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The use of nemabiome metabarcoding to explore gastro-intestinal nematode species diversity and anthelmintic treatment effectiveness in beef calves

Russell W. Avramenko [a], Elizabeth M. Redman [a], Roy Lewis [b], Murilo A. Bichuette [c], Bruna M. Palmeira [a], Thomas A. Yazwinski [d], John S. Gilleard [a,*]

[a] Department of Comparative Biology and Experimental Medicine, Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada
[b] Merck Animal Health, Calgary, Alberta, Canada
[c] São Paulo State University, Jaboticabal, São Paulo, Brazil
[d] Department of Animal Science, University of Arkansas, Fayetteville, AR, United States

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ABSTRACT

Next-generation deep amplicon sequencing, or metabarcoding, has revolutionized the study of microbial communities in humans, animals and the environment. However, such approaches have yet to be applied to parasitic helminth communities. We recently described the first example of such a method – nemabiome sequencing – based on deep-amplicon sequencing of internal transcribed spacer 2 (ITS-2) rDNA, and validated its ability to quantitatively assess the species composition of cattle gastro-intestinal nematode (GIN) communities. Here, we present the first application of this approach to explore GIN species diversity and the impact of anthelmintic drug treatments. First, we investigated GIN species diversity in cow-calf beef cattle herds in several different regions, using coproculture derived L3s. A screen of 50 Canadian beef herds revealed parasite species diversity to be low overall. The majority of parasite communities were comprised of just two species; *Ostertagia ostertagi* and *Cooperia oncophora*. *Cooperia punctata* was present at much lower levels overall, but nevertheless comprised a substantive part of the parasite community of several herds in eastern Canada. In contrast, nemabiome sequencing revealed higher GIN species diversity in beef calves sampled from central/south-eastern USA and Sao Paulo State, Brazil. In these regions *C. punctata* predominated in most herds with *Haemonchus placei* predominating in a few cases. *Ostertagia ostertagi* and *C. oncophora* were relatively minor species in these regions in contrast to the Canadian herds. We also examined the impact of routine macrocyclic lactone pour-on treatments on GIN communities in the Canadian beef herds. Low treatment effectiveness was observed in many cases, and nemabiome sequencing revealed an overall increase in the proportion of *Cooperia* spp. relative to *O. ostertagi* post-treatment. This work demonstrates the power of nemabiome metabarcoding to provide a detailed picture of GIN parasite community structure in large sample sets and illustrates its potential use in research, diagnostics and surveillance.

1. Introduction

There is a need to develop better tools with which to qualitatively and quantitatively assess the species composition of gastro-intestinal nematode (GIN) communities in both humans and animals. Approaches utilizing next-generation sequencing technologies, similar to those used to study the microbiome, hold massive potential in this regard but have yet to be seriously explored for helminths (Avramenko et al., 2015). GIN infections of livestock are an excellent system in which to explore such approaches because complex co-infections are common and cause both clinical disease and sub-clinical production loss worldwide (Stromberg and Gasbarre, 2006; Grisi et al., 2014). Control is largely reliant on the routine application of anthelmintic drugs, the high effectiveness of which has allowed farmers to maximize stocking rates and increase production returns for many years. However, these parasites are still often poorly controlled (Gasbarre et al., 2015). Results of a recent survey of the National Animal Health Monitoring System (NAHMS) in the United States (US), suggest that GINs are as preva-

* Corresponding author at: HSC 2531, 3330 Hospital Drive NW, Calgary, AB T2N 4N1, Canada. Fax: +1 403 210 6693.
E-mail addresses: rwavrame@ucalgary.ca (R.W. Avramenko), jsgillea@ucalgary.ca (J.S. Gilleard).

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lent in cattle today as they were before the wide-scale introduction of broad-spectrum anthelmintic use over 30 years ago (Stromberg et al., 2015). Furthermore, cattle parasites resistant to the macrocyclic lactone drugs are now emerging, making the reliance on chemical control increasingly unsustainable (Gasbarre et al., 2009; Kaplan and Vidyashankar, 2012).

There are many different GIN species that infect cattle, and many of these differ in their pathogenicity, production impact, epidemiology and drug sensitivity (Taylor et al., 2015). However, there is remarkably little contemporary data published on the prevalence and infection intensities of different parasite species in different geographical regions and production systems. Most information for North America is based on a small number of regional studies, many of which are over 40 years old, or on anecdotal information from veterinarians, parasitologists and diagnostic laboratories (Hitchcock, 1956; Zimmerman and Hubbard, 1961; Ciordia, 1975; Stromberg et al., 2015). Additionally, there is a need for more information regarding which parasite species are developing resistance to each drug class and how drug treatments are impacting parasite species diversity and community composition.

One of the main barriers to obtaining large-scale quantitative data on parasite species prevalence, infection intensities and the impact of anthelmintic use is the lack of appropriate tools. Although conventional PCR assays are increasingly used to detect cattle parasites, these are not quantitative and only indicate the presence or absence of different parasite species in a sample (Zarlenga et al., 2001). The more commonly available PCR assays also only allow discrimination to the genus level (Stromberg et al., 2015). A real time PCR assay has been developed for *Ostertagia ostertagi* and *Cooperia oncophora* but these tests require significant optimization between laboratories and only detect two of the many species commonly present in cattle parasite communities in different regions (Roeber et al., 2012; Höglund et al., 2013).

We have recently developed and validated a new approach, nemabiome sequencing or metabarcoding, which accurately quantifies the relative proportions of parasitic nematode larvae isolated from cattle feces (Avramenko et al., 2015). It is based on deep amplicon next generation sequencing of the internal transcribed spacer 2 (ITS-2) rDNA locus and is equivalent to 16S rDNA sequencing of bacterial communities used in microbiome studies (Avramenko et al., 2015). In this paper, we apply nemabiome metabarcoding to investigate the species composition of parasite communities, and how those are impacted by macrocyclic lactone treatments, in calves from 50 Canadian cow-calf beef herds. We also compare the species composition of parasitic nematode communities in Canadian beef cattle with those from the mid/southern US, as well as Sao Paulo State, Brazil. This work illustrates how nemabiome metabarcoding provides a powerful new approach to obtain an unprecedented amount of quantitative information on parasite community composition with many applications, not only in cattle, but in a variety of different host-parasite systems.

2. Materials and methods

2.1. Collection of fecal samples

2.1.1. Canada

Samples were collected from 50 beef herds from across Canada from June through December of 2012; British Columbia (BC) (1), Alberta (23), Saskatchewan (7), Manitoba (2), Ontario (16) and Quebec (1). 20 individual animal fecal samples were collected, either per rectum or as freshly voided on pasture, from groups of calves from 50 different herds either at pasture or at feedlot entry. An additional 20 fecal samples were collected from the same group (but not necessarily from the same animals) 14 days after anthelmintic treatments were applied by the producer using their own protocols. Fecal samples were collected under an approved Animal Use Protocol (Animal Care Committee, Study #AC13-0157, University of Calgary, Canada), which is in accordance with the principles outlined in the Guidelines of the Canadian Council on Animal Care. Fecal samples were placed in plastic bags, which following exclusion of air were sealed and shipped with cool packs. Fecal egg counts (FECs) were performed using the Modified Wisconsin Technique (Ito, 1980). Three grams of feces were used for each FEC with all eggs being counted providing a detection threshold of 0.33 eggs per gram of feces (epg). Coprocultures were set up as described in Roberts and O'Sullivan (1950), however vermiculite was used in place of sawdust, and incubated at room temperature (~20 °C) for 21 days. Larvae harvested from each individual fecal sample were pooled by herd (keeping pre-treatment and post-treatment samples separate). Larvae were fixed in 70% ethanol and stored at −80 °C in aliquots of ~1000–2000 larvae where possible. In the case of post-treatment fecal samples larvae were obtained from only 42 out of 50 herds assessed and, in some cases, fewer than 1000 larvae were obtained (range 100–1000).

2.1.2. US

Fecal samples from the US were collected from 38 individual stocker cattle entering feedlot operations throughout 2014 in Oklahoma (22), Arkansas (3) and Nebraska (13). Each calf was from a different stocker cattle delivery from farms across the mid/southern US, and thus likely to be derived from different farms. Fecal samples were collected per rectum, stored and subsequently cultured for 14 days using the same protocol as for the Canadian fecal samples. Larvae were fixed in 70% ethanol until needed.

2.1.3. Brazil

Fecal samples were collected from 26 farms, from animals 6–9 months of age, across Sao Paulo State in Brazil during August and September, 2015. Three fecal samples from each farm were collected, set up individually for coproculture and the resulting L3s pooled together. Coprocultures were set up as previously described at room temperature (~25 °C). Larvae were collected and fixed in 70% ethanol until needed.

2.2. Calculation of drug treatment effectiveness

For the Canadian samples, mean egg counts of each group were compared pre- and post-anthelmintic treatment. Drug treatment effectiveness was calculated using the eggCounts R Statistical package, using the web interface with default parameters (Torgerson et al., 2014). The percentage reduction in egg counts was calculated separately for both strongyle and *Nematodirus* egg counts. This produced a FEC reduction for each farm with 95% confidence intervals (CIs). In order to assess the effectiveness of the anthelmintic treatments against each nematode species, we partitioned the strongyle FECs into separate species by multiplying the total egg count by the fraction of each species in the sample as determined by the nemabiome sequencing data from the L3 cultures. A similar approach has been used by others using species proportions determined by visual morphological analysis of L3s from fecal cultures (Waghorn et al., 2006; McMahon et al., 2013). Drug treatment effectiveness was not assessed for species that had fewer than 1 epg pre-treatment. Percent effectiveness tests for each species were calculated by the following formula: (EggsPre − EggsPost)/(EggsPre) * 100. As the species analysis was completed on pooled data, calculation of S.D. and/or CIs was not possible on a per farm basis. Statistical differences between effectiveness for each species, across all farms, were determined with a repeated-measures ANOVA, with a Sidak confidence interval adjustment.

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