Anthelmintic resistance in nematodes infecting dairy heifers in Puerto Rico

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Abstract. The efficacy of the anthelmintics albendazole (ALB), doramectin (DOR) and ivermectin (IVM) were evaluated in Holstein heifers (n = 420; ~6 months of age; BW = 149 ± 39.6 kg) distributed in 21 dairy farms. All heifers were in pasture and have not been exposed to anthelmintics for 3 months. A subset of 10 dairy farms were randomly chosen to determine the predominant genus of gastrointestinal nematodes. At each dairy farm, fecal samples were collected directly from the rectum of ≥ 20 heifers. Morphological characteristics of cultured nematodes' larva were used to identify their genus. *Cooperia* spp. was the most predominant nematode (70.7 %), followed by *Oesophagostomum* spp. (19.9 %). For the fecal egg count reduction test (FECRT) the modified McMaster technique was used. At each dairy farm, a priori, groups of ≥ 18 heifers were randomly divided to be treated with IVM, or DOR, or ALB. Fecal samples were collected directly from the rectum 7 days before anthelmintic treatment (d-7), at the time of the treatment (d0), and 14 days after treatment (d14). Only heifers that had an average FEC 242 ≥ 100 eggs per gram of feces (average of FEC from day d-7 and d0) were used. Ivermectin (n = 148), and DOR (n = 130) were administered via subcutaneous injection at 0.2 mg/kg of body weight and ALB (n = 142) was administered orally at 7.5 mg/kg of body weight. Albendazole proved to be the most effective anthelmintic (P < 0.0005) with an efficacy of 67.47 % ± 8.43. The efficacy of DOR and IVM did not differ (P = 0.8713). While DOR (n = 130) exhibited an efficacy of 25.50 % ± 18.00, IVM exhibited an efficacy of -8.13 % ± 19.89 due to an increase in FEC after treatment. These results show that anthelmintic nematode resistance is a problem in Puerto Rico.

Key Words: helminths, parasitism, albendazole, doramectin, ivermectin

Resistencia antihelmíntica en nematodos infectando vaquillas lecheras en Puerto Rico

Resumen. Evaluamos la eficacia de los antihelmínticos albendazol (ALB), doramectina (DOR) e ivermectina (IVM) en vaquillas Holstein (n = 420; ~ 6 meses de edad; BW = 149 ± 39.6 kg) distribuidas en 21 lecherías. Todas las vaquillas pastoreaban y no habían sido tratadas con antihelmínticos en ≥ 3 meses. Se eligieron al azar 10 lecherías para determinar el género predominante de nematodos gastrointestinales, donde se recolectaron muestras fecales directamente del recto de ≥ 20 vaquillas. Se identificó el género de las larvas de nematodos cultivados morfológicamente. *Cooperia* spp. fue el género de nematodos más predominante (70.7%), seguido por *Oesophagostomum* spp., (19.9 %). Para la prueba de reducción del recuento de huevos fecales (RRHF), mediante la técnica de McMaster modificada, en cada lechería, a priori, grupos ≥ 18 vaquillas se dividieron al azar para ser tratadas con IVM, o DOR, o ALB. Se recogieron muestras fecales directamente del recto 7 días antes del tratamiento antihelmíntico (d-7), en el momento del tratamiento (d0) y 14 días después del tratamiento (d14). Solo vaquillas con un RRHF promedio ≥ 100 huevos por gramo de heces (promedio de RRHF del día d-7 y d0) fueron consideradas. Ivermectina (n = 148), y DOR (n = 130) se administraron a través de una inyección subcutánea a razón de 0.2 mg/kg de peso vivo. Albendazol (n = 142) fue administrado vía oral a razón de 7.5 mg/kg de peso vivo. De los tres antihelmínticos utilizados, ALB resultó ser el más eficaz (P < 0.0005) con una eficacia del 67.47 % ± 8.43. La eficacia de DOR y IVM no difirió (P = 0.8713). Mientras que DOR mostró una eficacia de 25.50 % ± 18.00; IVM mostró una eficacia de -8.13 % ± 19.89 debido a un aumento de RRHF después del tratamiento. Estos resultados muestran que la resistencia a los antihelmínticos es un problema en Puerto Rico.

Palabras clave: helmintos, parasitismo, albendazol, doramectina, ivermectina

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Introduction

Nematode gastrointestinal (GI) parasitism in dairy heifers can cause low weight gains, poor feed efficiency and delays in breeding age, all of which may eventually result in poor milk production (Eysker and Ploeger, 2000; Gasbarre, 2014). The control of GI nematodes relies mostly on the use of broad-spectrum anthelmintics and their indiscriminate use has resulted in the development of anthelmintic resistant GI nematode populations worldwide (Sutherland and Leathwick, 2011; Cotter et al., 2015).

Resistant populations of GI nematodes develop more quickly when the same anthelmintic has been used intensively and repeatedly without any other measure for parasite control (Martin et al., 1997; Cotter et al., 2015). There have been over 145 documented cases of GI nematodes anthelmintic resistance in cattle involving all three classes of the broad-spectrum anthelmintics, and at least ten species of nematodes (Sutherland and Leathwick, 2011). Resistance to the macrocyclic lactones has become common in *Cooperia* spp. in New Zealand (Familton et al., 2001) and in Argentina (Mejía et al., 2003). Benzimidazoles resistance has been found in *C. punctata*, *Ostertagia ostertagi* spp.

and *Haemonchus placei* spp. in Argentina (Mejía et al., 2003) and in *C. oncophora* in New Zealand (Winterrowd et al., 2003). In the US resistance was reported in *Cooperia* spp. and *Haemonchus* spp. (Gasbarre et al., 2004; Gasbarre, 2014).

There are only a few main classes of anthelmintics, and a limited number is available for the treatment of GI nematode parasitism in cattle (Gasbarre, 2014). Among the broad-spectrum anthelmintics commonly used in cattle are included: benzimidazoles, imidazothiazoles/hydropyrimidines and macrocyclic lactones (ivermectins and milbemycins) (Coles et al., 1992; Gasbarre, 2014). The discovery of new anthelmintics with different mechanisms of action is a slow process; therefore, the preservation of the effectiveness of currently available anthelmintics is indispensable for continuing increments in dairy cattle productivity and efficiency (Coles et al., 2006; Gasbarre, 2014). The objective in this study was to determine if dairy heifers in Puerto Rico had GI nematodes populations resistant to the broad-spectrum anthelmintics benzimidazoles, imidazothiazoles/hydropyrimidines, and macrocyclic lactones.

Materials and Methods

Selection of dairy farms and heifers within farms

The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Puerto Rico, Mayagüez Campus. Out of a total of 320 dairy farms active in Puerto Rico at the time of this research project, 25 dairy operations were selected for this field trial because animals had unique identifications within the herd and had written records of veterinary treatments. However, 4 dairy farms were eliminated from the study because their average parasitic infections (evaluated as fecal egg counts) before anthelmintic treatments was less than the lower critical value of 100 eggs per gram of feces needed to performed the Fecal Egg Count Reduction Test (FECRT). Although all heifers were in pasture, because this was a field trial and not a controlled experiment, between the 21 farms used in the study there was variability in the type and quantity of forage available to the heifers. However the predominant grass species available were *Cynodon nelenfuesis*, *Megathyrus maximus*, and *Brachiaria* spp. Although all heifers were receiving some type grain supplementation, quantities and qualities were highly variable between the 21 farms used in the study. At the time of the trial all heifers were required to be on pasture and not have been exposed to anthelmintic treatment for a

minimum of 3 months. All heifers were about 6 months old based on the farmers' records. In the field trial, a total of 420 Holstein type heifers (black and white) were evaluated using the FECRT analysis. Animals that phenotypically resemble other dairy breed (i.e. Jersey, Brown Swiss) or undetermined crosses (i.e. all black) were not used in the trial. In each dairy farm, a priori groups of at least 18 heifers were randomly allocated in balanced groups to be treated with ivermectin (IVM) (Ivomec®, Merial, Duluth, Georgia, USA), doramectin (DOR) (Dectomax®, Zoetis, New Jersey, USA), or albendazole (ALB) (Valbazen®, Zoetis, New Jersey, USA). Heifers were weighted using a Nasco Weight by Breed dairy management tape (Nasco, Fort Atkinson, WI, USA). Ivermectin (n = 148), and DOR (n = 130) were administered via subcutaneous injection at 0.2mg/kg of body weight and ALB (n = 142) was administered orally at 7.5 mg/kg of body weight.

Fecal Egg Count Reduction Test (FECRT)

Fecal samples were collected directly from the rectum 7 days prior to anthelmintic treatment (d-7), at the time of the treatment (d0), and 14 days after treatment (d14). Samples were individually collected in 50 mL centrifuge tubes and were analyzed within 4

hours from collection time. Only heifers that had an average FEC ≥ 100 eggs per gram of feces (average of FEC from day d-7 and d0) were considered in the FECRT. For each treatment, the observed drug efficacy (EFF) was calculated by using the formula: $\text{FECRT \%} = (T1 - T2) / T1 \times 100$ where T1 is pre-treatment egg count and T2 is post-treatment egg count (d14) (Kholik et al., 2019).

From each heifer, fecal sub-samples (3g) were diluted in 40 mL of distilled water. The fecal solution was then filtered through a piece of gauze to remove solid organic matter and centrifuged for 4 minutes at $4000 \times g$. The pellet was then suspended in 30 mL of a solution of sodium chloride (specific gravity = 1.2; 11.97M). Parasite fecal egg counts (FEC) were determined by the modified McMaster's technique as described by Coles, et al. (1992). Using this technique, the eggs per gram of feces (EPG) were calculated by counting the number of nematode eggs inside the McMaster slide frames and then multiplied by a factor of 33.

Identification of predominant species of GI nematodes in dairy heifers

Out of the 21 dairy farms participating in the study, a subset of 10 dairy farms were randomly chosen to evaluate which genus of GI nematodes were predominantly infecting dairy heifers in Puerto Rico. At each dairy farm, fecal samples were collected directly from the rectum of at least 20 dairy heifers that have not been treated with any anthelmintic for at least 3 months before the sampling. Samples were individually collected in 50 mL centrifuge tubes and

were sent within 4 hours from collection time, by overnight service, to the Laboratory of Dr. Ray M. Kaplan at the Department of Infectious Diseases, University of Georgia in Athens, GA. The fecal samples from each farm were pooled, mixed with vermiculite (Sargent-Welch, Buffalo, NY) and incubated at 25°C for 10 days (nematode cultures). The nematode cultures were examined and stirred every day and distilled water was added as needed to prevent nematode larval desiccation. After 10 days of incubation, nematode larva (L3stage) were recovered using the Baermann's technique (Dinaburg, 1942), in which the nematode cultures were suspended over a sieve inside a funnel with lukewarm water for 24 hours. Recovered larvae were stored at 10°C in deionized water. Subsequently, nematode larvae were stained using a 10 % Lugol's iodine solution. Afterward, the genus of recovered nematode larvae were identified using morphological characteristics of the anterior end, tail sheath extension, and overall body length (van Wyk, et al., 2004).

Statistical analysis

For the drug efficacy analysis, the arithmetic mean $\geq$ standard error were calculated using InfoStat® Statistical Software (Córdoba, Provincia de Córdoba, Argentina). Negative efficacies (increased EPG after treatment) were reported as negative values. The sole fixed effect in the model was treatment (IVM; DOR; ALB). The arithmetic means of the percentage of drug efficacy were analyzed by ANOVA (GLM) using InfoStat® statistical software and statistical significance was declared at $P < 0.05$.

Results and Discussion

Cooperia spp. was the most predominant nematode genus observed (70.7 %). *Oesophagostomum* spp., *Haemonchus*, and *Trichostrongylus* spp. were the next more predominant genus with 19.9 %, 4.6 %, and 4.2 % respectively (Figure 1). Other genus such as *Strongyloides* spp. and *Bunostomum* spp. were much less prevalent (0.25 %). Several other studies have identified *Cooperia* spp. as the most predominant GI nematode in cattle. For example, in Brazil, *Cooperia* spp. was confirmed to be the most predominant species, followed by *Haemonchus* spp. and *Oesophagostomum* spp. (Bricarello et al., 2007). In fecal cultures evaluated in Argentina, the predominant anthelmintic resistant nematode species was determined to be *Cooperia* spp. (Anziani et al., 2004).

The main objective of this investigation was to evaluate the presumptive anthelmintic resistance of GI nematodes infecting dairy heifers to three anthelmin-

tics commonly used in Puerto Rico. The mean FECR at each of the evaluated farms is presented in Figure 2. Overall, the treatments differed statistically ($P < 0.0005$). Of the three anthelmintic used, ALB ($n = 142$) proved to be the most effective ($P < 0.0005$) with a FECR of $67.47 \% \pm 8.43$. The efficacy of DOR ($n = 130$) and IVM ($n = 148$) did not differ ($P = 0.8713$). However, DOR exhibited a efficacy of $25.50 \% \pm 18.00$, whereas IVM efficacy was $-8.13 \% \pm 19.89$ due to an increase in FEC after treatment. These results show that anthelmintic nematode resistance is a problem in Puerto Rico.

Ineffective drug actions (increases in the number of nematode eggs after treatment) like the ones found in our study for IVM may arise from the establishment of new worm populations after the anthelmintic treatment alters the previous established population composed of susceptible and resistant individuals (Browman, 2009). Drug treatment results in the death of

susceptible members of established nematode populations and the vacant niche may stimulate resistant hypobiotic nematode larvae that are dormant in the mucosa (Bowman, 2009). Typically, these nematode larvae can develop into an adult worm in less than a week (Taylor et al., 2007), making possible the establishment of an adult worm population that is able to produce eggs that can be found during the FECRT procedure 14 days after treatment.

Figure 1: Predominant gastrointestinal (GI) nematode species found in fecal nematode larval cultures. Out of the 21 dairy farms participating in the study, a subset of 10 dairy farms were chosen to evaluate which species of GI nematodes were infecting dairy heifers in Puerto Rico. At each dairy farm, fecal samples were collected directly from the rectum of at least 20 dairy heifers.

Figure 2: Mean fecal egg count reduction (FECR) ($\pm$ standard errors) obtained with Doramectin (DOR), Ivermectin (IVM), and Albendazole (ALB). At each of 21 dairy operations groups of at least 18 heifers, predominantly Holstein or Holstein crossbreeds were used in the study. Parasite fecal egg counts (FEC) were determined by the modified McMaster technique. Of the three-anthelmintic used, ALB ($n = 142$) proved to be the most effective ($P < 0.0005$) with a FECR of $67.47\% \pm 8.43$. The efficacy of DOR ($n = 130$) and IVM ($n = 148$) did not differ ($P = 0.8713$). However, Doramectin exhibited a FECRT of $25.50\% \pm 18.00$, whereas IVM FECR was $-8.13\% \pm 19.89$ due to an increase in FEC after treatment.

The presence of anthelmintic resistance is determined if the observed efficacy of an anthelmintic in a FECRT is less than the cutoff value of 95% (Coles et al., 1992). Based on the use of this cutoff value, all the anthelmintic treatments tested did not provide an effective FECR, indicating that anthelmintic resistance is indeed a problem in Puerto Rico. Although ALB resulted in the highest FECR (67.47 %), it was still well below the cutoff value of 95 % FECR. The presence of anthelmintic resistance had been previously detected in two commercial dairy farms in the municipality of Hatillo, Puerto Rico (Rivera-Mujica et al., 2009). At Farm #1, while ALB achieved a FECR of 79.00 %, IVM and DOR, only achieved a FECR of 37.80 %, and 30.97 % respectively. At Farm #2, ALB achieved a FECR of 100 %, while IVM and DOR only achieved a FECR of 14.30 % and 42.28 % respectively. Those previous results agree with the present study where ALB showed a higher observed efficacy than DOR and IVM.

Although the FECRT analysis is a well established procedure in parasitism research, it is important to acknowledge its limitations. Some factors that may affect the accuracy of the FECRT could include that egg output by female worms might not strongly correlate to the actual worm burden (Eysker and Ploeger, 2000). Also, non-uniform distribution of nematode eggs in the feces might be possible, and distribution of nematode species might be variable in the members of the host population (Vidyashankar et al., 2007). However, the FECRT analysis is a simple and relatively affordable procedure that has been used for decades to evaluate the existence of anthelmintic resistance worldwide.

Anthelmintic resistance develops when "there is a greater frequency of individuals within a population that can tolerate doses of an anthelmintic when compared to the normal population" (Prichard et al., 1980). Repeated treatment using the same drug accounts for the selection of resistant populations of GI nematodes (James et al., 2009; Sutherland and Leathwick, 2011; Cotter et al., 2015). Moreover, resistance develops more quickly when the same anthelmintic agent has been used intensively without any other measure for parasite control like proper stocking density and pasture rotation (Martin et al., 1997; James et al., 2009; Gasbarre, 2014). Also, insufficient exposure to the drug (under dosing), is another factor involved in the accelerated development of resistant populations of GI nematodes (Coles, 2002). Differences in the nematode's genome can confer tolerance to drug by altering the nematode metabolism of the drug, resulting in the ability of the parasitic nematode to tolerate progressively higher doses than the normal population

(Barragry, 1987; James et al., 2009; Gasbarre, 2014). Incorrect parasitism control measures result in an increment in the frequency of alleles with genetic changes that confer resistance to a drug in a parasitic population (James et al., 2009; Vidyashankar et al., 2012). The frequency of this alleles continues to increase over time with repeated drug exposure, as the offspring of such individuals inherit the drug resistance capabilities (James et al., 2009; Vidyashankar et al., 2012). At this point the identity and frequency of genetic mutations that provides nematodes anthelmintic resistance in Puerto Rico is unknown.

The resistance of GI nematodes to broad-spectrum anthelmintics in ruminants is a world-wide phenomenon. Across the world there have been hundreds of demonstrated instances of anthelmintic resistance in parasitic nematodes that infect cattle (Sutherland and Leathwick, 2011). In at least ten species of nematodes, resistance has been documented for all classes of broad-spectrum anthelmintics (Sutherland and Leathwick, 2011). Most cases of anthelmintic resistance in cattle involve *Cooperia* spp. (Stromberg et al., 2012). Countries like Argentina (Anziani et al., 2001, 2004; Fiel et al., 2001) the United Kingdom (Stafford and Coles, 1999), and New Zealand (Winterrowd et al., 2003) have reported anthelmintic resistance in *Cooperia* spp. Indeed, resistance to the macrocyclic lactones has become common in *Cooperia* spp. infecting cattle in New Zealand (Familton et al., 2001) and in Argentina (Mejía et al., 2003). Specifically, in Argentina, resistance of *Cooperia* spp. to IVM have been reported with a FECR from 74.5 % (Mejía

et al., 2003), but as low as 13.00 % (Anziani et al., 2004). In Brazil, Soutello et al., (2007) reported resistance of *Cooperia* spp. to IVM with a FECR of 28.6 %. Nevertheless, in the US, anthelmintic resistance has also been documented *Haemonchus* spp. in addition to *Cooperia* spp. (Gasbarre et al., 2004; Gasbarre, 2014). Likewise, in Australia resistance of *Ostertagia ostertagi* to fenbendazole, levamisole, and ivermectin was documented similarly to *Cooperia oncophora* (Cotter et al., 2015). Moreover, in Argentina benzimidazoles resistance has been found in *Ostertagia ostertagi*, *Haemonchus placei* and *Cooperia punctata* infecting cattle (Mejía et al., 2003). Therefore, the presence of nematodes anthelmintic resistance across the world is well documented and our findings suggest that it is also a problem in Puerto Rico.

Parasitism can affect the performance of dairy heifers such as weight gain, feed efficiency, reproductive performance, and eventually milk production. The control of GI nematodes has relied mostly on the use of broad-spectrum anthelmintics, and indiscriminate use of these treatments has resulted in the development of resistant nematode populations around the world. In our study, using a cutoff value of 95 % for drug efficacy, as determined by a fecal egg count reduction test (FECRT), the presence of nematode anthelmintic resistance in dairy heifers seems to be a widespread problem in Puerto Rico. Dairy farms should implement integrated methods of parasitic nematode control to preserve the effectiveness of currently available anthelmintics and ultimately enhance dairy heifers' performance.

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