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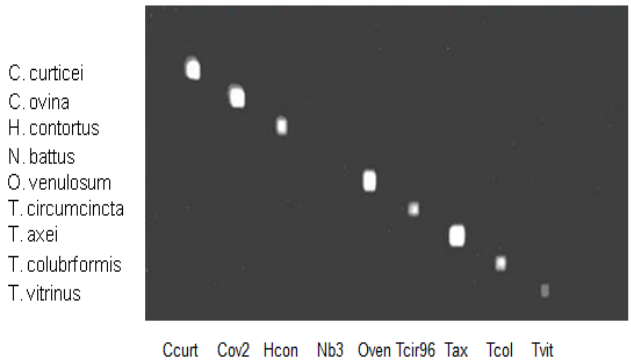
Molecular approaches to identifying nematode species and studying the genetic basis for resistance in ovine nematodes



Key external stakeholders:

Sheep producers, Parasitologists, Veterinary / diagnostic technicians, Pharmaceuticals

Practical implications for stakeholders:

Sheep are host to numerous species of helminth parasites. One of the major impediments to achieving effective control and management of these parasites is the lack of accurate diagnostic tests to identify parasite species responsible for disease that would inform tailored treatment decisions. Traditional methods of identifying parasitic nematodes are labour intensive and time consuming. DNA based assays have the potential to rapidly and reliably identify the species responsible for infection. In the present study the development towards a probe based assay to aid in the identification of the economically important nematodes infecting sheep was undertaken. Further development of the assay is warranted before this could have field application.

Results from a number of studies have indicated a high incidence (approximately 80%) of reduced susceptibility in ovine nematode populations on Irish farms to Benzimidazole (Bz). Tests to detect Bz resistance are cumbersome and lack sensitivity and specificity so a rapid and accurate test to detect Bz resistance would be preferred. It has been reported that single nucleotide polymorphisms at codon 167, 198 and/or 200 of the beta tubulin gene are responsible for BZ resistance. This has now been confirmed in Irish ovine parasitic nematode populations and will aid in the development of an accurate and rapid test to detect Bz resistance.

Results from the drench test survey conducted on commercial lowland flocks revealed drench failure of 100%, 50% and 27% to benzimidazole, levamisole and macrocyclic lactones respectively. Worthy of note that even though the participation in this survey was 48% (n=57) the number of samples that were suitable for inclusion was 33. The three principal reasons for exclusion of data from analysis were the flock faecal egg count sample pre-dosing was too low i.e. <200 eggs per gram, not adhering to the guidelines on the pre and post dosing interval required for the specific classes of anthelmintic or absence of a pre or post dosing sample.

Main results:

- This study has identified BZ resistant species in Ireland along with the gene and specific mutation responsible for BZ resistance in *Teladorsagia circumcincta*.
- A molecular tool to detect different genera of common ovine gastrointestinal nematodes simultaneously was designed. While the results were promising, further development to improve the assay's diagnostic capability is required.
- The results from the survey on drench failure in Irish flocks highlighted an emerging problem with drench failure to the macrocyclic lactone group of anthelmintics.

Opportunity / Benefit:

The confirmation of the genetic basis of BZ resistance in Irish *T. circumcincta* will aid in the development of an accurate and rapid test for BZ resistance

Increased industry awareness and measures to delay the development of macrocyclic lactone resistance need to be exploited.

Collaborating Institutions: UCD

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1. Project background:

Anthelmintic treatment has been an effective method of nematode control for over 40 years and current production systems rely heavily on such chemical methods of gastrointestinal nematode (GIN) control. Productivity is therefore threatened by the emergence of anthelmintic resistance (AR). In Ireland it has been demonstrated that 95% and 38 % of flocks tested showed evidence of BZ and LEV resistance in GIN populations. The situation with the ML group is less clear.

Current methods for differentiating GIN species relies chiefly on identification based on morphology using classical microscopy methods which is both time consuming, laborious and requires trained personnel. The development of a more accurate and rapid test based on molecular methods would aid in efficient diagnosis.The development of a reverse line blot hybridization assay that could detect different genera of common ovine GINs would facilitate diagnostic and epidemiological studies.

Current methods for AR detection, such as the faecal egg count reduction tests lack sensitivity, are labour intensive and time consuming. A molecular test based on the knowledge of specific genes and mutations leading to resistance would be an improvement on classical methods of AR detection. It has been demonstrated that resistance to Benzimidazole is commonly due to mutations in the gene encoding β-tubulin, although the precise codon affected varies from species to species and region to region. No information was available for Irish GIN species. In order to identify the mutations responsible for BZ resistance in Irish GIN populations, the coding region of the β–tubulin gene from BZ-resistant *T. circumcincta* was sequenced.

2. Questions addressed by the project:

- To identify BZ resistant species in Ireland along with the gene and specific mutation responsible for BZ resistance
- To develop rapid molecular diagnostic tools for important sheep nematode species in Ireland.
- Extent of drench failure in Irish flocks.

3. The experimental studies:

Experiment 1

- Development of a DNA based assay to diagnose gastrointestinal nematodes of economic importance in ovines. Reference parasitic material for the study was collected. SOP were identified and developed for DNA extraction from adult nematodes. The feasibility of using the internally transcribed spacer (ITS) regions and intergenic spacer (IGS) regions as targets for a molecular assay (based on species specific probes) to identify parasitic nematode species relevant to sheep in Ireland was examined.

Experiment 2

- The development of a reverse line blot hybridization assay that would simultaneously be able to distinguish between thirteen ovine gastrointestinal nematode species

Experiment 3

- To identify the mutations responsible for BZ resistance in Ireland

Experiment 4

- Drench test failure in Irish flocks and species identification

4. Main results:

Experiment 1

Nine species of parasitic sheep nematodes namely, *Cooperia curticei, Chabertia ovina, Haemonchus contortus, Nematodirus battus, Oesophagostomum colubianum, Teladorsagia circumcincta, Trichostrongylus axei. T. colubriformis, T. vitrinus and Oesophagostomum colubianum*, were used. DNA was extracted from a

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single adult worm from each species.

- The entire ITS-1, 5.8s, ITS-2 region and IGS region of the rDNA were amplified using various primer sets. Purified PCR products were then ligated into plasmids, transformed into *E.coli* cells, purified plasmids and then sequenced. The Align tool using the Smith-Waterman algorithm was used to compare the sequences.
- Amplification of the IGS region was unsuccessful for some species. Overall the sizes of the ITS-1 fragments ranged from 770bp (*Trichuris ovis)* to 368bp (*Chabertia ovina*) .The sizes of the ITS-2 fragments ranged from 409bp (*Trichuris ovis)* to 231bp (*Haemonchus contortus*). The GC content of the ITS-1 ranged from 40% to 57% while for ITS-2 it ranged between 32% and 62% for all species. Both ITS-1 and ITS-2 regions exhibit low levels of homology. The ITS-2 exhibited lower levels in 22/28 (79%) of the pairwise comparisons. The ITS-2 seems the most promising region for the design of discriminating probes for species specific identification in a reverse line blot (RLB) hybridization assay

Experiment 2

On the basis of the results from experiment 1, the ITS-2 region was subsequently selected as a genetic target for use in a reverse line blot hybridisation (RLB) assay. A set of 'universal' PCR primers were designed based on conserved regions flanking the ITS-2. Moreover species specific hybridisation probes were designed for nine species of nematodes based on areas of the ITS-2 sequences that were unique to each species. Reverse line blot hybridization assay was developed and optimised for the majority of species (e.g. see plate above: image of the RLB membrane after hybridisation and visualisation, results displaying the specificity of the probes designed.)However as inconsistencies in identification were observed between results from RLB and classical identification methods in particular to *N.battus,* further development of the assay is warranted before this could have field application.

Experiment 3

To identify the mutations responsible for BZ resistance in Irish nematode populations. Five farms with a prior history of BZ resistance were identified and contacted. Lambs (n=4) with a patent infection were purchased from four of these farms and transported to the Department of Agriculture Research Farm (Longtown, Co. Kildare).

BZ resistance was confirmed by 2 rounds of dosing with an anthelmintic containing Benzimidazole. Faecal egg counts were determined 10 to 14 days post-dosing to confirm parasite survival. Lambs were slaughtered at day 15/16 post the second round of BZ dosing to recover nematodes from the gastrointestinal tract which survived BZ treatment. Male nematodes were identified morphologically, placed in cryovials by species in batches of 1, 5 or 10 and snap frozen in liquid Nitrogen until further processing. Genomic DNA was extracted from individual adult male nematodes (up to 15 per farm) and the coding region of the β–tubulin gene was sequenced in order to determine the specific mutations responsible for BZ resistance

Experiment 4

Drench tests carried out in lamb flocks by commercial producers. Producers (n=120) (nominated by the sheep advisors) were contacted with the view to carrying out a drench test. Sample kits and detailed instructions were provided. Samples were returned by 57 participants, 33 were suitable for inclusion in that the faecal egg count (FEC) pre dosing was ≥200 eggs per gram (e.p.g.) and the post dosing sample was submitted at the recommended interval pre and post dosing for class of anthelmintic being tested. Results revealed inefficacy of 100%,(n=10) 50% (n=3) and 27% (n=4) to benzimidazole, levamisole and macrocyclic lactones respectively.

Once the FEC was determined, the remainder of the flock faecal sample was processed for coproculture to recover third stage larvae (L3) from which DNA was extracted to test (Reverse line blot assay). The predominant species observed pre and post dosing was *Teladorsagia circumcincta* (>90%).

5. Opportunity/Benefit:

- This research has advanced our knowledge on the genetic basis of benzimidazole resistance in Irish nematode populations
- This study has generated some preliminary data and SOPs towards the development of a molecular test that is capable of differentiating species generated from faecal samples
- An emerging problem of ML drench failure in Irish nematode populations is highlighted

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6. Dissemination:

International conference

Presented at the *Joint British Society Animal Science and Agricultural Research Forum Annual conference*, Queen University, Belfast. 12th April 2010

National Conferences Oral presentations at the joint. *Irish Society Parasitology and British Association Veterinary Parasitology Scientific meeting*, Backweston, Ireland 27/11/09 and at the *Irish Society Parasitology annual meeting, DCU*, Ireland 25/01/13.

Opendays

Presented at : Farm Fest 2008 Teagasc Athenry, Sheep 2010 Lyons Estate UCD , Sheep 2012 Teagasc Athenry and Lab open days/visits and In-service training days to advisors

Main publications:

Nabavi, R., Conneely, B., McCarthy, E., Good, B., Shayan, P., de Waal T. (2011) Comparison of internal transcribed spacers and intergenic spacer regions of five common Iranian sheep bursate nematodes. *Acta Tropica*

7. Compiled by: Drs. Barbara Good, Orla Keane

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