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Nematode control failure due to anthelmintic resistance in a sheep farm in Malaysia: First identification of the F200Y mutation in the isotype 1 β-tubulin gene

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Abstract. This paper reports total nematode anthelmintic resistance towards albendazole, fenbendazole, levamisole and ivermectin in a commercial sheep farm located in Terengganu, Malaysia. Faecal Egg Count Reduction Test (FECRT) was conducted on 25 sheep, where five sheep in each group were treated with the respective four anthelmintics based on live bodyweight. The balance of five sheep placed in the control group were not treated with any anthelmintics. At day 13 post-treatment, faecal egg count was conducted and nematode worm egg count reduction percentage was calculated to determine the resistance status towards the respective anthelmintics tested. Results showed that nematodes were resistant to all the anthelmintics tested, namely albendazole, fenbendazole, levamisole and ivermectin with reduction percentage of 87%, 46%, 94% and 68%, respectively. Subsequently, the third stage larvae of *Haemonchus contortus* and *Trichostrongylus colubriformis* recovered from post-treatment faecal cultures were subjected to allele-specific polymerase chain reaction (AS-PCR) assay to determine the presence of the benzimidazole resistance gene. This study reports the occurrence of the classical F200Y mutation in the isotype 1 β-tubulin gene, for the first time in Malaysia.

INTRODUCTION

In September 2017, it was recorded that the mortality of sheep in a particular farm located in Terengganu, Malaysia was 30 sheep in that month. In addition, other live sheep in that farm were observed to have symptoms of nematode infection, namely pale ocular mucous membrane (FAMACHA© score of 4), loose faeces and were weak. Total worm count was conducted on the abomasum of one dead sheep and a total of 4,800 *Haemonchus contortus* were found. Anthelmintic resistance was suspected since worm infection symptoms were still observed in some of the infected sheep despite being dewormed with fenbendazole in August 2017, approximately one month before. This farm only used fenbendazole for worm control and used ivermectin for ectoparasite control. The frequency of deworming on this farm is twice in 6 months. They depended on the officers from the Department of Veterinary Services for

treating the animals. This study was carried out to investigate the status of nematode anthelmintic resistance towards different anthelmintics, namely fenbendazole, albendazole, levamisole and ivermectin in this affected sheep farm.

MATERIALS AND METHODS

Screening

Prior to the Faecal Egg Count Reduction Test (FECRT), rectal faecal samples were taken from 70 sheep to screen for nematode worm egg count. Modified McMaster Method (M.A.F.F., 1986) was used to determine nematode worm egg count. Animals with nematode worm egg count higher than 200 eggs per gram of faeces (e.p.g.) were recruited for further study, as suggested by Coles *et al.* (1992). The larval culture and identification (M.A.F.F., 1986) was performed simultaneously to determine the genus of nematode present in the sheep. Animals selected in this study were not treated with any anthelmintics in the previous 8 weeks as suggested by Coles *et al.* (1992).

Treatment

Faecal Egg Count Reduction Test (FECRT) was conducted following the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines methods for the detection of anthelmintic resistance in nematodes of veterinary importance (Coles *et al.*, 1992) on 25 sheep selected based on the egg count screening results. The sheep were randomly divided into five groups, with five sheep per group for Control, Albendazole, Fenbendazole, Levamisole and Ivermectin. The anthelmintics were chosen based on the availability of anthelmintics at the Health Unit, Department of Veterinary Services Terengganu because they were responsible for deworming sheep and goats in Terengganu following the requests from farmers.

The sheep from the five groups were weighed individually and treated accordingly based on their groups. The dosage for each anthelmintic treatment was calculated based on the live weight of individual sheep and following the manufacturer's recommended dosage. Sheep from the control group were not treated with any anthelmintics. Information on each anthelmintic used in this study is summarised in Table 1.

Post-treatment

On day 13 after treatment, rectal faecal sample was obtained from each animal. Three grams of faeces from each animal were subjected to modified McMaster method (M.A.F.F., 1986) while 1 gram of faeces from each animal were pooled following their respective groups and were used in larval culture (M.A.F.F., 1986) for L_3 identification up to genus level (M.A.F.F., 1986).

Data analysis

Nematode worm egg count reductions were calculated following the formula provided by Coles *et al.* (1992) to determine the resistance status towards the anthelmintics tested;

Table 1. Information on the anthelmintics - brands, active ingredients, dosages and manufacturers used in this study

Anthelmintic brand	Active ingredient	Dosage per body weight	Company, country
Bexton Albendazole 10%	100 mg albendazole/ml	0.5 ml/10 kg	Cipla Ltd., India
Fenben 10%	100 mg fenbendazole/ml	0.1 ml/1kg	Nova Laboratories Sdn. Bhd., Malaysia
Coopers Nilverm	32 g levamisole hydrochloride/L	0.25 ml/1kg	Coopers Animal Health, Australia
Kelamectin 1%	10 mg ivermectin/ml	1 ml/50kg	Kela Laboratoria NV, Belgium

Faecal Egg Count Reduction Test (FECRT %) = $(1 - X_t / X_c)$, where
X_t: arithmetic mean of post-treatment e.p.g of the treated group
X_c: arithmetic mean of post-treatment e.p.g of the control group

Resistance to a particular anthelmintic was considered to be present based on Coles (1992), when (1) the percentage reduction in egg count was less than 95% and (2) the 95% confidence level was less than 90%. If only one of the two criteria was met, resistance was classified as suspected.

F200Y mutation of isotype 1 β-tubulin gene detection

Benzimidazole resistance gene detection was conducted according to protocol described by Coles *et al.* (2006) with some modifications. The third stage infective larvae (L_3) harvested from control group were ex-sheathed using 3.5% sodium hypoclorite. A total of 40 unidentified larvae were randomly selected from harvested larval culture and subjected to individual DNA extraction, followed by molecular species identification, and resistance gene detection. Larva in 2 µl distilled water was transferred to 200 µl tube containing 5 µl of Tris-EDTA buffer, pH 8 (Axon Scientific, Malaysia) and 5 mg/ml proteinase K (Invitrogen, USA). The larva was then incubated overnight at 65°C, followed by 95°C for 10 min.

The digested larva was subjected to nested-PCR to amplify the isotype 1 β-tubulin gene using two pairs of primers. The first PCR was conducted in a 12.25 µl reaction volume consisting of 6.5 pmol of primer Pn1 (5'-GGC AAA TAT GTC CCA CGT GC-3') and Pn2 (5'-GAA GCG CGA TAC GCT TGA GC-3'), respectively, 1X MyTaq™ Red Mix (BIOLINE, UK) and 7 µl of digested larva. The PCR cycling programme consisted of 94°C for 3 min, followed by 20 cycles of denaturation (94°C for 55s), annealing (57°C for 55s) and extension (72°C for 55s), finally 72°C for 10 min. For the second PCR, 12.5 pmol of each primer namely Pn3 (5'-GTG CTG TTC TTG TTG ATC TC-3') and Pn4 (5'-GAT CAG CAT TCA GCT GTC CA-3'), respectively, 1X MyTaq™ Red Mix (BIOLINE, UK), and 1 µl first PCR product made up a 25 µl reaction mixture. The second PCR was similar to the first PCR, with the exception of an increase in the number of PCR cycles to 33.

To determine the occurrence of altered target site responsible for benzimidazole resistance, allele-specific polymerase chain reaction (AS-PCR) assay developed by Coles *et al.* (2006) was adopted to screen for the F200Y mutation in the isotype 1 β-tubulin gene of *H. contortus* and *Trichostrongylus colubriformis*. The AS-PCR involved separate reactions for the resistant allele (RA), and susceptible allele (SA). For *H. contortus*, 25 µl of RA/SA reaction mixture comprised of 1.5 µl of the second PCR product, 8.5 pmol of the primers Ph1 (5'-GGA ACG ATG GAC TCC TTT CG-3') and Ph2 (5'-GGG AAT CGA AGG CAG GTC GT-3'), respectively, 25 pmol of resistant allele primer Ph3 (5'-CTG GTA GAG AAC ACC GAT GAA ACA TA-3') or susceptible primer Ph4 (5'-ATA CAG AGC TTC GTT GTC AAT ACA GA-5'), and 1X MyTaq™ Red Mix (BIOLINE, UK). The reaction mixture was subjected to 94°C for 4 min, followed by 33 cycles of 94°C for 55 s (denaturation), 55°C for 55 s (annealing), 72°C for 55 s (extension), and a final step at 72°C for 10 min. Two precent gel electrophoresis aided by Sybr Safe DNA stain (Invitrogen, USA), was performed with 90V for 45 min in TAE buffer to determine the presence of fragments for resistant and susceptible genotypes. For species identification, digested larvae which were negative to the target base pair for *H. contortus* were further subjected to PCR with the specific primers for *T. colubriformis*.

The AS-PCR of *T. colubriformis* involved 8.5 pmol of the primers Pc1 (5'-GGA ACA ATG GAT TCC GTT CG-3') and Pc2 (5'-GGG AAT CGG AGG CAA GTC GT-3'), respectively, 25 pmol Pc3 resistant allele primer (5'-CTG GTA GAG AAT ACC GAT GAA ACA TA-3') or Pc4 susceptible allele primer (5'-ATA CAG AGC TTC GTT ATC GAT GCA GA-3'), 1X MyTaq™ Red Mix (BIOLINE, UK) and 1.5 µl of second PCR product which

made up 25 µl of reaction volume. The same AC-PCR protocol for *H. contortus* was performed. The AS-PCR for these two species amplified an internal control at ~750 bp in both RA and SA reactions, ~250 bp for resistance allele (RA) and ~550 bp for susceptible allele (SA).

RESULTS

Worm egg count reduction percentages
Nematode worm egg count reduction percentage for albendazole, fenbendazole, levamisole and ivermectin were 87%, 46%, 94% and 68%, respectively (Table 2).

Pre- and post- treatment third stage infective larvae of nematodes
The predominant nematode species from pre-treatment pooled faecal samples were *Haemonchus contortus* (80%), *Trichostrongylus colubriformis* (19%) and *Cooperia* sp. (1%) (Table 3). However, only *H. contortus* and *T. colubriformis* were recovered from post-treatment faecal samples (Table 3).

Benzimidazole resistance gene detection
The AS-PCR fragments were amplified in 37 digested larvae (14 *H. contortus*; 23 *T. colubriformis*) in the Control group. The homozygous resistance (RR) genotype was found in high frequency (71.4% or 10 of 14) followed by the homozygous susceptible (SS) genotype (28.6% or 4 of 14) (Table 4). As for *T. colubriformis*, the F200Y mutation was detected in all AS-PCR positive individuals. The RR was the predominant genotype (73.9% or 17 of 23), followed by RS (26.1% or 6 of 23). Interestingly, homozygous susceptible (SS) genotype was not observed in both species. In three samples which were tested negative to primers designed for *H. contortus* and *T. colubriformis*, they could be of other species such as *Cooperia* sp. which was reported in 6% of the worm population in this farm.

Table 2. Worm egg count reduction (%) for all treatment groups

	Albendazole	Fenbendazole	Levamisole	Ivermectin
Reduction	87%	46%	94%	68%
Upper 95% confidence limit	99%	96%	99%	97%
Lower 95% confidence limit	-28%	-655%	42%	-218%

Table 3. Pre-treatment and post-treatment third stage larvae proportion (%) for treatment and control group

Pre-treatment	Nematode	Third stage larvae proportion (%)				
		Control				
	Haemonchus contortus	80				
	Trichostrongylus colubriformis	19				
	Cooperia sp.	1				
Post-treatment	Nematode	Third stage larvae (%)				
		Control	Albendazole	Fenbendazole	Levamisole	Ivermectin
	Haemonchus contortus	51	60	65	80	70
	Trichostrongylus colubriformis	43	40	35	20	30
	Cooperia sp.	6	0	0	0	0

Table 4. Genotypes and allele frequencies of isotype 1 β-tubulin in *Haemonchus contortus* and *Trichostrongylus colubriformis* larvae

	N	Genotype						Allele frequency	
		SS		RS		RR		S	R
		n	%	N	%	n	%		
H. contortus	14	0	0	4	28.6	10	71.4	0.14	0.86
T. colubriformis	23	0	0	6	26.1	17	73.9	0.13	0.87
Total	37	0	0	10	27.0	27	73.0	0.14	0.86

DISCUSSION

Results from this study demonstrated anthelmintic failure due to resistance of nematodes towards anthelmintics tested on a commercial sheep farm. This finding is not the first case in Malaysia since total anthelmintic failure due to nematode resistance in farms has been reported previously by many authors (Chandrawathani *et al*., 2004, 2013; Khadijah *et al*., 2006a, b; Nor-Azlina *et al*., 2011; Premaalatha *et al*., 2014; Abubakar *et al*., 2015; Basripuzi *et al*., 2012).

According to Coles *et al*. (1992), resistance towards certain anthelmintics occurs when the anthelmintic fails to reduce at least 95% of the nematode worm egg count. In this current study, nematode worm egg count reduction percentage for albendazole, fenbendazole, levamisole and ivermectin were 87%, 46%, 94% and 68%, respectively, indicating resistance. However, based on the guidelines of Wood *et al.* (1995), effectiveness of anthelmintics is indicated by the percentage of worm egg count reduction where >98% is considered highly effective, 90–98% as effective, 80–89% as moderately effective and <80% as insufficiently effective. Hence, the anthelmintic efficacy based on this recommendation showed that levamisole is still effective, albendazole is moderately effective while fenbendazole and ivermectin are insufficiently effective in this farm.

Haemonchus contortus was found to be resistant to all anthelmintics tested in this farm and this nematode was predominant in all post-treatment larval culture. This finding is expected, and in agreement with the findings of other authors in Malaysia. Resistance of *H. contortus* towards benzimidazoles was firstly reported in Malaysia by Dorny *et al.* (1993) followed by Chandrawathani *et al.* (2004, 2013), Nor Azlina *et al.* (2011), Premaalatha *et al.* (2014), Abubakar *et al.* (2015) and Basripuzi *et al.* (2012). In addition, *H. contortus* was also reported to be resistant to levamisole (Basripuzi *et al.*, 2012; Premaalatha *et al.*, 2014) and ivermectin (Nor-Azlina *et al.*, 2011; Basripuzi *et al.*, 2012).

In this study, *T. colubriformis* was found to be resistant to all the anthelmintics tested. This finding is similar to those reported by other authors in Malaysia. Sivaraj *et al.* (1994) and Dorny *et al.* (1994) firstly reported on resistance of this nematode towards benzimidazoles, and later other authors reported resistance of this nematode to levamisole (Sivaraj *et al.*, 1994; Basripuzi *et al.*, 2012; Chandrawathani *et al.*, 2003) and ivermectin (Nor-Azlina *et al.*, 2011).

Albendazole and Fenbendazole

In this study, the nematodes on this farm are resistant to albendazole and fenbendazole. This result is similar to those reported by Pandey and Sivaraj (1994) and Chandrawathani *et al.* (1999, 2014). For this farm, resistance to fenbendazole is most likely due to the use of this anthelmintic for nematode control since the establishment of the farm in 2006. Exposure of nematodes on this farm to the same anthelmintic for a long period of time caused selection of resistant nematodes to this particular anthelmintic, as suggested by Dorny *et al.* (1994). Besides that, deworming of sheep at

this farm has been frequent; twice in a period of six months. It was suggested by Taylor and Hunt (1989) that frequent use of anthelmintics from the same group may result in the development of anthelmintic resistance, explaining resistance to both albendazole and fenbendazole on this farm. It has been suggested that anthelmintic resistance can develop even when only two or three treatments were given annually (Coles *et al.*, 1995).

Another possible cause of resistance could be the sub-optimal dosage given to the animals during deworming because the farmers did not weigh their animals before deworming. Under dosing is likely to favour the survival of heterozygous, enhancing the selection pressure for resistance (F.A.O., 2004) and the most frequent cause of under dosing is probably by incorrect guessing of the animal's weight (Coles and Roush, 1992).

Several single nucleotide polymorphisms such as F167Y, E198A and F200Y in the isotype-1 α-tubulin gene have been found to be associated with benzimidazole resistance (Zhang *et al.*, 2016). Specifically, F200Y is by far the most common SNP linked to benzimidazole resistance (Kotze *et al.*, 2014). Over-reliance of both albendazole and fenbendazole on this farm may also contribute to benzimidazole resistance caused by the altered target site in isotype 1 α-tubulin, namely the F200Y point mutation. In this study, occurrence of this point mutation was recorded in both *H. contortus* and *T. colubriformis* for the first time in Malaysia. Identification of this specific point mutation can serve as an alarming marker on the extensive use of benzimidazole which inevitably elicits different levels of resistance. However, further studies with larger sample size from wider sampling areas, are warranted to determine the current distribution of F200Y resistance alleles, and to uncover the actual cause of anthelminthic treatment failure in Malaysia.

Levamisole

For the Levamisole group, 94% reduction was recorded in nematode worm egg counts similar to those reported by Pandey and Sivaraj (1994) and Khadijah *et al.* (2006a; b). While this anthelmintic can be considered effective according to interpretations of Wood *et al.* (1995), reduction percentage of 94% indicates that 6% of the nematodes were resistant towards levamisole. The higher percentage of reduction for levamisole could be due to the fact that the sheep in this farm have not been treated with this anthelmintic. Thus, the 6% resistant nematodes might have been originated from previous sheep farms. The sheep in this farm were originated from various farms in the states of Perak, Kelantan and Pahang, in which sheep from these states were reported to harbour nematodes resistant to levamisole (Chandrawathani *et al.*, 1999; 2004; Khadijah *et al.*, 2006a; b).

Ivermectin

For the Ivermectin group, there was only 13% reduction in nematode worm egg counts, indicating resistance. Resistance status of nematodes on this farm is similar to those reported by Chandrawathani *et al.* (1999; 2014) in sheep. In this farm, ivermectin was regularly used for ectoparasite control because this drug belongs to the macrocyclic lactones group and was reported to be effective for endoparasites (Rahman, 1997) and ectoparasites (Zamri-Saad *et al.*, 1990) control. Thus, exposure of existing nematodes in sheep towards macrocyclic lactones may have influenced the resistance level for this anthelmintic. Selection for resistant nematodes can develop more rapidly when the sheep are treated regularly and particularly with the same anthelmintics over an extended period causing the anthelmintics to be ineffective (Dorny *et al.*, 1994).

As a conclusion, this study reports resistance of nematodes to al anthelmintics tested. Results on this study is the first report on the detection of the F200Y mutation in the isotype 1 α-tubulin gene in Malaysia, and this may be one of the resistance mechanisms contributing to nematode control failure due to anthelmintic resistance on the studied sheep farm. Monitoring of anthelmintic resistance status

should be conducted as a routine practice in small ruminant farms to reduce the burden of economic losses, particularly for the low-income farmers. In addition, the use of anthelmintics in this studied farm should be minimized. Alternatively, zero grazing can be conducted on this farm to avoid re-infection from contaminated pastures. Feeding sheep with plants that have anthelmintic properties like Neem leaves (*Azadirachta indica*) is also recommended.

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