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A multiplex PCR-based method to identify strongylid parasite larvae recovered from ovine faecal cultures and/or pasture samples

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ABSTRACT

A multiplex PCR-based method was developed to overcome the limitations of microscopic examination as a means of identifying individual infective larvae from the wide range of strongylid parasite species commonly encountered in sheep in mixed sheep-cattle grazing situations in New Zealand. The strategy employed targets unique species-specific sequence markers in the second internal transcribed spacer (ITS-2) region of ribosomal DNA of the nematodes and utilises individual larval lysates as reaction templates. The basic assay involves two sets of reactions designed to target the ten strongylid species most often encountered in ovine faecal cultures under New Zealand conditions (viz. *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *Trichostrongylus vitrinus*, *Cooperia curticei*, *Cooperia oncophora*, *Nematodirus spathiger*, *Chabertia ovina*, and *Oesophagostomum venulosum*). Five species-specific primers, together with a pair of "generic" (conserved) primers, are used in each of the reactions. Two products are generally amplified, one by the generic primer pair regardless of species (providing a positive PCR control) and the other (whose size is indicative of the species present) by the appropriate species-specific primer in combination with one or other of the generic primers. If necessary, any larvae not identified by these reactions can subsequently be tested using primers designed specifically to detect those species less frequently encountered in ovine faecal cultures (viz. *Ostertagia ostertagi*, *Ostertagia leptospicularis*, *Cooperia punctata*, *Nematodirus filicollis*, and *Bunostomum trigonocephalum*). Results of assays undertaken on >5500 nematode larvae cultured from lambs on 16 different farms distributed throughout New Zealand indicated that positive identifications were initially obtained for 92.8% of them, while a further 4.4% of reactions gave a generic but no visible specific product and 2.8% gave no discernible PCR products (indicative of insufficient or poor quality DNA template). Of the reactions which yielded only generic products, 91% gave positive identifications in an assay re-run, resulting in a failure rate of just ~0.4% for reactions containing amplifiable template. Although the method was developed primarily to provide a reliable way to identify individual strongylid larvae for downstream molecular applications, it potentially has a variety of other research and practical applications which are not readily achievable at present using other methods.

1. Introduction

Most domestic livestock raised in pastoral grazing situations in temperate countries such as New Zealand harbour mixed-species burdens of parasitic nematodes. Within

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these burdens, the nematode species present can vary considerably in their epidemiology, pathogenicity, tolerance of different anthelmintics, and propensity to develop anthelmintic resistance. Moreover, in New Zealand where it is common for sheep and cattle to be farmed on the same property, the range of nematode species encountered in lambs can often be very diverse (Vlassoff and McKenna, 1994). The ability to reliably identify the species involved when nematodosis or anthelmintic resistance is suspected can be of considerable importance in determining how best to manage the situation.

The most widely used non-invasive (*ante mortem*) means of assessing the composition of nematode burdens in livestock under such circumstances remains the preparation of faecal cultures and subsequent differential identification and enumeration of infective larvae recovered from them (Coles et al., 2006; Van Wyk and Mayhew, 2013). For many years this has relied almost entirely on the microscopic examination of a range of diagnostic features such as overall length, tail shape and length, structural features of the cephalic extremity, number of intestinal cells and number of caudal tubercules (Soulsby, 1968; MAFF/ADAS, 1986; Lancaster and Hong, 1987; McMurtry et al., 2000; Van Wyk et al., 2004; Van Wyk and Mayhew, 2013). However, while such microscopic examination can provide a reasonably reliable indication of the identity of strongylid larvae at the generic level, determining the species involved is more problematic even when the work is carried out by an experienced operator. Indeed, even at the generic level, morphological and morphometrical differences between larvae can sometimes be small and, in a proportion of cases, measurements may overlap. For example, amongst ovine nematode larvae, those of *Teladorsagia* and *Trichostrongylus* are often particularly difficult to differentiate for this reason (Waghorn et al., 2014), and this can lead to incorrect diagnoses and in some cases inappropriate advice being given by animal health advisors.

Recent advances in molecular biology have provided a means to identify nematode parasite eggs and/or larval stages with much greater reliability than has generally been possible in the past. The first and second internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (rDNA) have proved to be particularly useful sources of species- and/or genus-specific markers for this purpose (see reviews by Gasser, 1999, 2006; Roeber et al., 2013b) because they generally show moderate levels of sequence variability between species but relatively low levels of variability within species. Moreover, as pointed out by Hung et al. (1999), rDNA is particularly amenable to amplification using polymerase chain reactions (PCRs) because multiple copies are present in the genome of most organisms.

A number of studies have thus utilised ITS-1 and/or ITS-2 sequence information in PCR-based assays designed to detect the presence of particular nematode species or genera in domestic or feral livestock (e.g. Schnieder et al., 1999; Zarlenga et al., 2001; von Samson-Himmelstjerna et al., 2002; Wimmer et al., 2004; Bott et al., 2009; Roeber et al., 2011; Sweeny et al., 2011, 2012; Höglund et al., 2013). Furthermore, some recent studies have focussed on assessing the potential of one of the above assays (Bott et al., 2009) to replace larval culture in ovine faecal egg count reduction tests (Roeber et al., 2012a), on developing a high-throughput platform to undertake the assay (Roeber et al., 2012b) and on identifying more efficient methods to isolate nematode DNA from sheep faeces (Roeber et al., 2013a).

However, most of the above assays have been designed to diagnose the presence of a relatively limited number of the nematode species or genera likely to infect livestock, and none appears to have the capacity to identify individual larvae of the wide range of strongylid parasite species frequently encountered in ovine faecal cultures and/or pasture samples in mixed sheep-cattle grazing situations.

The need for of a suitable method to reliably identify individual nematode larvae recovered from ovine faecal cultures for downstream research applications (including on-going work to investigate genetic relationships between various candidate molecular markers and particular anthelmintic resistance phenotypes in the field), provided the motivation for the present work. Such molecular genetics studies generally rely on the use of oligonucleotide primers and/or probes which tend to be species-specific. The ability to accurately identify larvae recovered from faecal cultures for such purposes is thus essential (Barrére et al., 2013). The aim of the present study was therefore to establish a relatively straightforward and reliable PCR-based method to identify individual infective larvae of the wide range of strongylid nematode species often encountered in naturally-infected field-grazed sheep under New Zealand conditions.

2. Materials and methods

2.1. Nematode material used to generate ITS-1 and ITS-2 sequence data

Although ITS-1 and ITS-2 sequence information for many of the common parasitic nematode species found in sheep in New Zealand was already available in Genbank™, this was not the case for all species of interest to us. Furthermore, it was not clear to what extent ITS sequences from New Zealand parasitic nematode populations might vary from those available in Genbank™. We thus initially amplified, cloned and sequenced ITS-1 and ITS-2 fragments from samples of individual worms from local populations of each of the nematode species of interest.

Adult male gastrointestinal nematode parasites of the order Strongylida (including *Haemonchus contortus*, *Teladorsagia circumcincta*, *Trichostrongylus axei*, *Trichostrongylus colubriformis*, *Trichostrongylus vitrinus*, *Cooperia curticei*, *Nematodirus spathiger*, *Nematodirus filicollis* and *Oesophagostomum venulosum*) were recovered at necropsy from the abomasum and small and large intestines of five naturally infected lambs obtained from three different properties in the central region of the North Island of New Zealand. Adult male *T. circumcincta* representing three laboratory strains maintained at AgResearch Grasslands were also included. Additional ITS-2 sequences were subsequently generated from infective larvae of most of the species above from a wide range of different field sources within New Zealand.

Other strongylid species more commonly associated with cattle (and/or farmed red deer) in New Zealand (Bisset,