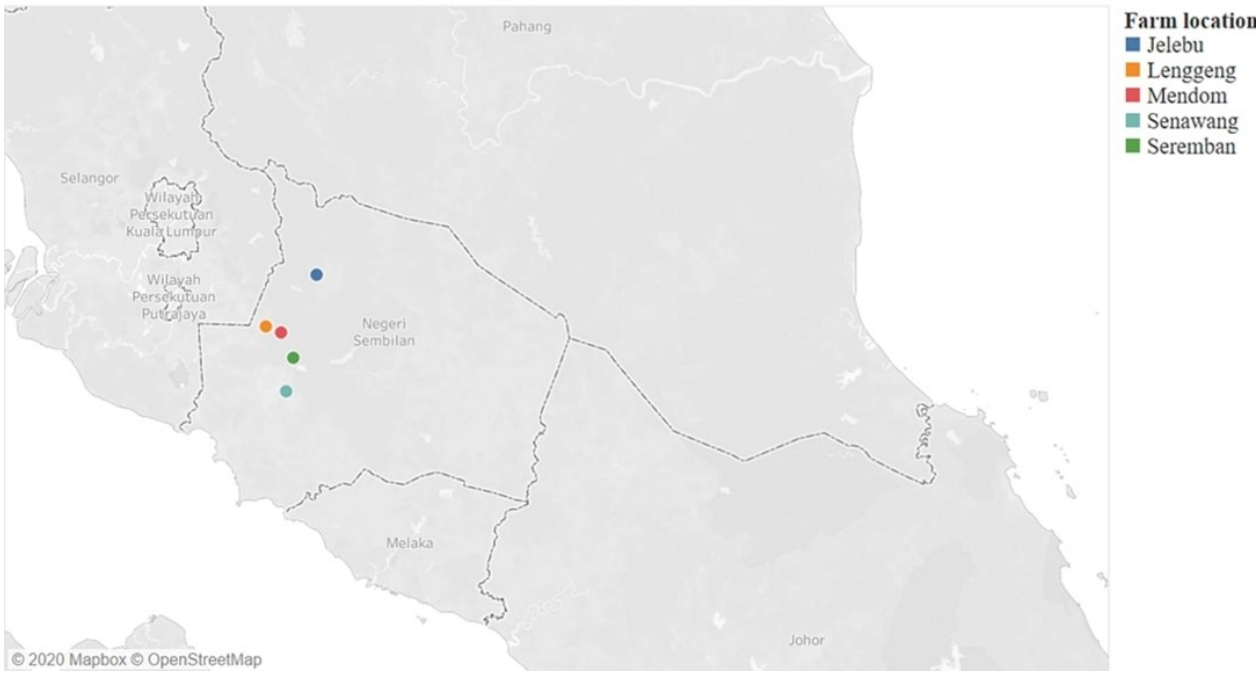

Figure 1 Map of Negeri Sembilan showing the sampling areas.

Results

A total of 341 sheep (n=84) and goats (n=257) were examined in Jelebu (n=60), Lenggeng (102), Senawang (n=55), Seremban (n=75) and Mendom (n=49). Microscopic examination of blood smears revealed that 173 (50.7%; 95% CI=45.45 - 56.00) individual small ruminants in all the herds were positive for *M. ovis* (Table 1). The prevalence of mild infection (67.6%) was significantly (p<0.05) higher than moderate (24.9%) and severe (7.5%) infections. Moreover, the mean packed cell volume was significantly lower (p<0.05) in

severe infections (22.69±1.77) relative to mild (29.11±0.55) and moderate (30.53±1.06) infections (Table 2). Univariable analysis revealed an association between haemotropic *Mycoplasma ovis* infection and breed ($\chi2$= 9.035; p= 0.029), physiological status ($\chi2$= 8.786; p= 0.032), farm ownership ($\chi2$= 5.764; p= 0.016), management system ($\chi2$= 5.764; p= 0.016), frequency of vector control ($\chi2$= 6.039; p= 0.049) and frequency of deworming ($\chi2$= 6.039; p= 0.049) and other exposure factors in Table 3. The multiple logistic regression model revealed 3.97 and 2.23 times more likelihood of *M. ovis* infection among breeds of Malin sheep ($p<0.05$; OR=3.97; 95% CI=1.34-11.72) and Boer goats ($p<0.05$; OR=2.23; 95% CI=1.19-4.18) compared to exotic White Dorper sheep. Likewise, the pregnant ($p<0.05$; OR=2.89; 95% CI=1.23-6.81) and lactating ($p<0.05$; OR=2.23; 95% 1.04-4.79) females had 2.89 and 2.23 times more likelihood of *M. ovis* infection compared to immature animals. Conversely, the Saanen goat breed (p=0.141; OR=1.98; 95% CI=0.79-4.91) and Mating stock (p=0.343; OR=1.33; 95% CI=0.74-2.39) fitted the final model but were not significantly associated (p>0.05) with *M. ovis* infection (Table 4).

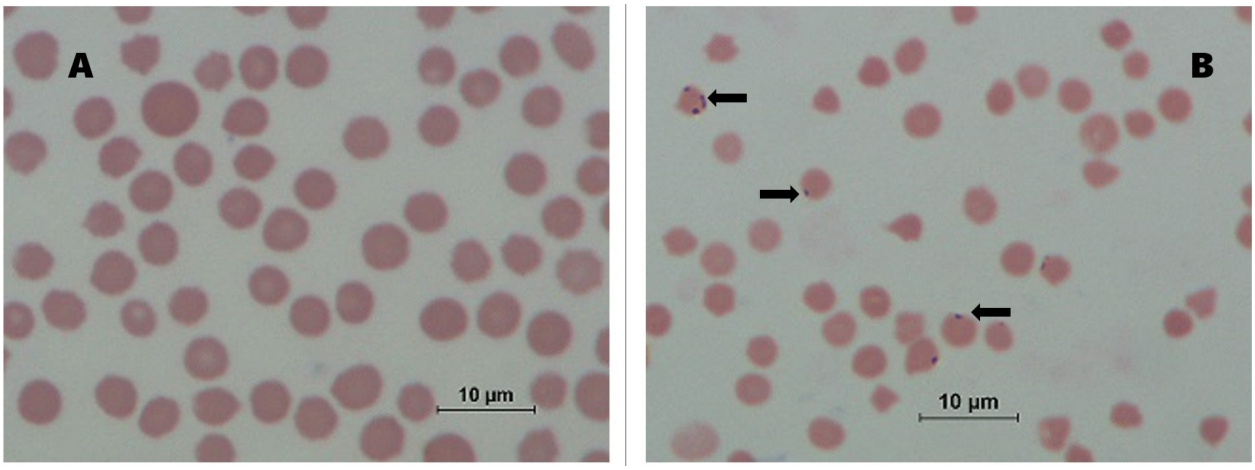

Figure 2 Giemsa stained peripheral blood smears. Plate A (Negative smear) and Plate B (Positive smear) showing *M. ovis* infected red blood cells (arrows).

Table 1 The overall prevalence of haemotropic *M. ovis* in different small ruminant farms.

Farm locations	No of examined	Prevalence (%)	95% CI*
A-Jelebu	60	22 (36.7)	25.62 - 49.32
B-Lenggeng	102	53 (52.0)	42.37 - 61.41
C-Senawang	55	28 (50.9)	38.08 - 63.62
D-Seremban	75	42 (56.0)	44.75 - 66.67
A-Mendom	49	28 (57.1)	43.27 - 69.97
Overall	341	173 (50.7)	45.45 - 56.00

*confidence interval.

Table 2 Distribution of *M. ovis* infection severity and mean PCV in small ruminants.

Severity	N (%)	PCV	
		Mean ± SE	Range
Mild infection	117(67.6)	29.11 ± 0.55[a]	16 – 48
Moderate infection	43(24.9)	30.53 ± 1.06[a]	17 – 46
Severe infection	13(7.5)	22.69 ± 1.77[b]	14 – 36

Mean PCV with superscripts ([a, b]) in column are significantly different (p<0.05).

Table 3 Results of univariable analysis between *M. ovis* infection and exposure factors.

Variables	No. Examined (%)	No. Positive (%)	Chi-square (χ2)	*P*-value
Species				
Sheep	84 (24.6)	39 (46.4)	0.826	0.363
Goats	257 (75.4)	134 (52.1)		
Breed				
White dorper Sheep	60 (17.6)	22 (36.7)	9.035	0.029*
Saanen Goat	33 (9.7)	16 (48.5)		
Boer Goat	224 (65.7)	118 (52.7)		
Malin Sheep	24 (7.0)	17 (70.8)		
Gender				
Male	75 (22.0)	36 (48.0)	0.287	0.592
Female	266 (78.0)	137 (51.5)		
Age				
Young	66 (19.4)	28 (42.4)	2.261	0.133
Adult	275 (80.6)	145 (52.7)		
Body condition score				
Fat	37 (10.9)	16 (43.2)	2.010	0.366
Thin	47 (13.8)	21 (44.7)		
Average	257 (754)	136 (52.9)		
FAMACHA Score				
Severely anaemic	08 (2.3)	4 (50.0)	0.093	0.993
Mildly anaemic	135 (39.6)	68 (50.4)		
Anaemic	189 (55.4)	96 (50.8)		
Non-anaemic	09 (2.6)	5 (55.6)		
[1]PCV Category				
Non-anaemic	290 (85.0)	142 (49.0)	2.424	0.119
Anaemic	51 (15.0)	31 (60.8)		
Physiological status				
Immature	64 (18.8)	27 (42.2)	8.786	0.032*
Mating Stock	179 (52.5)	86 (48.0)		
Lactating	58 (17.0)	32 (55.2)		
Pregnant	40 (11.7)	28 (70.0)		
Farm locations				
A-Jelebu	60 (17.6)	22 (36.7)	6.450	0.168
B-Lenggeng	102 (29.9)	53 (52.0)		
C-Senawang	55 (16.1)	28 (50.9)		
D-Seremban	75 (22.1)	42 (56.0)		
E-Mendom	49 (14.4)	28 (57.1)		
Farm ownership				
Government	60 (17.6)	22 (36.7)	5.764	0.016*
Individual	281 (82.4)	151 (53.7)		
Production purpose				
Dairy	33 (9.7)	16 (48.5)	0.074	0.786
Meat	308 (90.3)	157 (51.0)		
Management system				
Intensive	60 (17.6)	22 (36.7)	5.764	0.016*
Semi-intensive	281 (82.4)	151 (53.7)		
Prophylactic treatment				
No	157 (46.0)	81 (51.6)	0.086	0.769
Yes	184 (54.0)	92 (50.0)		
Frequency of vector control				
Every three months	60 (17.6)	22 (36.7)	6.039	0.049*
Every six months	232 (68.0)	123 (53.0)		
Occasional	49 (14.4)	28 (57.1)		
Frequency of deworming				
Every three months	60 (17.6)	22 (36.7)	6.039	0.049*
Selective	232 (68.0)	123 (53.0)		
Every six months	49 (14.4)	28 (57.1)		
Distance to a water body				
500 – 1000 metres	190 (55.7)	92 (48.4)	0.918	0.338
< 500 metres	151 (44.3)	81 (53.6)		
Distance to forest				
500 – 1000 metres	190 (55.7)	92 (48.4)	0.918	0.338
< 500 metres	151 (44.3)	81 (53.6)		

[1]PCV Category (Jackson and Cockcroft, 2007), *significant (p<0.05)

Table 4 Results of logistical regression analysis showing the risk factors of *M. ovis* infection.

Variables	Categories	B	S E	Wald	df	*P*-value	AOR	95% CI
Breed	Saanen goat	0.683	0.463	2.172	1	0.141	1.98	0.79 - 4.91
	Boer goat	0.803	0.321	6.265	1	0.012*	2.23	1.19 - 4.18
	Malin sheep	1.379	0.553	6.224	1	0.013*	3.97	1.34 - 11.72
Physiological status	Mating stock	0.284	0.299	0.899	1	0.343	1.33	0.74 - 2.39
	Lactation	0.801	0.391	4.199	1	0.040*	2.23	1.04 - 4.79
	Pregnancy	1.062	0.437	5.896	1	0.015*	2.89	1.23 - 6.81

AOR=adjusted odds ratio, 95% CI=95% confidence interval, *P*-values with asterisk (*) are significant.

Discussion

This study investigates the overall prevalence and risk factors of haemotropic *Mycoplasma ovis* in selected smallholder sheep and goat flocks in Negeri Sembilan, Malaysia. Microscopic examination of blood smears revealed an overall prevalence of 50.7% among individual animals and 100% infection in five small ruminant herds. The observed prevalence of haemotropic *M. ovis* in this study is equivalent to the 50% reported in Japan (Tagawa *et al.,* 2012) and 51.5% reported in Hungary (Hornok *et al.,* 2009) and is also analogous to the 45% reported in China (Wang *et al.,* 2017) and Australia (Mason *et al.,* 1989). The high prevalence of *M. ovis* in this study could be linked to the prevailing environment and management practices which favour mechanical transmission via hematophagous arthropods such as Stomoxys, Mosquitoes, Ixodid ticks and sucking lice (Neimark *et al.,* 2001; Hornok *et al.,* 2009; Machado *et al.,* 2017; Wang *et al.,* 2017). Although we did not find ticks and lice on sheep and goats in this study, the prevalence of *M. ovis* may be explained by the results of earlier studies, which reported a high density of biting dipterid fly vectors in Peninsula Malaysia (Chin *et al.,* 2010; Khadijah *et al.,* 2014; Saleeza *et al.,* 2013). Tropical conditions are favourable for the propagation of haematophagous arthropods and account for the high prevalence of vector-borne diseases in the tropics and subtropics (Jongejan and Uilenberg, 2004; Paul *et al.,* 2017). In general, we observed that environmental factors such as high rainfall, temperature, humidity, and the proximity of farms to dense vegetation and water bodies and the crowding of animals in pens without flyscreen are favourable for vector biology and disease prevalence in the study area. Previous studies also showed that management practices such as flock immunisation, ear-tagging, marking, mulesing and wool shearing, which cause bleeding, promote the iatrogenic transmission of *M. ovis* within a flock (Campbell *et al.,* 1971; Philbey *et al.,* 2006). Therefore, the common practice of grazing under semi-intensive management systems, shared needles and tag applicators, inadequate herd health programs and poor knowledge of the disease among the smallholder farmers were significant predisposing factors of *M. ovis* in the study area.

The current prevalence of infection is however considerably lower than previous reports in sheep (Souza *et al.,* 2019), captive deer (Grazziotin *et al.,* 2011[a]) and free-range deer (Grazziotin *et al.,* 2011[b]) due to differences in sensitivity of laboratory diagnostic methods (Hampel *et al.,* 2014), geographical location and possibly species differences in susceptibility to infection. In the past, fatal outbreaks of haemotropic *Mycoplasma ovis* affecting entire flocks of small ruminants have occurred in Australia (Campbell *et al.,* 1971), Germany (Neimark *et al.,* 2004), Hungary (Hornok *et al.,* 2009), Argentina (Aguirre *et al.,* 2009), Japan (Tagawa *et al.,* 2012), Malaysia (Jesse *et al.,* 2015), Tunisia (Rjeibi *et al.,* 2015), Turkey (Aktas and Ozubek, 2017), China (Wang *et al.,* 2017), Iraq (Faraj and Kamal, 2017) and, most recently, in Mexico (Martínez-Hernández *et al.,* 2019). Such outbreaks usually result from immunosuppressive conditions such as malnutrition, handling stress, unfavourable weather conditions and the presence of concurrent infectious diseases (Boes and Durham, 2016; Paul *et al.,* 2020).

This study further showed that breed and physiological status of small ruminants are associated with a higher risk of *M. ovis* infection. Breed and physiological status are important intrinsic factors determining host susceptibility to infectious agents and their role in disease dynamics (Thrusfield, 2005). The higher exposure of indigenous Malin sheep agrees with a previous study that reported breed differences in *M. ovis* infection prevalence in small ruminants (Hampel *et al.,* 2014). Similarly, the higher risk of *M. ovis* infection observed in pregnant and lactating animals in this study was previously linked to stress caused by negative nutrition and weak immune states experienced by females during pregnancy and lactation (Gulland *et al.,* 1987b; Fitzpatrick *et al.,* 1998; Hornok *et al.,* 2009).

The higher prevalence of mild subclinical *M. ovis* infection observed among small ruminants in this study is consistent with Hampel *et al.* (2014), who reported a high prevalence of the subclinical disease in apparently healthy sheep. Usually, field infections of small ruminants are chronic and characterised by mild bacteraemia and regenerative anaemia due to low pathogenicity of *Mycoplasma ovis* (Gulland *et al.,* 1987b; Fitzpatrick *et al.,* 1998; Hornok *et al.,* 2009; Porter and Kaplan, 2011; Machado *et al.,* 2017). As previously reported, *M. ovis* severity has been associated with the mean PCV of small ruminants such that a lower PCV was recorded in severely infected animals. Haemolytic anaemia is recognised as a significant manifestation of hemotropic *M. ovis* (Neitz *et al.,* 1934; Campbell *et al.,* 1971; Daddow, 1977, 1979) due to distortion of the erythrocyte membrane (Gulland *et al.,* 1987a), increased membrane fragility (Hampel *et al.,* 2014; Norris *et al.,* 1987), agglutination of red blood cells (Kanabathy and Nachiar, 2004), erythrophagocytosis (Philbey *et al.,* 2006), oxidative injury, enzymatic lysis and the disruption of cell functions (Theiss *et al.,* 1996).

The high prevalence of haemotropic *Mycoplasma ovis* among smallholder flocks in Negeri Sembilan is associated with sheep and goat breed and physiological status. Selective breeding of resistant

stock and proper nutritional management of pregnant and lactating animals combined with vector control, routine deworming and prophylactic treatment will reduce the impact of *M. ovis* on production outcomes of sheep and goats in the study area.

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