Sustainable Worm Control on Scottish Dairy Farms with 'PASTURE' (Parasite Amplicon Sequencing to Understand Resistance Emergence)

Paul Campbell[1], Alastair Antonopoulos[1,2], Jennifer McIntyre[1], Kerry O'Neill[1], Andrew Forbes[1], Kathryn Ellis[1], Roz Laing[1]

[1] School of Biodiversity, One Health, and Veterinary Medicine, University of Glasgow, Glasgow, Scotland, UK

[2] Kreavet, Kruibeke, Belgium

The cost of helminth infections in dairy cattle in Europe and the UK was recently estimated at €941 million per annum, due to reduced productivity and the cost of anthelmintic treatment (1). The negative impact of helminth infections on the livestock industry is amplified by the global emergence of anthelmintic resistance to all three broad spectrum anthelmintic classes licensed for use in cattle against gastrointestinal and pulmonary nematodes and a widely used flukicide, triclabendazole (2-5), leaving farmers with reduced options once resistance arises. While resistance in gastrointestinal worms of cattle has received much less attention than in sheep, initial reports concerning reduced drug efficacy of pour-on products were published in 2009 (6) and resistance to all classes is now thought to be widespread (3, 7-8).

Diagnosis of resistance in the field currently relies on the faecal egg count reduction test (FECRT). However, this is an indirect measurement of worm survival and can only detect late-stage resistance (9). Furthermore, the FECRT is impractical for dairy youngstock in particular, which are often treated with a long acting anthelmintic over the first grazing season, when they are most likely to be affected by parasitic gastroenteritis. Genetic markers are highly sensitive and can be used to assess resistance status without animal handling or drug treatment by genotyping worms collected from faecal samples. Genetic markers are currently only available for the benzimidazole class, where variants in the *β-tubulin isotype-1* gene (primarily F200Y) confer resistance (10). We recently identified a genetic marker for levamisole resistance in a helminth of sheep, *Haemonchus contortus*, where the S168T variant in the *acr-8* gene confers resistance and is absent in drug-sensitive populations (11-14). The genetic variants conferring macrocyclic lactone resistance are currently unknown in any parasitic helminth species, although multiple resistance genes have been implicated.

As a first step in developing a sensitive diagnostic test for multi-drug resistance in parasitic helminths of cattle, a DNA amplicon sequencing panel was designed. Established and putative anthelmintic resistance genes were identified in the most pathogenic gastrointestinal nematode in UK cattle, *Ostertagia ostertagi,* using genomic and transcriptomic resources provided by the Welcome Sanger Institute (Tree of Life) and Moredun Research Institute (15) respectively. Next-generation sequencing (NGS) primers were designed to amplify ten validated or putative resistance loci (Table 1) and three neutral loci not expected to be under anthelmintic selection, then incorporated with published speciation primers (internal transcribed spacer-2 (ITS-2) region) for simultaneous surveillance of parasitic helminth community composition. This panel of 14 primers were used to generate PCR amplicons from pooled populations of ~1,000 nematode larvae from eight Scottish dairy farms, collected as part of an ongoing study (University of Glasgow PhD project, funded by a James Herriot Scholarship). For five of these farms, populations were collected at two or three time points over the grazing season (Table 2). Amplicons from the resulting 14 sample populations were Illumina sequenced at Glasgow

Polyomics and analysis was undertaken using DADA2 software (16) to identify genetic variants at each locus in each population.

First, the ITS-2 sequences were used to determine the species composition of each pooled population (Figure 1). In total, 13 parasitic nematode species were identified: *Chabertia ovina*, *Cooperia oncophora*, *Haemonchus contortus*, *Oesophagostomum radiatum*, *Oesophagostomum venulosum, Ostertagia leptospicularis, Ostertagia ostertagi, Nematodirus helvetianus, Teladorsagia circumcincta, Trichostrongylus axei, Trichostrongylus colubriformis*, and *Trichostrongylus vitrinus.* The most prevalent species were *C. oncophora* and *O. ostertagi,* which were detected in all 14 populations. *Cooperia oncophora* was the most abundant species in the dataset, followed by *O. ostertagi*, the latter representing a minimum of 26.4% of reads in every population. The next most abundant species were *T. axei, T. colubriformis*, and *N. helvetianus*, which accounted for 2.7%-3.7% of the dataset. Other parasitic helminths that are typically found in sheep, such as *H. contortus* and *T. circumcincta*, were observed but were present in less than 2% of the dataset.

For the benzimidazole class, sequencing of the *β-tubulin isotype-1* locus in *O. ostertagi* found the F200Y resistance variant in one population only, after benzimidazole treatment on an organic farm. This variant was present at high frequency (60.5% of *β-tubulin isotype-1* reads in this population). Other variants associated with resistance, such as E198L, were not detected in any *O. ostertagi* populations in this study. However, due to high sequence conservation at the locus, our primers also amplified the *β-tubulin isotype-1* locus in *T. colubriformis* and identified the resistant E198L variant at a frequency of 100% on the same farm as the resistant *O. ostertagi*. No resistance mutations were identified in the *β-tubulin isotype-2* locus.

For levamisole, resistance marker S168T in the *acr-8* gene was identified in *O. ostertagi* populations from two farms from different regions in Scotland. This variant was present at a very low frequency (0.47 – 1.27% *acr-8* reads in each population) and neither farm had reported levamisole use in the last seven years. However, prior historical use of the drug could not be ruled out.

There are no established genetic variants for macrocylic lactone resistance in *O. ostertagi* or any parasitic nematode species. However, we included putative resistance genes from the literature in the panel to assess for evidence of drug selection, which would implicate a role in anthelmintic resistance. Analysis of the putative resistance loci is ongoing, in concert with whole genome sequencing of a subset of populations from farms pre- and post- ivermectin and moxidectin treatment, to ensure every resistance locus is included and revealed in the analysis. This whole genome approach has been made possible by very recent advances in the genome assembly of *O. ostertagi*, and by the isolation of significant numbers of *O. ostertagi* eggs and larvae post-ivermectin and post-moxidectin treatment on multiple Scottish farms.

The preliminary results of this study suggest that benzimidazole resistance is present in Scottish dairy calves but is not currently widespread; this may reflect the greater reliance on macrocyclic lactones rather than benzimidazoles in conventional herds. However, the detection of resistance mutations at a high frequency on one farm should be a clear reminder to farmers that resistance variants are rapidly selected by drug treatment and clinical resistance (drug failure) will follow. Similarly, the presence of a levamisole resistant variant on two farms in different regions, also suggests that resistance is emerging to this class. This is the first report of S168T in UK cattle

and while the variant is currently at an extremely low frequency on both farms, if the situation in cattle mirrors that of sheep, clinical resistance due to S168T can be expected with future levamisole use. Most conventional dairy farmers in Scotland rely heavily on the macrocyclic lactones and the identification and validation of resistance markers for this anthelmintic class is a pressing need, which we are actively pursuing.

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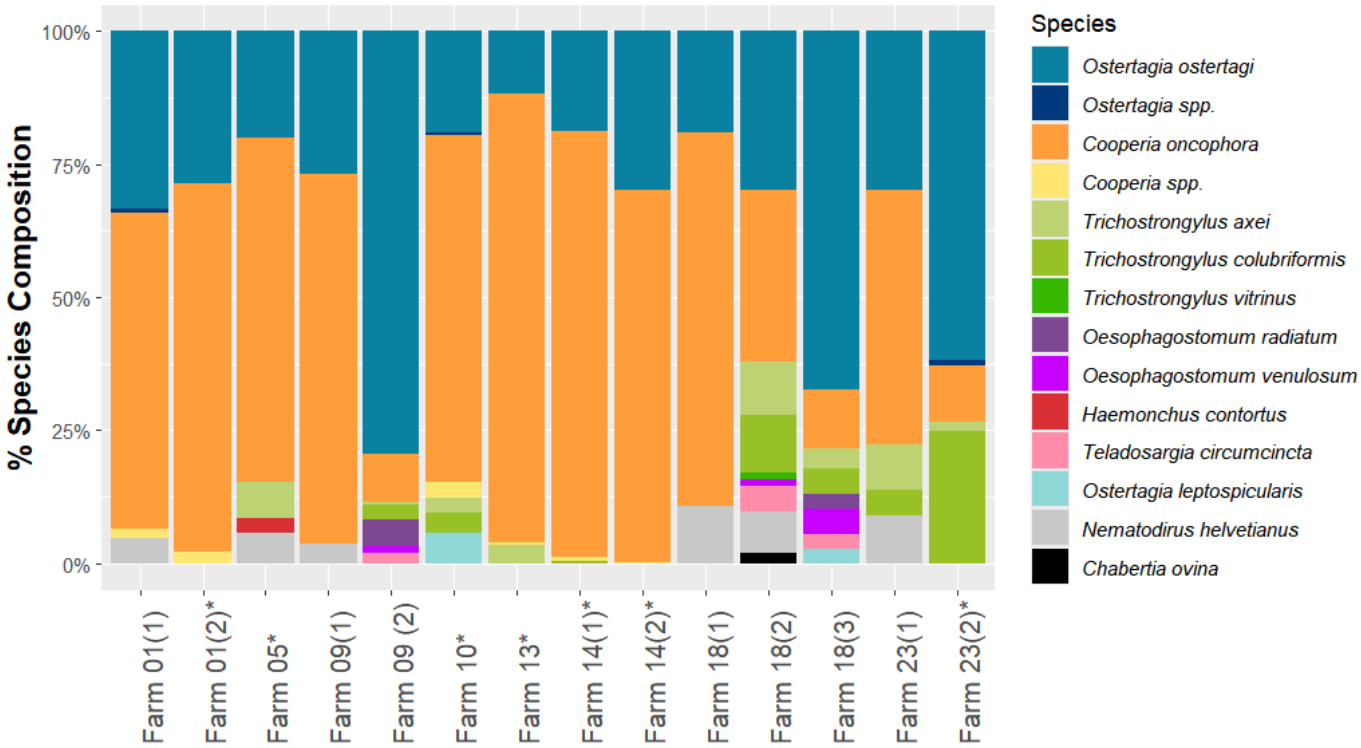
Table 1. Established and putative anthelmintic resistance loci included in the study.

Anthelmintic class	PASTURE amplicon locus	Status as resistance marker
Benzimidazole	*β-tubulin-isoype-1*	Established
	β-tubulin-isotype-2	Established
Levamisole	*acr-8*	Established (sheep nematodes)
	unc-29	Putative
	unc-63	Putative
Macrocyclic lactone	*avr-14*	Putative
	avr-15	Putative
	Checkpoint kinase	Putative
	cky-1	Putative
	pgp-9	Putative

Table 2. Farm populations included in the study. Anthelmintic treatments were administered by farmers as part of their own worm control strategy as this was part of a wider observational longitudinal study during the 2022 grazing season. Farms 18 and 23 are organic premises.

Farm	Number of populations (treatment status)	Anthelmintic class used on farm
Farm 01	2 (pre-treatment and post-treatment)	Ivermectin
Farm 05	1 (post-treatment)	Ivermectin
Farm 09	2 (untreated)	-
Farm 10	1 (post-treatment)	Ivermectin
Farm 13	1 (post-treatment)	Moxidectin LA
Farm 14	2 (both post-treatment)	Moxidectin LA
Farm 18	3 (untreated)	-
Farm 23	2 (pre-treatment and post-treatment)	Fenbendazole

Figure 1. Relative species abundance of gastrointestinal nematode communities, determined by ITS-2 sequencing. Populations marked with an asterisk were collected post-treatment with ivermectin (Farms 01(2), 05, 10), moxidectin LA (Farms 13, 14(1), 14(2)), or fenbendazole (Farm 23(2)).



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