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Short Communication

Anthelmintic resistance in hair sheep farms in a sub-humid tropical climate, in the Huasteca Potosina, Mexico

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ABSTRACT

This is the first report about anthelmintic resistance (AR) in hair sheep farms determined in a sub-humid tropical climate, in an area known as Huasteca Potosina, Mexico. Faecal egg count reduction tests (FECRT) and egg hatch *in vitro* tests were conducted to identify the level of AR against benzimidazole (BZ) in parasitic gastrointestinal nematode (GIN) populations. An allele-specific polymerase chain reaction (AS-PCR) was performed to obtain a 250 bp band, indicating resistance, and a 550 bp band, indicating susceptibility to BZ. Macrocyclic lactones (ML) and imidazothiazole (IMZ) drugs were also tested with the FECRT. A PROBIT analysis was conducted using SAS to determine the 50% lethal doses (LD_{50}) of the drugs according to the egg hatch *in vitro* test. Resistance to BZ and ML was found on all farms (0–70% effectiveness), whereas the susceptibility of nematodes to IMZ was detected with the FECRT (93–100% effectiveness). The LD_{50} was higher than the discriminating dose (0.1 $\mu g\,ml^{-1}$) for BZ and confirmed AR to this anthelmintic; we also confirmed a high AR frequency with AS-PCR. Therefore, we suggest that strategic deworming should be performed to avoid the development of resistance to imidazothiazole.

1. Introduction

Anthelmintic drugs are commonly used to increase sheep productivity by controlling gastrointestinal nematodes (GIN) in flocks. The frequent use of these products causes genetic changes, which induce parasitic nematode populations to survive, producing the well-known problem of anthelmintic resistance (AR). Currently, worldwide, AR dispersion is indicated for almost all commercial products against GIN in ruminants (Van den Brom et al., 2015).

The anthelmintic resistance problem is reducing flock productivity due to the mortality of lambs by gastrointestinal parasitism, discouraging ovine production (Torres-Acosta et al., 2012; Goolsby et al., 2017). Due to increasing concerns for AR in tropical areas in Mexico (Herrera-Manzanilla et al., 2017), it has become necessary to record AR in areas with a high prevalence of GIN, like the sub-humid regions of Mexico, to contribute to the epidemiological study of AR in these areas and to have a proper deworming programme and integrate other husbandry procedures to support the agricultural economy.

A widely used method to detect AR is the faecal egg count reduction test (FECRT). However, alternative diagnosis methods such as bioassays have been designed (Almeida et al., 2013) to provide relevant information to obtain the LD_{50} (the lethal dose of the drug that prevented 50% of hatching eggs) that enables the discrimination of GIN isolates with or without resistance (Coles et al., 2006). In addition, tools such as PCR tests provide information on the genes that confer resistance to benzimadazole (BZ). Resistance to these compounds has been associated with a single nucleotide polymorphism (SNP) in codons 167 (F167Y) and 200 (F200Y) of the isotype I gene of β-tubulin (Lambert et al., 2017).

The objective of this study was to determine AR to BZ, macrocyclic lactones (ML) and imidazothiazoles (IMZ) in GIN using FECRT and *in vitro* assays in naturally infected hair sheep in farms localised in the sub-humid region known as Huasteca Potosina in Mexico, as well as to use allele-specific polymerase chain reaction (AS-PCR) to confirm AR to BZ.

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2. Materials and methods

This study was conducted in the three municipalities (Ciudad Valles, Tamuín and Tanquian) of a sub-humid region in Mexico known as Huasteca Potosina (altitude between 20 and 900 m above sea level), with a temperature of around 20–26 °C and 1000–2000 mm of rainfall annually (INEGI, 2010).

Adult ewes and some rams were selected from farms; they were not treated with anthelmintics (AH) for 2 months before the experimental assay. The selected hair breeds were Dorper, Pelibuey, Kathadin, Blackbelly and their crosses. This study was conducted in June, during the beginning of the rainy season.

A FECRT was conducted (Coles et al., 2006). Three anthelmintics that are commonly used in the region were tested: (1) benzimidazole (BZ, Febendazole, Paradex L 10% Biochem, 5.8 mg kg^{-1}) by an oral route; (2) ivermectin (IVM, Endovet, Rivefarma, 0.2 mg kg^{-1}) by a subcutaneous route; and (3) levamisole (LEV, Ripercol L 12%, Zoetis, 7.5 mg kg^{-1}) by an intramuscular route. Sheep with at least 150 nematode eggs per gram of faeces (EPG) were selected to form the treatment groups. One group of 9–10 sheep without anthelmintic treatment was used as a control and three groups of 9–10 sheep were dewormed. Individual sheep were weighed to determine the correct dose of each anthelmintic drug. The number of EPG was determined using the McMaster technique.

Fourteen days after the anthelmintic treatments, individual faecal samples and FEC were obtained to calculate the arithmetic mean, 95% confidence interval and percentage of reduction of FEC. The GIN population was considered resistant for a given anthelmintic class when the FECRT reduction percentage was < 95% and the lower limit of the 95% confidence interval (CI) was < 90%; a GIN population was considered suspected when one of the two above-mentioned criteria was identified and a GIN population was considered susceptible to any AH when neither of the above-mentioned criteria were observed (Coles et al., 2006).

An *in vitro* egg hatch test (EHT) test was conducted with faecal samples that were positive for GIN. To obtain nematode eggs, the faeces were mixed with saturated saline solution and processed, as described by Muñiz-Lagunes et al. (2015). Populations of nematodes were considered resistant at $LD_{50} \geq 0.1$ μg ml^{-1} (Coles et al., 2006). One hundred nematode eggs were placed in each well in polystyrene plates (Nunc, Maxisorp™) and incubated at room temperature for 24 h. BZ was used at a minimum concentration of 12.54–0.05 mg ml^{-1}. The control group contained only nematode eggs suspended in distilled water. The reaction was halted at 24 h by placing 10 μl of Lugol's iodine solution (Hycel, Mexico) into each well. The proportion of unhatched eggs and nematodes in the first larval stage (L_1) were counted. Data were analysed to obtain the LD_{50} and LD_{99} using a logistic regression model (PROBIT) in the SAS program (SAS, 2004).

Approximately 50 L_3 GIN were collected from the faecal cultures. Genomic DNA (gDNA) extraction and AS-PCR were performed to determine the polymorphisms at codon 200 of isotype I of the β-tubulin gene of *Haemonchus* spp. (Encalada-Mena et al., 2014). A β-tubulin allele with 250 and 550 bp indicate the presence of a resistant and susceptible nematode population to BZ, respectively. Also, *Haemonchus* spp., *Oesophagostomum* spp., *Cooperia* spp., and *Trichostrongylus* spp., were genotyped, according to the method of Encalada-Mena et al. (2014).

3. Results

Our results showed a low level of FEC for all sheep naturally infected with GIN from all farms (Table 1). The number of infected sheep with > 150 EPG was < 50%, and 41% of the sheep could not be used (across all the farms) for the three anthelmintic groups.

This study is the first evidence of widespread AR to BZ on sheep farms of a sub-humid region of Mexico, using the FECRT and confirmed with PCR. The three groups treated with BZ exceeded the number of EPG excreted, in comparison to control sheep after deworming; therefore, there were no reductions in EPG (Table 2). In addition, resistance to BZ was confirmed *via in vitro* assays with the EHT, in which no egg hatching from the treated groups was observed. Also, high anthelmintic doses were required to achieve mortality between 50% and 99% and the PROBIT equation had a non-significant slope (β1; $P > .05$) in most cases (Table 2). The AS-PCR technique for codon 200 (TTC/TAC) was associated with nucleotide changes of the β-tubulin gene, showing a 250 bp allele, confirming resistance to BZ (Fig. 1).

Table 1
Percentage of sheep infected by gastrointestinal nematodes on the tested farms.

Farm	Total	Positive sheep		Eggs per gram of faeces (EPG)			
	Sheep	> 150 EPG	Percentage	Mean	SD	SE	Median
1	80	38	47.5	244	405	45	50
2	77	38	49.4	551	1087	124	100
3	80	36	45.0	209	301	34	100
4	31	19	61.3	492	577	104	250
5	79	37	46.8	267	450	51	100
6	80	20	25.0	176	342	38	0
7	55	19	34.5	608	2088	282	0
8	78	19	24.4	129	288	33	0
Mean	70	28.3	41.7	335	692	89	75

EPG = eggs per gram; SD = standard deviation; SE = standard error.

For four sheep farms, a FECRT with IVM, was conducted (Table 3). IVM resistance led to a FEC reduction of around 67% for the four farms, which is a big problem for nematode control. In contrast, treatment with LEV showed high efficacy in two cases; one farm showed resistance and the other showed < 95% effectiveness and was classified as suspect (Table 3).

The blood feeding nematode *Haemonchus* spp. was predominantly on all farms, followed by *Trichostrongylus* spp., *Cooperia* spp. and *Oesophagostomum* spp. (Fig. 2).

4. Discussion

BZ is the most widely used anthelmintic to control GIN infections in small ruminants, and the abuse on BZ use is associated with AR (Zanzani et al., 2014). In Mexico, many reports indicate similar results with BZ (Liébano-Hernández et al., 2015). In the present study, the reduction of BZ efficacy was similar to those observed by Herrera-Manzanilla et al. (2017) and Alcalá-Canto et al. (2016), who found a reduction from 0 to 48%, concluding that high resistance occurred in many flocks.

The PCR test is a technique that helps to confirm the results of the FECRT in less time than coproculture. This technique is already widely used for the determination of resistance (Paraud et al., 2016). In the present study, the PCR test confirmed the low efficacy of BZ in the FECRT of nematodes like *Haemonchus* spp., which were observed on all farms.

The lethal doses obtained with the EHT were very high on all farms and exceeded the discriminating dose of 0.1 μg ml^{-1} (Coles et al., 2006). In many cases, the curve could not be constructed because the product was inefficient at the highest doses used and high concentrations were required to achieve an LD_{50}, which together with the FECRT results, indicate a high resistance to these products in the studied area. This coincides with another study in which the continuous use of anthelmintics caused resistance to BZ (dos Santos et al., 2014).

In Mexico, few studies have been conducted to evaluate levamisole resistance in flocks; however, resistance have been reported in southern Mexico (Herrera-Manzanilla et al., 2017) and in other regions of the Americas (García et al., 2016; Chaparro et al., 2017). This drug has little effect on nematodes in immature stages, which easily leads to the development of resistance if it is used indiscriminately. Because of this,

Table 2
Results of the faecal nematode egg count reduction test (FECRT) and egg hatch test (EHT) to determine anthelmintic resistance in hair sheep using a benzimidazole (febendazole) in a sub-humid tropical climate in Mexico.

Farm	Control group (n = 10)	Faecal egg count reduction test			*In vitro* test			
		Treated (n = 10)	Reduction (%) (CI)	Result	β_0	β_1	LD_{50} (mg ml^{-1})	LD_{99} (mg ml^{-1})
1	1450	2669	0 (0–70)	Resistant	Ns	Ns	0.01	–
2	861	640	26 (0–77.3)	Resistant	Ns	Ns	15.5	27.9
3	533	405	24 (0–67.7)	Resistant	*	Ns	–	–
4	1406	795	43 (0–74.6)	Resistant	Ns	Ns	–	–
5	614	225	63 (8.1–85.4)	Resistant	Ns	Ns	–	–
6	363	650	0 (0–31.2)	Resistant	**	*	40.6	99.7
7	1383	2595	0 (0–58.8)	Resistant	**	*	25.1	-
8	1533	465	70 (0–91.6)	Resistant	Ns	Ns	52.5	88.1

β_0: intercept; β_1: slope; Ns: not significant ($P > .05$); *: significant ($P < .05$); **: high significant ($P < .01$). LD_{50}: Lethal doses at 50%; LD_{99}: Lethal doses at 99%. CI. Confidence intervals in brackets.

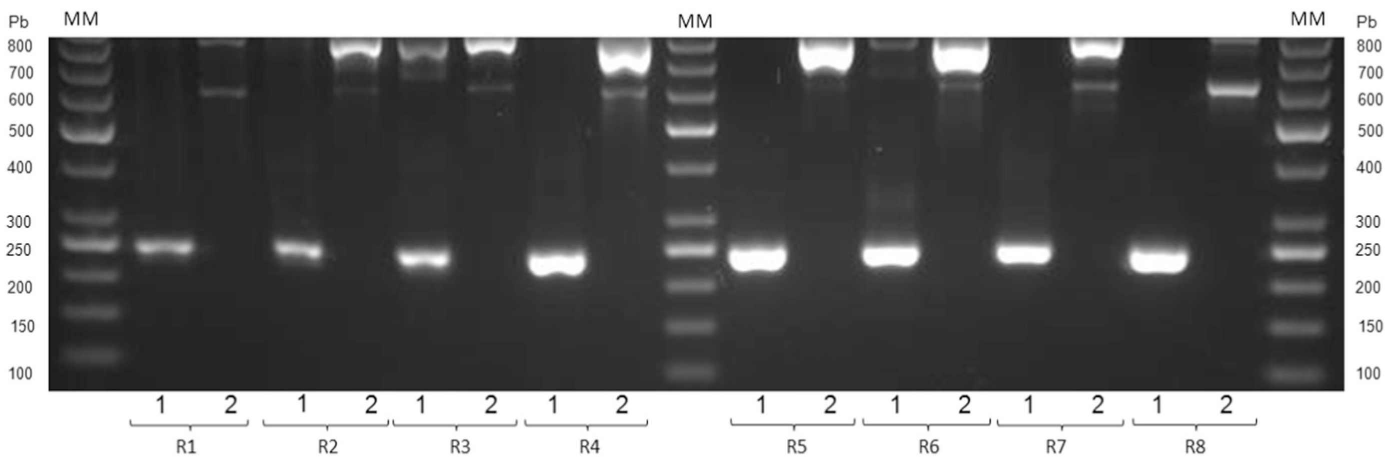

Fig. 1. Amplification of the PCR products by endpoint corresponding to the fragments of susceptibility (550 bp, S) or resistance (250 bp, R). MM: Molecular marker. R1 corresponds to farm 1, R2 to farm 2 and so on for each ranch.

Table 3
Results of faecal nematode egg count reduction tests (FECRT) to determine anthelmintic resistance in hair sheep using a macrocyclic lactone (ivermectin) and an imidazothiazole (levamisole) in a sub-humid tropical climate in Mexico.

Farm	Control group (n = 10)	Faecal egg count reduction test – Ivermectin			Faecal egg count reduction test – Levamisole		
		Treated (n = 10)	Reduction (%) (CI)	Result	Treated (n = 10)	Reduction (%) (CI)	Result
1	1450	2656	0 (0–55.1)	Resistant	640	98 (88.4–99.6)	Suspect
2	861	456	47 (0–79.2)	Resistant	0	100 (100 – 100)	Susceptible
3	533	417	22 (0–66.9)	Resistant	39	93 (64.4–98.5)	Resistant
5	614	200	67 (0–92.1)	Resistant	10	98 (92.0–99.7)	Susceptible

CI. Confidence intervals in brackets.

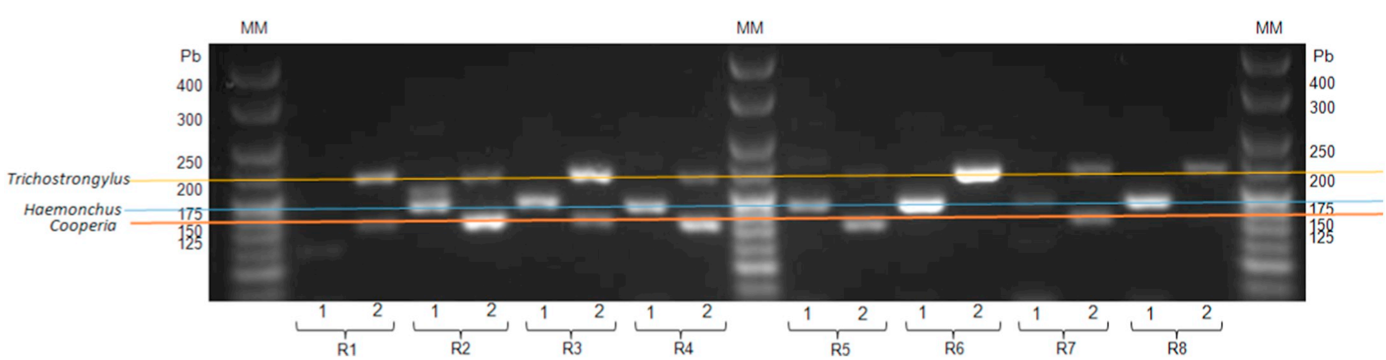

Fig. 2. Genotypes of larvae-infecting gastrointestinal nematodes before anthelminthic treatment. *Ostertagia* spp. 257 bp, *Haemonchus* spp. 176 bp, *Oesophagostomum* spp. 329 bp, *Trichostrongylus* spp. 243 bp, *Cooperia* spp. 151 bp.

it is recommended that anthelmintics, and specifically levamisole, only be used at critical times, and that selective deworming be performed to help reduce the damaging impact of nematodes and decrease the populations of resistant nematodes.

Ivermectin resistance is an international concern due to its endectocide activity, which is highly appreciated by farmers. Among the main GIN, *Haemonchus* spp. and *Cooperia* spp. have shown serious problems to being treated with IVM and BZ. However, the β-tubulin gene is considered a candidate molecular marker for early diagnosis of BZ resistance (Ramünke et al., 2016). In contrast, IVM requires further study because more genes can be used for diagnosis and it is necessary to conserve the toxicity of these anthelmintic drugs.

In conclusion, in the sub-humid region known as Huasteca Potosina, there is a high presence of anthelmintic resistance to both benzimidazole and ivermectin among the gastrointestinal nematodes of hair sheep farms. However, nematodes are still susceptible to levamisole. Therefore, strategic deworming should be performed to avoid the development of levamisole resistance.

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Conflict of interest

There were no conflicting interests that could have influenced the conduct and reporting of this study.

Ethical statement

This study considered the international guiding principles for biomedical research involving animals.

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