Malays. Appl. Biol. (2022) 51(3): 107-115
https://doi.org/10.55230/mabjournal.v51i3.2260

MULTIPLE ANTHELMINTIC RESISTANCE AMONG DORPER SHEEP DETECTED WITH PHENOTYPIC MARKERS AGAINST PARASITIC GASTROENTERITIS

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Accepted 18 August 2022, Published online 30 September 2022

ABSTRACT

Parasitic gastroenteritis (PGE) is a significant disease that affects small ruminant production. PGE is controlled exclusively by chemical anthelmintics but restricted by anthelmintic resistance. Hence, dependence on anthelmintics needs to be reduced. This study aimed to investigate the anthelmintic resistance status of a Dorper sheep farm while determining the phenotypic markers of resistance to PGE. Sheep that met the criteria of the Faecal Egg Count Reduction Test (FECRT) were divided into a control and four treatment groups of 11 to 13 animals per group. Faecal and blood samples at pre- and post-treatments were subjected to faecal egg counts (FEC), faecal culture, packed cell volume (PCV) and peripheral eosinophil counts (PEC). The data were analysed by Spearman rank correlation and two-way ANOVA. FECRT showed resistance towards albendazole, levamisole, fenbendazole and ivermectin which was predominated by *Haemonchus contortus*. Significant negative correlations were observed between FEC and PCV in control (r=-0.88, p<0.01), fenbendazole (r=-0.58, p<0.01) and ivermectin (r=-0.69, p<0.01) groups. Significant positive correlations were detected between FEC and PEC in control (r=0.95, p<0.01) and levamisole (r=0.56, p<0.01) groups. This study shows multiple anthelmintic resistance with promising resistant characteristics against PGE among sheep.

Key words: Anthelmintic resistance, parasitic gastroenteritis, phenotypic markers, faecal egg count, packed cell volume, peripheral eosinophil counts

INTRODUCTION

Parasitic gastroenteritis (PGE) is known to be one of the major threats in small ruminant production worldwide due to high morbidity and mortality. However, indiscriminate use of anthelmintics has led to the development of anthelmintic resistance with rapid escalating trends (Chandrawathani & Nurul Aini, 2012; Premaalatha *et al*., 2014; Mat Yusof & Md Isa, 2016; Peña-Espinoza, 2018; & Kelleher *et al*., 2020) Thus, sustainable control approaches of PGE such as the adoption of resistant host breed could be implemented to reduce the dependence on chemical anthelmintics.

In Malaysia, sheep were reported to have the highest infection rate of gastrointestinal nematode (91.4%) compared to goats (83.7%) and deer (25%) (Tan *et al*., 2012). Recent studies conducted in small ruminant farms in Penang and Perak, Malaysia have also shown that gastrointestinal nematode was the most common parasite found in goat and sheep populations (Khor *et al*., 2018; Azima *et al*., 2020). The predominant gastrointestinal nematode among small ruminants in this country is *Haemonchus contortus* (Khadijah *et al*., 2006). Loss of body weight, anaemia, lethargy, inappetence, dehydration, oedema and death in heavy nematode burden are the common signs of this bloodsucker infection (Besier *et al*., 2016; Emery *et al*., 2016).

Studies in the early 1990s revealed benzimidazole resistance on 33 out of 96 goat farms throughout Peninsular Malaysia (Dorny *et al*., 1994) and four sheep farms (Pandey *et al*., 1994) in Malaysia. Another nematode resistance against benzimidazole was also shown on 50% of the sheep farms located in Perak, Kelantan, Kedah, Terengganu, Johor, Pahang and Penang and 75% of the goat farms in Perak,

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Kelantan, Johor and Negeri Sembilan in late 1990s (Chandrawathani *et al*., 1999). Three years study on a government farm in Kedah, Malaysia from 1997 to 2000 reported that nematode resistance among small ruminants was worsened towards benzimidazole, levamisole, and ivermectin, including suspected resistance to moxidectin (Chandrawathani *et al*., 2003).

A study conducted on a sheep farm owned by a government agency in Johor, Malaysia showed suspected gastrointestinal nematode resistance to ivermectin, closantel and cydectin with complete resistance to fenbendazole (Sabariah *et al*., 2017). Later on, a commercial sheep farm in Terengganu, Malaysia has also been reported to develop total nematode resistance towards albendazole, fenbendazole, levamisole and ivermectin (Khadijah *et al*., 2018).

Apart from Peninsular Malaysia, complete nematode resistance to benzimidazole, imidazothiazole, macrocyclic lactone and salicylanilide anthelmintics in sheep and goat farms has been found in Sabah (Chandrawathani *et al*, 2004). Besides, studies showing nematode resistance to common anthelmintics among goat populations have been reported in neighbouring states namely Kelantan and Perak (Basripuzi *et al*., 2012; Premaalatha *et al*., 2019).

Dorper is among the sheep breeds that are frequently evaluated for resistant traits against PGE. From the previous studies, this breed has been reported to be either resilient (Baker *et al*., 2003), resistant (Burke *et al*., 2002) or susceptible (Matika *et al*., 2004; Mugambi *et al*., 2004) to PGE. Thus, the resistance status of this breed against PGE is still unclear for both pure and crossbreeds.

The correlations of faecal egg count (FEC) as the most important phenotypic marker of resistance against PGE with packed cell volume (PCV) and peripheral eosinophil counts (PEC) have been studied among naturally and deliberately infected Boer goats reared in tropical and temperate regions, respectively (Basripuzi *et al*., 2018; Hayyan *et al*., 2020). PCV is used for the measurement of red blood cells to determine anaemia. Low PCV is commonly related to high FEC due to the blood-sucking activity of adult *H. contortus* in the abomasa of infected ruminants (Baker *et al*., 2003). PEC represents the immune responses against parasite infection in the circulating blood. In Scottish Blackface sheep, high PEC is associated with low FEC due to a high immune response against *Teladorsagia circumcincta*, the most predominant gastrointestinal nematode in temperate countries (Stear *et al*., 2002). However, high PEC is associated with high FEC in Boer goats that were experimentally infected with *H. contortus* (Basripuzi *et al*., 2020). These studies suggest that PEC plays an important role in combating PGE in sheep but not in goats.

This study was conducted to investigate the anthelmintic resistance status in a semi-intensive Dorper sheep farm located in Perlis, the northeast and smallest state of Peninsular Malaysia where anthelmintic resistance status is unknown. The correlations between the phenotypic markers of resistance to gastrointestinal nematode infection that include FEC, PCV and PEC were also determined. This is because positive correlations are expected to occur between FEC and PCV; while negative correlations are expected between the FEC and PEC among resistant host breeds.

MATERIALS AND METHODS

Study site and animals

This study was conducted on a Dorper sheep farm with a population of 200 sheep. The farm is located in Perlis, a state in Northern Malaysia. The sheep were imported from Australia, used as breeders and raised in a semi-intensive management system where the flock were released to the grazing area during the daytime and kept in a slatted floor house with a shed at night.

The application for animal ethical clearance of this study was approved by the Animal Ethics Committee of Universiti Malaysia Kelantan (UMK/FPV/ACUE/RES/2/2020). Forty per cent of the sheep population on the farm (n=80) were selected For Faecal Egg Count Reduction Test (FECRT) according to the method outlined by the World Association for the Advancement of Veterinary Parasitology (Coles *et al*., 1992; 2006). This includes the criteria of sheep with no history of anthelmintic treatments for the previous eight weeks before the commencement of the study.

For the past four years of the herd health program on the farm, the flock were treated with chemical anthelmintics namely fenbendazole, albendazole and ivermectin to control PGE. The detailed frequency of anthelmintics used in this farm is presented in Table 1.

Faecal egg count

Faecal samples were collected per rectum from 80 Dorper sheep and screened for the presence of nematode eggs. The original McMaster's technique (Gordon & Whitlock, 1939) was modified to determine the FEC. Each faecal sample weighed 3 g was mixed with 45 mL saturated sodium chloride solution (SG=1.2), filtered and used to fill two McMaster chambers. The gastrointestinal nematode eggs were identified as having broad ellipse, thin-shelled, barrel-shaped side walls and containing blastomeres. The eggs were identified and enumerated under the microscope within the ruled areas of both McMaster chambers (Chandrawathani *et al*., 2019).

Table 1. Anthelmintics and frequency of administration in the farm from 2017 to 2020

Year	Anthelmintic	Frequency of administration
2017	Fenbendazole	Once
2018	Fenbendazole	Twice
	Albendazole	Once
	Ivermectin	Once
2019	Fenbendazole	Once
	Albendazole	Twice
	Ivermectin	Once
2020	Albendazole	Once
	Ivermectin	Twice

Faecal egg count reduction test

The method for Faecal Egg Count Reduction Test (FECRT) was conducted following Coles *et al*. (1992; 2006). Fifty-eight out of 80 sheep were detected with more than 150 epg of faeces and thus subjected to FECRT. The sheep were then divided into a control and four treatment groups namely albendazole, fenbendazole, levamisole and ivermectin with 11 to 13 animals in each group. The average mean of FEC for each group was approximately similar that were ranged between 979 and 1295 epg. The dosages and modes of administration of the anthelmintics are shown in Table 2.

The second faecal sampling was conducted after 14 days to obtain post-treatment FEC. The percentage of FECRT was calculated as 100 (1- Xt / Xc) where Xt is the mean epg of post-treatment in the treated group and Xc is the mean epg of post-treatment in the control group. The gastrointestinal nematode population will be classified as 'resistant' if the Faecal Egg Count Reduction percentage (FECR%) is less than 95% and the confidence level of 95% is less than 90% (Coles *et al*., 1992). In addition, resistance is considered critical, severe and moderate when FECR% is less than 50%, 50-90% and 91-95% respectively (Khadijah *et al*., 2006; Basripuzi *et al*., 2012).

Faecal culture, identification and enumeration of L3

The preparation of pre-and post-treatment faecal culture for control and treatment groups was following Hayyan *et al*. (2020). The identification and enumeration of L3 were conducted according to the Ministry of Agriculture, Fisheries and Food of Great Britain (1986).

Peripheral eosinophil counts

Carpentier's eosinophil counting solution was prepared by adding 3 mL of 40% formaldehyde saturated with calcium carbonate, 2 mL of 2% aqueous solution of Eosin Y and 95 mL of distilled water. A blood sample of 10 µL was preserved in 90 µL of Carpentier's solution and stored at room temperature. The PEC was counted in a haemocytometer under a microscope with one cell calculated as 5.6 cells per µL of whole blood (Stear *et al*., 2002).

Packed cell volume

The PCV was measured by filling the blood into glass capillary tubes and sealed with cristaseal. The tubes were centrifuged at 220 rcf for 5 min and the volume was measured by a rotoreader (Basripuzi *et al*., 2018).

Statistical analysis

R software version 4.1.1 was used for statistical analysis. Data of the study were analyzed for descriptive statistics such as means, standard deviations, variance, minimum values, maximum values and skewness of FEC, PCV and PEC. Shapiro-Wilk test was used to check the normality of the data. Correlations between the means of FEC, PCV and FEC of each group were analysed using Spearman rank correlation. Kruskal-Wallis test was used to determine the presence of a significant difference in FEC between groups. Then, the Wilcoxon rank sum test was used for pairwise comparison between pre- and post-treatments of albendazole, fenbendazole, levamisole, ivermectin and the control group at a significance level of $p<0.05$.

Table 2. Anthelmintic dosages and modes of administration for Faecal Egg Count Reduction Test

Group	Anthelmintic used	Dosage	Mode of administration
1	Control	-	-
2	Albendazole	7.5 mg/kg	Oral
3	Fenbendazole	5 mg/kg	Oral
4	Levamisole	7.5 mg/kg	Oral
5	Ivermectin	0.2 mg/kg	Subcutaneous

Table 3. Descriptive statistics and distributions of overall mean faecal egg count, packed cell volume and peripheral eosinophil counts

Phenotypic marker	SD[1]	Minimum	Maximum	Prob (Norm)[2]	Skewness
FEC[3]	1003.38	0.0	5450.0	<0.001	2.0
PCV[4]	5.97	13	42	0.039	0.2
PEC[5]	63.14	0.0	453.6	<0.001	3.9

[1]Standard deviation; [2]Probability that the distribution was significantly different from normal distribution by Shapiro-Wilk test (p<0.05); [3]Faecal egg counts; [4] Packed cell volume; [5] Peripheral eosinophil counts

RESULTS

Faecal egg counts, packed cell volume and peripheral eosinophil counts

The pre-and post-treatment of FEC, PCV and PEC ranged from 0 to 5450 epg, 13 to 42% for PCV and 0 to 453.6 cells/ µL respectively (Table 3). The FEC and PEC showed a highly significant (p<0.001) difference from a normal distribution and showed positive skewness. The PCV was also significantly different from the normal distribution (p<0.05) but showed a slight positive skewness.

Faecal egg count reduction test

The mean FEC of all anthelmintic groups reduced after the treatment except in the ivermectin group (Table 4). However, all anthelmintics tested had 95% lower confidence limits that were less than 90%. Albendazole and levamisole showed FECR% between 50 and 90% indicating a severe degree of resistance. Fenbendazole and ivermectin showed FECR% of less than 50% indicating the critical level of resistance.

FEC decreased in all treatment groups except for the ivermectin group which has the same trend as the control group (Figure 1); suggesting ivermectin as the most ineffective anthelmintic in this study. The figure also shows that levamisole group has the lowest post-treatment FEC. Furthermore, the post-treatment FEC of the levamisole group does not overlap with its pre-treatment FEC suggesting levamisole as the most effective anthelmintic in this study.

In addition, the Kruskal-Wallis test showed that there were significant differences in FEC between pre- and post-treatments ($H(1)=5.29$, $p=0.022$) and FEC between different anthelmintic groups (control, albendazole, fenbendazole, levamisole, ivermectin) ($H(4)=10.20$, $p=0.04$). The pairwise comparisons by the Wilcoxon rank sum test showed a nearly significant difference between the pre-and post-treatment FEC of the levamisole group in comparison to the control

Table 4. Status and level of anthelmintic resistance in the sheep farm

Treatment group	N[1]	Status	Level	Mean FEC[2]		FECR[5] (%)	95% CI[6]	
				Pre-[3]	Post-[4]		Lower	Upper
Levamisole	12	Resistance	Severe	1192	146	88	70	96
Albendazole	11	Resistance	Severe	1295	609	52	-1	77
Fenbendazole	12	Resistance	Critical	1175	725	43	-31	75
Ivermectin	11	Resistance	Critical	1294	1841	-45	-250	40

[1]No of animals; [2]Faecal egg counts; [3]Pre-treatment; [4]Post-treatment; [5]Faecal egg count reduction; [6]CI = confidence interval

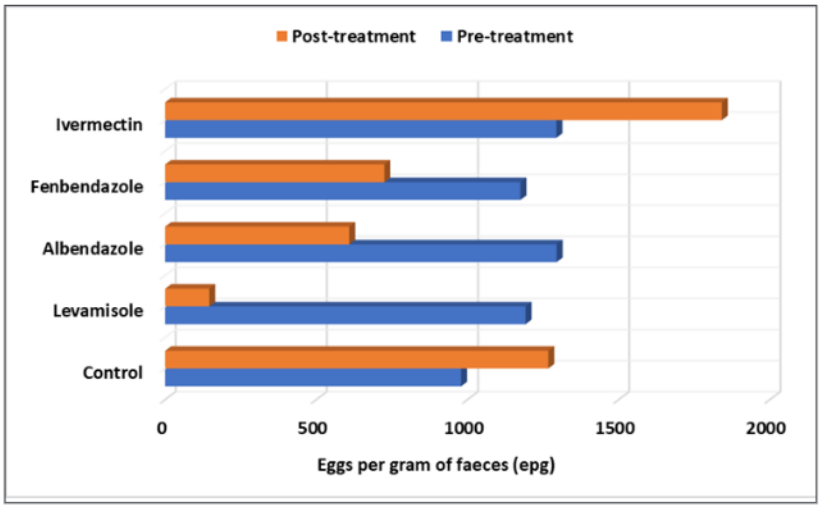

Fig. 1. Pre-treatment and post-treatment faecal egg counts of Dorper sheep treated with different anthelmintics.

and ivermectin groups (p=0.052). However, there was no significant difference between the pre-and post-treatments of FEC for albendazole, fenbendazole, and ivermectin groups in comparison to the control group.

Identification of L3 from pre- and post-treatment faecal culture

Pre-treatment faecal culture showed that *H. contortus* was the most predominant L3 (88%) followed by *Trichostrongylus* sp. (6%) and *Oesophagostomum* sp. (6%). The L3 percentage reduction for *H. contortus* were 10% and 22% following fenbendazole and levamisole treatment respectively (Figure 2). However, the percentage remained the same after albendazole treatment (93%) and increased by 1% in ivermectin group during post-treatment. The percentage of *Trichostrongylus* sp. L3 decreased by 2% and 1% in the albendazole and ivermectin group during post-treatment respectively, but increased to 9% in the fenbendazole group and maintained 8% in the levamisole group after the treatment. On the other hand, the L3 *of Oesphagostomum* sp. rose by 2%, 7% and 22% following albendazole, fenbendazole and levamisole treatments but remained the same after ivermectin treatment (5%).

Correlations between faecal egg counts, packed cell volume and peripheral eosinophil counts

There were significant correlations among FEC, PCV and PEC in the same groups (Table 5). FEC showed significant negative correlations to PCV in control, fenbendazole and ivermectin groups at -0.88,

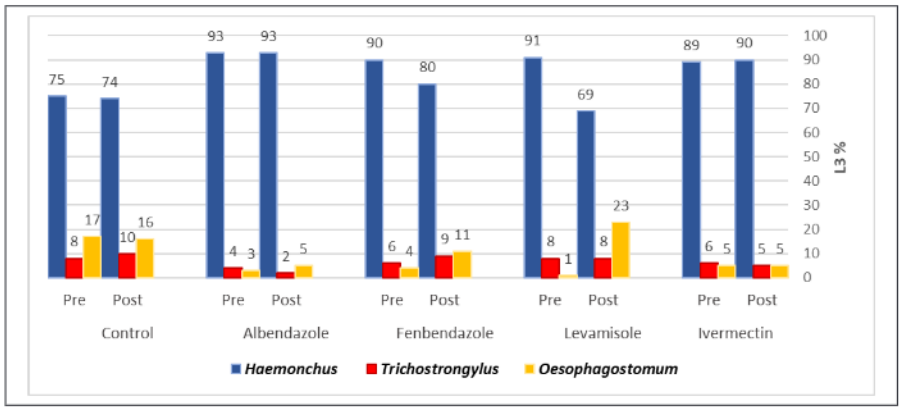

Fig. 2. Pre-treatment and post-treatment percentages of different L3 genera in control and treatment groups.

Table 5. Spearman rank correlation of post-treatment faecal egg count, packed cell volume and peripheral eosinophil counts in control and treatment groups

	F1[4]	F2[5]	F3[6]	F4[7]	F5[8]	P[2]1	P2	P3	P4	P5	E[3]1	E2	E3	E4
F2	0.01													
F3	0.73 **	0.21												
F4	0.43	-0.35	0.36											
F5	0.67 **	0.28	0.77 **	0.01										
P1	-0.88 **	0.15	-0.78 **	-0.51	-0.51									
P2	-0.37	-0.30	-0.80 **	-0.34	-0.43	0.51								
P3	-0.50	-0.18	-0.58 *	-0.05	-0.31	0.55 *	0.32							
P4	-0.81 **	-0.18	-0.72 **	0.01	-0.78 **	-0.71 **	0.27	0.69 **						
P5	-0.54 *	-0.09	-0.78 **	-0.12	-0.69 **	0.53 *	0.42	0.62 *	0.71 **					
E1	0.95 **	0.13	0.78 **	0.49	0.74 **	-0.89 **	-0.44	-0.38	-0.75 **	-0.55 **				
E2	-0.71 **	0.31	-0.34	-0.53 *	-0.31	0.70 **	0.11	0.03	0.36	-0.01	-0.78 **			
E3	-0.01	0.04	0.25	-0.29	0.37	0.05	-0.04	-0.16	-0.25	-0.71 **	0.04	0.37		
E4	0.68 **	-0.02	-0.91 **	0.56*	0.55*	-0.81**	-0.72 **	-0.56 *	-0.59 *	-0.75 *	0.74 **	0.41	0.26	
E5	-0.88 **	0.06	-0.42	-0.21	-0.53 *	0.69*	0.04	0.48	0.75 **	0.37	-0.78 **	0.59 *	0.04	-0.36

[1]Faecal egg counts; [2]Packed cell volume; [3]Peripheral eosinophil counts; [4]Control group; [5]Albendazole group; [6]Fenbendazole group; [7]Levamisole group; [8]Ivermectin group; *p<0.05; **p<0.01

-0.58 and -0.69 respectively ($p<0.01$) and significant positive correlations to PEC in control and levamisole groups at 0.95 ($p<0.01$) and 0.56 ($p<0.05$). PCV in the control group had a high negative correlation with PEC in the control and levamisole groups at -0.89 ($p<0.01$) and -0.81 ($p<0.05$), respectively. There were significant negative correlations between PEC in the levamisole group and PCV in all treatment groups. (-0.81, -0.72, -0.56, -0.59, -0.75; $p<0.05$). The same trend of correlations was also displayed by FEC in the fenbendazole group and PCV in all treatment groups at -0.78, -0.80, -0.58, -0.72 and -0.78 ($p<0.01$).

DISCUSSION

The phenomenon of multiple anthelmintic resistance among small ruminants has been alarming not only in Malaysia but also in other parts of Asia (Bihaqi *et al*., 2020). This has been shown in the present study which strongly displayed the consequences of frequent and improper use of fenbendazole, albendazole and ivermectin in this farm where all anthelmintics tested were confirmed with resistant status (Table 4). The sheep have been treated three to four times a year with at least two types of anthelmintics since 2018 (Table 1). According to Abbott *et al*. (2012), the more frequent treatment given, the faster anthelmintic resistance develops. This is because frequent treatment gives less opportunity for the susceptible nematodes to produce eggs and eventually leads to pasture contamination with eggs from resistant nematodes.

Additionally, anthelmintics from benzimidazole and macrocyclic lactone groups were used for three consecutive years on this farm. It has been shown by Khadijah *et al*. (2018) that exposure of nematodes to the same class of anthelmintic for a long period resulted in the selection of resistant nematodes. This explains the results of critical to severe degree of resistance to fenbendazole, albendazole and ivermectin. Other factors that may contribute to this problem were the under-dosing of hosts and prophylactic mass treatment that were commonly conducted on this farm. Sub-optimal doses allow the survival of heterozygous resistant nematodes while mass treatment with more than 80% of the sheep population contributes to the rapid development of anthelmintic resistance (Shalaby, 2013).

Nevertheless, the status of levamisole is questionable as this anthelmintic has never been used for a parasite control program on this farm. Since the Dorper sheep raised on this farm were originated from Australia, there was a possibility that the sheep have been treated with imidazothiazoles based products in their originated farm before the importation to Malaysia which eventually led to the resistant status of levamisole in the present study.

Identification of the L3 genus from the post-treatment faecal culture suggests that levamisole was the only effective anthelmintic in combatting PGE that was due to *H.contortus* infection. A study conducted by Basripuzi *et al*. (2012) in Kelantan, Malaysia also showed that levamisole has the highest effectiveness against *H. contortus* as opposed to the other anthelmintics such as benzimidazole, ivermectin and closantel. *Trichostrongylus* sp. was the only L3 genus that was susceptible to albendazole treatment in this study. However, *Oesophagostomum* sp. was resistant to all anthelmintic groups. Hence, the effectiveness of the other class of anthelmintics such as salicylanilides should be investigated to combat *Oesophagostomum* sp. infection in this farm.

As expected, correlation analysis shows that high FEC is associated with low PCV and high PEC within the same treatment group. Low PCV among sheep with high FEC is due to the pathogenicity of *H. contortus* from their haematophagous activity that subsequently causes anaemia (Alam *et al*., 2020). The immune response of eosinophilia among induced infected sheep plays an important role to fight against the gastrointestinal nematodes, particularly *H. contortus* (Balic *et al*., 2006; Terefe *et al*., 2009). However, the mechanisms of eosinophil action were reported to vary between host species (Jenvey *et al*., 2020; Hayyan *et al*., 2020) and it is species-specific (Henderson *et al*., 2006).

Since the Dorper sheep were co-infected by *H. contortus*, *Trichostrongylus* sp. and *Oesophagostomum* sp., interactions between the phenotypic markers of resistance (eg. FEC, PCV & PEC) and the variables of adult nematodes (eg. length and number) could be investigated. In Boer goats, negative relationships were found between the composite burden and number of *H. contortus* with the length of *Trichostrongylus colubriformis* thus affecting the egg deposition of both nematode species for FEC (Basripuzi *et al*., 2020).

CONCLUSION

In conclusion, multiple anthelmintic resistance against PGE has been detected in a semi-intensive farm in the Malaysian northern region where the imported sheep were kept as breeders. Ineffective control of anthelmintic resistance that leads to PGE could result in the loss of body weight among the sheep thus producing incompetent breeders. Nonetheless, the Dorper sheep breed has a potential resistance against PGE based on the findings on phenotypic markers but further investigation is needed for confirmation. New control strategies should be implemented on the farm to tackle the anthelmintic resistance problems. Semi-intensive systems could be replaced with an intensive system where the sheep were to be kept 24 hours in their shed to prevent infection during grazing. Instead of carrying unnecessary anthelmintic treatments, the animal should be treated with Strategic Prophylactic Treatments (SPTs) based on FEC monitoring and

FAMACHA chart to reduce the risk of resistance (Abbott *et al*., 2012). In the future, the criteria for sheep importation must be evaluated as well to prevent the occurrence of anthelmintic resistance that arise from the country or farm of animal origin.

ACKNOWLEDGEMENTS

This study was supported by the UMK Fundamental Research Grant (UMK-FUND) from Universiti Malaysia Kelantan (R/FUND/A0600/00520A/001/2020/00740). We would also like to acknowledge the Federal Land Development Authority (FELDA) for its collaboration in this research.

ETHICAL STATEMENT

This study was approved by the Animal Ethics Committee of Universiti Malaysia Kelantan (UMK/FPV/ACUE/RES/2/2020)

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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