Prevalence, fecal egg counts, and species identification of gastrointestinal nematodes in replacement dairy heifers in Canada

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ABSTRACT

Information is scarce regarding the epidemiology of gastrointestinal nematodes in Canadian dairy heifers. The objectives of this study were to estimate the prevalence and fecal egg counts of gastrointestinal nematodes in dairy heifers, and using a novel deep-amplicon sequencing approach, to identify the predominant gastrointestinal nematode species in Canadian dairy replacement heifers. Fresh environmental fecal samples ($n = 2,369$) were collected from replacement heifers on 306 dairy farms across western Canada, Ontario, Québec, and Atlantic Canada. Eggs per gram of feces (EPG) were determined using a modified Wisconsin double-centrifugation sugar flotation technique. Predominant nematode species at the farm level were identified by deep-amplicon nemabiome sequencing of the internal transcribed spacer-2 rDNA locus of nematode third-stage larvae. Generalized estimating equations were used to estimate predicted parasite prevalence and mean EPG in all heifers and by province, allowing for clustering within herds. Individual heifer egg counts ranged from 0 to 141 EPG (median: 0 EPG; interquartile range: 0 to 71 EPG). Gastrointestinal nematodes were detected in 20.9% (95% confidence interval: 17.2 to 24.6%) of heifers, and the predicted mean strongylid EPG accounting for clustering on farms was 1.1 (95% confidence interval: 0.6 to 1.6). The predominant parasite species were *Cooperia oncophora* and *Ostertagia ostertagi*. This is the first study in Canada to use a combination of deep-amplicon nemabiome sequencing and a traditional egg count method to describe the epidemiology of gastrointestinal nematodes in dairy heifers.

Key words: gastrointestinal nematode, heifer, nemabiome, Canada

INTRODUCTION

Gastrointestinal nematodes (GIN) are widely prevalent in cattle worldwide and in temperate climates. *Ostertagia ostertagi* is considered to be the most pathogenic and, together with *Cooperia oncophora*, the most common species (Shaw et al., 1998a). Young, growing animals, such as dairy replacement heifers, are at an increased risk of GIN infections mainly from a lack of previous exposure to parasites and a naïve immune system (Fox, 1997; Yazwinski et al., 2013). Dairy replacement heifers represent the future of a dairy farm; they account for a substantial amount of the enterprise costs but are also more susceptible to GIN infection compared with adult cows (Zwald et al., 2007; Lang, 2012; Bellet et al., 2018). Although some studies report earlier onset of puberty, a reduced culling rate during first lactation and increased mammary tissue development in heifers treated with anthelmintic products, suggesting that overall lifetime productivity may be reduced in untreated heifers, ambiguity remains whether these effects are because of reduced GIN levels or from other pharmacological drug effects on the metabolism or immune system of the treated animal (Purvis and Whittier, 1996; Mejía et al., 1999, 2009; Perri et al., 2013). Furthermore, the long-term effects of GIN infection as a heifer on productivity as an adult cow have not been assessed well and the actual economic effects of GIN infection on cattle farms are more challenging to establish because multiple management factors, socio-psychological values, and local input and output costs affect farm-level economics and cattle health (Corwin, 1997; Charlier et al., 2014, 2016; Bellet et al., 2018; Rushton and Bruce, 2019). Nonetheless, it is important to have an understanding of the GIN prevalence

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and infection intensity in cattle production systems to further direct research and extension interventions in a region or country to better mitigate potential effects on health and production. The importance of knowing GIN prevalence, infection intensity, and predominant species is emphasized further by growing concerns about the effects that livestock intensification, changing climates, and anthelmintic resistance have on the epidemiology of ruminant GIN including infection pressure, environmental survival, seasonality, and changes to species diversity (Sutherland and Leathwick, 2011; Morgan et al., 2013; Skuce et al., 2013; Rushton and Bruce, 2019).

In Canada, studies on GIN infection in dairy heifers are scarce, are limited to regional investigations, and have not been conducted in decades (Cox and Lemiski, 1989; Nødtvedt et al., 2002). Furthermore, identification of the species diversity using a deep-amplicon sequencing approach has never been done in Canadian dairy heifers. In light of the lack of a current basic understanding of the GIN epidemiology in Canadian dairy heifers, the aim of this study was to better describe the extent and characteristics of GIN infection in that population and to generate information that could be useful to guide future research and extension initiatives in the Canadian dairy industry. Therefore, the main objectives of this survey were to determine the GIN prevalence, fecal egg count (FEC) intensity, and using a novel deep-sequencing assay, to quantify the predominant GIN species in replacement heifers on Canadian dairy farms.

MATERIALS AND METHODS

Herd Selection

The data for this study were collected as part of a large multi-institutional Canadian National Dairy Study (NDS). The details of this study have been previously described (Bauman et al., 2018). Figure 1 shows the herd selection process through the NDS. (Source for number of farms and heifers: Canadian Dairy Information Centre, 2017.) National Dairy Study resources were defined by financial and geographical limitations; sample collection was conducted by 14 university students from across Canada and limited to the period of May to August 2015 (Bauman et al., 2018). As part of the NDS, all registered Canadian dairy producers (~12,000) were invited to participate in a phase I survey in the spring of 2015. The survey asked about dairy cattle husbandry, management, and welfare practices. Phase II (May to August 2015) included a site visit for collection of benchmarking data for herd-level disease testing on bovine leucosis, Johne's disease, digital

dermatitis, and mastitis pathogens, as well as the fecal samples collected for this study. The number of farms selected from each province was based on the percentage of dairy farms in that province relative to the total number of dairy farms in Canada. Therefore, the aim was to have approximately the following regional distribution of selected farms: 43% from Québec (QC), 35% from Ontario (ON), 13% from western Canada [British Columbia (BC), Alberta (AB), Saskatchewan (SK), and Manitoba (MB)], and 8% from Atlantic Canada [Newfoundland (NL), Prince Edward Island (PE), Nova Scotia (NS), and New Brunswick (NB)]. Holstein Friesians made up 94% of the registered Canadian national dairy herd in 2015 and this distribution was the same for all provinces; therefore, specific information on dairy breed was not collected (Canadian Dairy Information Centre, 2019).

Figure 1. Sampling scheme for collecting fecal samples from replacement dairy heifers in Canada as part of the National Dairy Study (NDS; Bauman et al., 2018) and corresponding data tables and figures.

Fecal Sample Collection

Fresh environmental fecal samples were collected from individual replacement heifers between May and August 2015. The sampling plan called for collection of fecal samples from bred heifers or replacement heifers present on the farm and which, at the time of sampling, were under observation for heat with intent to be bred. This definition was expected to exclude cows while also providing an explanation of the acceptable lower age of heifers to be included. Although that lower age was expected to vary by farm, the definition of “heifer under observation for heat with intent to be bred” was supposed to be specific enough to limit sample collection to breeding-age heifers. Breeding-age replacement heifers were targeted primarily because of their increased risk of gastrointestinal parasitism compared with older animals and because of a scarcity of information on GIN in this population in Canada.

All breeding-age replacement heifers on each farm were sampled up to a maximum of 10 heifers per farm. When the number of breeding-age heifers as described above was less than 10, samples were collected from younger replacement heifers co-housed with the target group to bring the number of samples collected to 10 per farm. Each farm was sampled once during the study period. The age range of the sampled heifers and whether they had access to pasture was collected when possible. Fecal samples were collected immediately after defecation in leak-proof containers and shipped in coolers with ice (not in direct contact with ice) by courier to the laboratory at the Western College of Veterinary Medicine in Saskatoon, SK, Canada. This study was approved by the University of Saskatchewan Committee on Animal Care and Supply and the Animal Research Ethics Board (approval number: 20150037).

Fecal Egg Counts

Fecal egg counts were performed on each individual sample using the modified Wisconsin double-centrifugation sugar flotation technique. Briefly, 5 g of feces was mixed with 12 mL of water and strained through cheesecloth into a 15-mL test tube. The pellet was mixed with Sheather's solution (specific gravity: 1.27) and centrifuged twice at $360 \times g$ (20°C) for 5 min, a coverslip was placed on top of the test tube, which was allowed to rest for at least 20 min on the benchtop to maximize egg flotation. This technique has a reported diagnostic sensitivity of <10 eggs per gram of feces (EPG) based on 5 g of feces (Zajac and Conboy, 2012). Samples were examined using light microscopy at 40× magnification, and the number of strongylids, *Nematodirus* spp., and *Trichuris* spp. eggs were recorded

separately based on the unique morphology of the latter 2 species. Strongylids were indistinguishable from one another and included *Ostertagia* spp., *Haemonchus* spp., *Cooperia* spp., *Oesophagostomum* spp., *Trichostrongylus* spp., and *Bunostomum* spp. A sample was considered positive for GIN if at least one strongylid, *Nematodirus* spp., or *Trichuris* spp. egg was observed under the microscope. Eggs per gram of feces were calculated by dividing the egg counts by the original weight of the sample.

Larval Coproculture

The coproculture protocol was modified from Roberts and O'Sullivan (1950). Regardless of FEC results of individual heifers, fecal samples from up to 10 heifers per farm were pooled. Approximately 8 g of fecal material from each heifer was combined to a maximum of 80 g and mixed with vermiculite in a culture glass. Tap water was added until the mixture reached a paste-like consistency. The coproculture was incubated for 10 to 12 d at 27°C to allow hatching of eggs and subsequent development to third-stage larvae (L3); coprocultures were checked daily and sprayed with water when needed to ensure a slightly moist environment. After 10 to 12 d, the culture glasses were filled with water, inverted into a Petri dish, and allowed to stand overnight without light; the L3 were then collected from the supernatant in the Petri dish, semi-quantified using a grading scheme (+: 1 to 20 larvae, ++: 21 to 50 larvae, +++: >50 larvae), and stored in 2-mL cryotubes in 70% ethanol at 4°C. Before DNA analysis, the number of L3 in each sample was estimated by removing the ethanol in the cryotube down to 300 µL, counting the number of larvae in a 10-µL aliquot, and multiplying by 30.

Larval Species Identification by Deep-Amplicon Sequencing

The proportions of GIN species present in fecal samples were determined using a deep-amplicon sequencing nemabiome assay targeting the internal transcribed spacer (ITS)-2 rDNA genomic locus using the NC1 and NC2 primers outlined by Avramenko et al. (2015). Previous molecular diagnostic techniques for GIN have been described using ITS-1 and ITS-2 loci of nematode larvae, including conventional and real-time PCR (Roeder et al., 2013). While these techniques are useful to describe the species present in a sample, the advantage of using next-generation sequencing technologies such as the deep-amplicon sequencing nemabiome assay is the ability to quantify the relative species proportions of larvae or eggs in a fecal sample.

Briefly, L3 larvae were digested by heating for 15 min at 95°C and freezing at -80°C for 60 min before adding 10 µL of lysis buffer [50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl₂, 0.45% Nonidet P-40, 0.45% Tween 20, and 0.01% (wt/vol) gelatin], and 120 µg/mL proteinase K (Thermo Scientific, Waltham, MA) and subjected to mechanical agitation to prepare DNA lysates. Initial PCR amplification was performed using the following cycling conditions: 95°C for 3 min, followed by 98°C for 20 s, 62°C for 15 s, 72°C for 15 min for 35 cycles, and a final extension of 72°C for 2 min. The PCR products were purified using magnetic bead purification according to the manufacturer's instructions (AMPure, XP Magnetic Beads, Beckman Coulter Inc., Brea, CA). An agarose gel electrophoresis was performed to confirm successful amplification. Unique combinations of Illumina index tags were added to each amplicon using a second limited-cycle PCR amplification performed with the following thermocycling conditions: 98°C for 45 s, followed by 98°C for 20 s, 63°C for 30 s, and 72°C for 2 min for 7 cycles. The next generation deep-amplicon sequencing assay was performed using Illumina MiSeq Desktop Sequencer, with the 2 × 250 reagent kit (Illumina Inc., San Diego, CA).

Statistical Analysis

Data were entered into a commercial spreadsheet program (Excel 2013, Microsoft Corp., Redmond, WA) and imported into a statistical software package (Stata SE version 14, Stata, College Station, TX). Where known, for each farm the age of the heifers available at the time of sampling was divided into 3 categories based on the quartiles of the age distribution of all sampled heifers from which age was known: 6 to 12 mo (25% of all heifers), 13 to 15 mo (50% of all heifers), or >16 mo (25% of all heifers). Access to pasture was recorded as a binary variable where that information was available. Number of farms and samples collected per farm were reported by province. The distribution of the number of samples received during the study months was reported. Descriptive summary statistics for the raw (crude) GIN prevalence (and exact 95% CI) and geometric mean EPG (±SD, range, median, and interquartile range) in all heifers were calculated separately for all strongylids, *Nematodirus* spp., and *Trichuris* spp. and reported by region: western Canada (BC, AB, SK, and MB); ON; QC; and Atlantic Canada (NL, PE, NS, and NB). For the purpose of this analysis, prevalence was defined at the animal level as a binary variable by categorizing the FEC for each heifer as 0 when EPG was 0 and as 1 when at least one GIN egg was identified on microscopy (i.e., EPG >0). Mean EPG was treated as discrete count data.

Generalized estimating equations (GEE) were used to provide population-averaged estimates of the predicted GIN prevalence and mean EPG in all heifers while allowing for clustering within herds (Dohoo et al., 2003). In all models, either GIN prevalence or mean EPG were the outcome variables of interest (dependent variables). Possible risk factors (independent variables) for both outcomes were evaluated as fixed effects in separate models: one that included only region, and one that included region, age, and pasture access on only a subset of the data where that information was available. The initial null or intercept-only (no independent variable) GEE model for the overall predicted prevalence used a binomial distribution and logit link function with an exchangeable within-group correlation structure and robust standard error. The overall mean strongylid, *Nematodirus* spp., and *Trichuris* spp. EPG were also determined using a null GEE model with a negative binomial family and log link function with an exchangeable within-group correlation structure and robust standard error.

Due to the overall low crude prevalence of *Nematodirus* spp. and *Trichuris* spp., subsequent analyses were restricted to strongylids only. The effect of region on the strongylid prevalence and mean EPG was assessed as a fixed effect introduced in the above GEE models.

Where the information was available, age and pasture access were also explored as possible risk factors for the GIN prevalence and mean EPG, while also accounting for region as a fixed effect. This was not a primary objective of this study, and therefore, that information was not collected systematically; however, because both age and pasture access are known risk factors for GIN infection in cattle, it was deemed valuable to briefly explore this with the subset of samples on which that information was available (Sanchez et al., 2002; O'Shaughnessy et al., 2015; Jelinski et al., 2016). For this, an additional GEE model was computed with the subset of the data where age and pasture access were known. Independent variables (region, mean age, pasture access) were forced into the model as fixed effects. When estimating the GIN prevalence and mean EPG accounting for region, age, and pasture access, interaction terms considered biologically plausible (pasture access and mean age; province and pasture access) were evaluated and retained in the models if statistically significant based on a Wald's test at a *P*-value ≤0.05. The effects of independent variables on the predicted GIN prevalence and mean EPG of strongylids were assessed using post-hoc pairwise comparison with a level of significance set at a *P*-value ≤0.05.

Bioinformatic analysis was performed on the ITS-2 rDNA Illumina raw sequence data to assign nematode species identity to individual Illumina sequence reads

(Avramenko et al., 2017). If a sample contained fewer than 2,000 reads, it was discarded and treated as a failed sample. The number of species-specific sequence reads divided by the total number of reads per sample, and multiplied by the appropriate correction factor to account for species-specific biases in the assay (Avramenko et al., 2015), provided the relative nematode species proportions. Nematode species proportions were reported with the corresponding geometric strongylid FEC and estimated starting number of L3 larvae for those farms. A detection threshold was calculated for each sample by dividing 100% by the starting number of L3 in that sample; species detected in the sample below this threshold were removed from further analysis. A detailed summary of the analysis methods can be found at www.nemabiome.ca.

RESULTS

Descriptive Summary of Sampled Populations and Samples Collected

A total of 357 dairy farms were sampled across Canada; however, fecal samples from only 306 of these farms could be included in the analyses because of sampling errors (such as sampling the same animal multiple times and including non-fresh fecal samples from environmental fecal pats). The 306 farms were distributed across all 10 Canadian provinces and included 122 (40%) farms from ON, 84 (27%) farms from QC, 55 (18%) farms from western Canada (18 each from AB and BC, 10 from SK, 9 from MB), and 45 (15%) farms from Atlantic Canada (17 from PE, 13 from NS, 12 from NB, and 3 from NL). A total of 2,369 replacement heifers were sampled from these 306 farms. Seven percent ($n = 155$), 35% ($n = 834$), 33% ($n = 778$), and 25% ($n = 602$) of all 2,369 samples were received in May, June, July, and August, respectively. Samples from MB and BC were only received in June and August, respectively, whereas samples from all other provinces were received throughout the study period. Median number of heifers in the target group per farm at the time of sampling was 16 (range: 1 to 80 heifers), and median number of heifers sampled per farm was 10 (range: 1 to 11 heifers). Age information was available for 1,881 heifers (1,881/2,369; 79.4%); mean age was 14 mo (SD: 2) with an overall range of 6 to 26 mo. The mean number of days between sample collection and fecal egg counting was 11.4 d (range: 2 to 22 d). Information on access to pasture was available from 1,163 heifers from all 10 provinces. Across all 4 regions, 275 heifers had some type of pasture access, whereas 888 heifers did not. Samples from heifers with pasture access were

submitted in June (4%; 12/257), July (34%; 93/275), and August (62%; 170/275), whereas no samples from pastured heifers were received in May.

Crude and Null-Model-Derived Prevalence and Mean FEC

Individual heifer egg counts ranged from 0 to 141 EPG (median: 0 EPG; interquartile range: 0 to 71 EPG). The crude GIN prevalence and mean FEC of all heifers for strongylid, *Nematodirus* spp., and *Trichuris* spp. are depicted in Table 1. In each region, strongylids were the most prevalent nematodes. Overall, strongylids were identified in 19.9% (95% CI: 18.4–21.6%) of heifers, whereas *Nematodirus* spp. and *Trichuris* spp. were identified in 4.0% (95% CI: 3.3–4.8%) and 10.2% (95% CI: 9.1–11.5%) of heifers, respectively. Fecal egg counts were low; the highest overall FEC was 1.4 (SD: 1.9) strongylid EPG of feces. The null-model-derived predicted overall prevalence and mean strongylid FEC are shown in Table 2 and were very similar to the raw results. The null-model-derived predicted prevalence and mean EPG for *Nematodirus* spp. and *Trichuris* spp. were also similar to the calculated crude results (data not shown).

Effect of Region on the Predicted Strongylid Prevalence and Geometric Mean EPG

The GEE-derived predicted strongylid prevalence by region is reported in Table 2. Region was not a significant risk factor in this model ($P = 0.17$). Heifers from ON had the highest strongylid prevalence (26.0%; 95% CI: 19.4–32.6%), whereas heifers from western Canada had the lowest prevalence (16.5%; 95% CI: 9.4–23.7%); prevalence among regions tended to be different (lowest $P = 0.07$). Region was also not a significant risk factor for the predicted mean EPG in this model ($P = 0.14$), although the predicted mean EPG ranged from 0.6 (95% CI: 0.2–1.0) in QC to 1.6 (95% CI: 0.7–2.5) in ON and differed between these 2 regions ($P = 0.03$).

Effect of Region, Mean Age, and Pasture Access on the Predicted Strongylid Prevalence and Geometric Mean EPG

When pasture access (yes/no) and mean age of sampled heifers were forced into the GEE model by region, the number of available observations decreased to 1,059 heifers. These heifers came from 138 different farms (data not shown) from all 4 regions. No significant interactions were observed between pasture access and age ($P = 0.17$) or province and pasture access ($P =$

Table 1. Crude prevalence (95% CI) and geometric mean fecal egg count ($\pm$ SD) measured in eggs per gram of feces (EPG) of strongylids, *Nematodirus* spp., and *Trichuris* spp. in fresh environmental fecal samples collected in 2015 from breeding-age heifers (n = 2,396) from 306 Canadian dairy farms, by region

Item	Province (no. of farms)				
	Ontario (n = 122)	Québec (n = 84)	Atlantic Canada ¹ (n = 45)	Western Canada ² (n = 55)	Overall (n = 306)
No. of heifers	900	602	360	507	2,369
Strongylids					
Prevalence (%)	25.8	18.1	14.4	15.8	19.9
(95% CI)	(23.0–28.7)	(15.2–21.4)	(11.1–18.5)	(12.8–19.2)	(18.4–21.6)
Mean EPG ($\pm$ SD)	1.7 (2.2)	1.1 (1.7)	1.2 (1.7)	0.9 (1.6)	1.4 (1.9)
<i>Nematodirus</i> spp.					
Prevalence (%)	3.4	5.0	2.5	4.7	4.0
(95% CI)	(2.4–4.9)	(3.5–7.0)	(1.3–4.7)	(3.2–7.0)	(3.3–4.8)
Mean EPG ($\pm$ SD)	0.5 (3.4)	0.8 (6.7)	0.4 (2.9)	0.5 (2.8)	0.6 (4.2)
<i>Trichuris</i> spp.					
Prevalence (%)	9.3	9.3	5.0	16.5	10.2
(95% CI)	(7.6–11.4)	(7.2–11.9)	(3.2–7.8)	(13.6–20.1)	(9.1–11.5)
Mean EPG ($\pm$ SD)	0.4 (2.6)	0.5 (3.9)	0.4 (2.2)	0.4 (2.3)	0.4 (2.7)

¹Atlantic Canada included Nova Scotia, Prince Edward Island, New Brunswick, and Newfoundland.

²Western Canada included British Columbia, Alberta, Saskatchewan, and Manitoba.

0.59), and the final model for the predicted prevalence included the fixed effects region ($P = 0.57$), pasture access ($P < 0.01$), and mean age ($P = 0.43$). The final model for the mean FEC was similar, including region ($P = 0.02$), pasture access ($P < 0.01$), and mean age ($P = 0.96$) without significant interaction terms ($P = 0.21$ for province and pasture access; $P = 0.16$ for pasture access and age).

Predicted GIN prevalence for each region by age and pasture access is depicted in Table 3. No significant difference was observed in the prevalence between different regions ($P \geq 0.25$). Overall, there was also no significant difference in the predicted prevalence in cattle of different ages ($P \geq 0.22$). However, overall, the prevalence was higher in heifers that had access to pasture compared with those that did not ($P < 0.01$, Figure 2).

Predicted mean EPG differed between some regions (Table 3). The mean EPG was significantly higher in ON compared with QC ($P = 0.03$) and western Canada had a significantly lower mean EPG compared with ON ($P = 0.01$) and Atlantic Canada ($P = 0.02$). Overall, no difference was observed in the predicted mean EPG in heifers from different age groups ($P \geq 0.77$), but similar to the predicted prevalence, the predicted mean EPG was higher in heifers with pasture access compared with those that had no pasture access (all $P < 0.01$, Figure 2).

Larval Species Identification Using NembioAssay

The L3 were harvested from 58 of the 306 larval cultures (one pooled culture per farm); all provinces were

Table 2. Predicted prevalence (95% CI) and geometric mean fecal egg count (FEC; 95% CI) of strongylids, accounting for clustering by herds, derived from the null-model (overall column) and from the generalized estimating equation model accounting for the effect of region, in 2,369 Canadian replacement dairy heifers on 306 farms in 2015

Item	Province (no. of farms)				
	Ontario (n = 122)	Québec (n = 84)	Atlantic Canada ¹ (n = 45)	Western Canada ² (n = 55)	Overall ³ (n = 306)
No. of heifers	900	602	360	507	2,369
Prevalence	26.0	18.5	16.8	16.5	20.9
(95% CI)	(19.4–32.6)	(11.9–25.1)	(8.0–25.5)	(9.4–23.7)	(17.2–24.6)
Geometric mean FEC	1.6*	0.6*	1.0	0.7	1.1
(95% CI)	(0.7–2.5)	(0.2–1.0)	(0–2.2)	(0–1.5)	(0.6–1.6)

¹Atlantic Canada included Nova Scotia, Prince Edward Island, New Brunswick, and Newfoundland.

²Western Canada included British Columbia, Alberta, Saskatchewan, and Manitoba.

³Overall predicted prevalence derived from the null (intercept-only) model.

*Statistical significance ($P \leq 0.05$).

represented except NL (Figure 3). All other samples either contained no larvae or contained free-living stage nematode larvae only. The estimated number of L3 larvae in each sample was recorded; the samples containing the lowest number of larvae were estimated to have between 1 to 30 larvae (Figure 3C). All farms contained <1,000 larvae, except farm PE1, which contained 3,360 L3 larvae (Figure 3C). The ITS-2 rDNA locus was successfully amplified from 47 of the L3 populations harvested from the 58 farms. All farms had a mean FEC <10 EPG except ON24 (21.2 EPG), PE1 (48.4 EPG), and QC11 (20.1 EPG; Figure 3A).

The nematode species found were *Ostertagia ostertagi* (detected in 100% of the samples), *Cooperia oncophora* (100%), *Trichostrongylus axei* (100%), *Oesophagostomum radiatum* (80.4%), *Haemonchus placei* (45.1%), *Cooperia punctata* (33%), *Oesophagostomum venulosum* (16%), and *Trichostrongylus colubriformis* (8%). The predominant species that accounted for over 50% of the L3 larvae in over 90% of the samples were *Ostertagia ostertagi* and *Cooperia oncophora* (Figure 3B). *Oesophagostomum radiatum* was detected on 80% of the farms, but it was the dominant species (>50%) on 3 farms (Figure 3B). *Nematodirus* spp. and *Trichuris* spp. were also recorded during the egg count procedure, but as expected, were not detected by deep-amplicon sequencing of larval cultures.

DISCUSSION

This is the first study in decades that determined the GIN prevalence, FEC intensity, and species diversity using the same methodology in Canadian dairy replacement heifers from all 10 provinces. Proportional species diversity on farms was determined with a novel deep-amplicon sequencing assay that highlighted the predominance of *O. ostertagi* and *C. oncophora* on most farms from all regions. The prevalence and FEC intensity were overall low in this population of heifers. This is a potentially salient finding because blanket whole-herd treatments with an anthelmintic product are common on many dairy farms but may in fact not be warranted in all animals and increase the risk of anthelmintic resistance development (Charlier et al., 2010; Scott et al., 2019). It is also a relevant finding because it suggests that clinical and economic effects of GIN in this population of heifers may not be of significant concern. However, while the results of this study are meaningful in providing a basic understanding of the minimum extent, caution must be taken not to over-interpret the results in these dairy heifers because of the characteristics of fecal shedding intensity and GIN infections. Although GIN infection was detected in about one-fifth of Canadian dairy replacement heifers, the true prevalence is likely higher. Similarly, the

Table 3. Predicted prevalence (95% CI) and predicted geometric mean fecal egg count (95% CI) of strongylids derived from a generalized estimating equation model, accounting for clustering by herds, in 1,059 Canadian replacement dairy heifers on 138 farms in 2015, by region, mean age, and pasture access (yes/no) of sampled heifers

Item	Province (no. of farms)				
	Ontario (n = 56)	Québec (n = 46)	Atlantic Canada ¹ (n = 20)	Western Canada ² (n = 16)	Overall (n = 138)
Overall prevalence (95% CI)	23.8 (16.8–31.0)	18.3 (12.5–24.1)	24.1 (10.4–37.9)	16.1 (3.2–29.1)	21.3 (17.1–25.5)
Prevalence by age (95% CI)					
6–12 mo	25.9 (17.1–34.7)	20.2 (10.5–29.9)	26.2 (11.8–40.5)	18.0 (3.9–32.0)	23.2 (16.0–30.4)
13–15 mo	25.1 (16.7–33.5)	19.5 (12.8–26.2)	25.4 (10.8–40.0)	17.3 (3.6–31.0)	22.4 (16.6–28.3)
≥16 mo	18.5 (8.8–28.2)	13.8 (6.2–21.4)	18.7 (4.5–32.9)	12.0 (0–24.7)	16.3 (8.3–24.2)
Prevalence by pasture access (95% CI)					
No	10.1 (4.2–16.0)	6.4 (1.8–10.9)	10.3 (0–21.0)	5.2 (0–11.9)	8.4 (4.5–12.2)*
Yes	71.1 (54.7–87.7)	60.0 (44.3–75.6)	71.6 (45.2–97.9)	54.6 (18.5–90.7)	65.6 (51.6–79.7)*
Overall mean EPG ³ (95% CI)	2.2 (0.5–3.9) ^{ab}	0.8 (0.3–1.2) ^a	1.9 (0.1–3.7) ^c	0.4 (0–0.8) ^{bc}	1.7 (1.4–2.0)
Mean EPG by age (95% CI)					
6–12 mo	2.2 (0–4.7)	0.8 (0–1.6)	1.9 (0–4.3)	0.4 (0–0.9)	1.5 (0–2.9)
13–15 mo	2.3 (0.1–4.5)	0.8 (0.4–1.2)	2.0 (0–4.0)	0.4 (0–0.9)	1.5 (0.5–2.6)
≥16 mo	2.0 (0.5–3.5)	0.7 (0–1.4)	1.7 (0–3.7)	0.4 (0–0.8)	1.3 (0.4–2.3)
Mean EPG by pasture access (95% CI)					
No	0.4 (0–0.9)	0.1 (0–0.2)	0.3 (0–0.7)	0.1 (0–0.2)	0.3 (0–0.5)*
Yes	8.4 (1.7–15.1)	2.9 (1.3–4.6)	7.2 (0–14.6)	1.5 (0–3.1)	5.7 (2.4–8.9)*

^{a–c}Means within a row with the same superscripts differ ($P \leq 0.05$).

¹Atlantic Canada included Nova Scotia, Prince Edward Island, New Brunswick, and Newfoundland.

²Western Canada included British Columbia, Alberta, Saskatchewan, and Manitoba.

³EPG = eggs per gram of feces.

*Means between rows differ for these predictor variables ($P \leq 0.05$).

Figure 2. Predicted strongyloid prevalence of heifers on pasture (●) or not on pasture (○) as well as predicted mean strongyloid fecal egg count [FEC; eggs per gram of feces (EPG)] of heifers on pasture (□) or not on pasture (■), based on mean heifer age and access to pasture ($n = 1,059$), accounting for clustering on farm. Error bars indicate 95% CI. *Statistical significance between heifers in the same age category but with or without pasture access ($P < 0.01$) is indicated by an asterisk.

mean EPG were overall low but comparable to previous studies from Canada where dairy heifers had a mean FEC of 1.1 (arithmetic mean), 16 (geometric mean), and 28 (geometric mean) EPG (Cox and Lemiski, 1989; Elsener et al., 2001; Nødtvedt et al., 2002). However, the mean EPG here were considerably lower compared with other countries, most notably Europe, where mean (arithmetic) FEC ranging from 116 to 280 EPG were found in dairy heifers (Areskog et al., 2013; Geurden et al., 2015). There are several possible reasons why the detected prevalence and the FEC intensity were overall low in this study. The deworming history of the sampled heifers was not available and it is possible that some heifers had been under the influence of an anthelmintic product at the time of sample collection. In terms of prevalence, it is generally acknowledged that GIN infections are widespread and that virtually every cow is likely infected (Charlier et al., 2016). The inability to accurately demonstrate this in many situations, however, is because of the lack of a perfect and practical diagnostic test in the live animal. The modified Wisconsin flotation technique was consistently able to detect <10 EPG, with a 100% sensitivity when the true EPG was >2.65 , and a sensitivity of 93% when the true EPG was <1.44 (Egwang and Slocombe, 1982; Zajac and Conboy, 2012). Although the test is sensitive to detect low egg counts, some heifers with a very low egg count may have been missed, resulting in a slight

underestimation of the prevalence. This also means that the true mean FEC might be somewhat higher than the reported mean 1.1 EPG; however, it would likely still be ≤ 2.65 EPG. It is also worth considering that there was an average of 11 d between sample collection and laboratory processing, which may have reduced the FEC since hatched eggs would not have been detected by fecal flotation. This does not take into account other factors that also affect FEC, such as intermittent egg shedding, and which could also result in a false-negative result. Therefore, dairy producers in this study should expect a slightly larger number of their heifers to harbor GIN than what was detected here. Although other tests, such as, for example, serum pepsinogen determination, may be more accurate at determining individual animal risk of disease or potential production effects, they are also more expensive and lack standardization (Charlier et al., 2016). In contrast, FEC methods are relatively easy to perform, inexpensive, and do not require specialized equipment. Despite these shortcomings, FEC methods are valuable and commonly used to advance our understanding of GIN epidemiology, and as this was the objective of this study, fecal flotation was deemed an acceptable approach to estimating GIN prevalence and FEC intensity (Eysker et al., 2000; Sargison, 2013).

Management and environmental conditions also influence the GIN prevalence and FEC intensity (Eysker

et al., 1998; Wilson et al., 1998; Beck et al., 2015). Although epidemiologic studies on GIN larvae survival and transmission patterns from Canada are scarce, it is possible that climate differences (e.g., long, cold winters; dry hot summers) reduce the risk of GIN infection in cattle and contribute to the lower FEC intensity in the heifers here compared with studies in Europe (Areskog et al., 2013; Geurden et al., 2015). Access to pasture is a significant risk factor for GIN infection in cattle and grazing management can greatly influence this (Stromberg and Averbek, 1999). Although access

to pasture was only known for a subset of heifers in this study, the increased prevalence and FEC intensity in the heifers with pasture access highlighted this epidemiologically important risk factor; in most studies on GIN prevalence in Europe, dairy heifers were grazed and this could be another reason for the differing GIN epidemiology between studies (Areskog et al., 2013; Geurden et al., 2015). Furthermore, although access to pasture was captured for some heifers, information on duration of pasture turnout or general pasture/grazing management before sampling was not. The risk of GIN

Figure 3. (A) Geometric mean fecal egg counts [FEC; eggs per gram of feces (EPG); raw data]. (B) Nematode species from deep-sequencing nemabiome assay of the internal transcribed spacer (ITS)-2 DNA region of third-stage (L3) larvae. Bars represent 100% of the larvae in the sample. (C) Estimated starting number of L3 larvae for each farm; asterisks indicate samples with <30 larvae (note: PE1 contained 3,360 L3 larvae). All samples (n = 47) are denoted by province (AB = Alberta; BC = British Columbia; MB = Manitoba; NS = Nova Scotia; ON = Ontario; PE = Prince Edward Island; QC = Québec; SK = Saskatchewan) and farm number.

infection varies throughout the grazing season in temperate regions and samples earlier in the study months from heifers with pasture access could be expected to have a lower FEC compared with samples collected later in the grazing season (Eysker et al., 2000). However, no samples from heifers with pasture access were received in the first month of the study (May) and most were received in the last month of the study (August); therefore, the month of sample collection itself was unlikely to affect the FEC in heifers with pasture access. However, had the sample collection been extended into September or October, it is possible that the FEC, and therefore also the prevalence, might have been higher in pastured heifers.

The mean FEC intensity warrants further discussion. Although FEC methods may not accurately depict the actual adult worm burden in the host or characterize the severity of infection, FEC intensity has been associated with clinical and subclinical effects (Shaw et al., 1998b; Eysker et al., 2000; Kunkle et al., 2013). However, clinical parasitic gastroenteritis was more commonly identified in cattle with >200 EPG, which is higher than even the highest individual EPG (141 EPG) in the heifers here, and several studies reported a FEC as high as 300 or 400 EPG in calves without evidence of clinical disease (Eysker et al., 1998; Shaw et al., 1998b; Forbes et al., 2007). However, the risk for a potential production effect in some heifers, although considered small, can presently not be discarded based on the available information from this study or the literature, especially given the potential for slight FEC underestimation and because subclinical infections and even FEC similarly low to those found in this study have been associated with reduced growth in some studies (Shaw et al., 1998a; Forbes et al., 2000; Kunkle et al., 2013). For the Canadian dairy replacement producers in this study, the FEC results, therefore, suggest several conclusions: despite a possible slight underestimation of the FEC in the heifers here, clinical disease would not be expected to occur while some subclinical effects cannot entirely be ruled out. Any effect may vary between regions as several provinces differed in the predicted FEC of their heifers. This is likely because differences in climate (such as temperature, precipitation, humidity) and management (such as grazing and pasture management, use of anthelmintic products) affect the risk of GIN infection but were not captured in this study. For example, the risk of parasite transmission in cattle was less in the southeastern part of AB, Canada, compared with the northwestern part of that province (Beck et al., 2015). However, the magnitude of FEC variations between the statistically significantly different values was small. It is unknown whether these would be sufficient to result in a significantly different production

or economic effect, especially because comprehensive and integrated approaches to whole-farm economics are needed to accurately determine the ultimate (subclinical) effect of GIN infection in cattle (Charlier et al., 2014, 2016; Bellet et al., 2018; Rushton and Bruce, 2019). Furthermore, the effect of GIN also depends on the predominant species, with *O. ostertagi* generally considered most pathogenic (Shaw et al., 1998a). Nonetheless, these results also highlight that future studies must consider regional and farm-level risk factors in more detail to better determine the epidemiology of GIN in Canadian dairy heifers and to assist the industry in mitigating risks of GIN infection.

With regard to heifer age, there was no difference in FEC among the age groups with or without access to pasture. While younger heifers are expected to have a higher FEC than older heifers, most heifers in this study were <2 yr of age as expected based on the target population in this study, and 50% fell within a desirable age at first breeding of 13 to 15 mo; therefore, the overall age range may have been too narrow to observe differences in FEC, but this was also not the objective of the study.

The proportional GIN species diversity of L3 populations isolated from fecal cultures was determined using a novel deep-amplicon nemabiome sequencing assay of the ITS-2 rDNA locus. Better characterizing the GIN infection in cattle by determining the predominant species is important in assessing the relevance of the detected GIN prevalence and FEC as GIN vary in their pathogenicity and fecundity (Coyne et al., 1991; Gasbarre, 1997). Of the 306 farms, L3 were successfully harvested from only 58 farms. This was not unexpected, given the low egg counts and known variability in the efficiency of larvae fecal cultures. Of the 58 samples to which ITS-2 rDNA deep-amplicon nemabiome sequencing was applied, DNA amplification was unsuccessful for 11 samples, which may be attributed to low DNA concentrations or poor DNA quality (i.e., low number of L3 larvae in the sample, insufficient DNA extraction combined with contaminants that inhibit PCR). One caveat to interpreting the nemabiome data relates to the challenge of working with samples with low FEC. Consequently, care should be taken when interpreting the nemabiome results on an individual farm basis. Although *O. ostertagi* and *C. oncophora* were clearly the predominant species overall, higher numbers of larvae would be required for more accurate species proportions to be determined on each farm and to detect rarer species present in samples. The predominant species *O. ostertagi* and *C. oncophora* often co-inhabit grazing cattle in temperate climates (Dimander et al., 2003). Both species have previously been predominantly identified in Canadian dairy cattle, and were also recently

found to be the predominant GIN species in Canadian beef herds (Nødtvedt et al., 2002; Avramenko et al., 2017). It is noteworthy that *O. ostertagi* was the predominant species detected in the larval populations harvested from fecal samples for several farms even though it has a much lower fecundity than the other species identified. This suggests that large numbers of adult worms may be infecting the host (Coyne et al., 1991). Given the lower fecundity of *O. ostertagi* relative to *C. oncophora*, the results presented here suggest that the former parasite is likely the most predominant in terms of adult worm populations. This is relevant as *O. ostertagi* is generally considered the most pathogenic cattle GIN in temperate regions (Shaw et al., 1998a). However, determining the predominant species alone also does not provide any information on the magnitude of the GIN infection. For this reason, the ITS-2 rDNA nemabiome assay was used in conjunction with the FEC to better interpret the relevance of both.

The predominance of *C. oncophora* is important because there have been several documented cases of anthelmintic resistance of *C. oncophora* to macrocyclic lactones in northern Europe and the Midwestern United States (Edmonds et al., 2010; Areskog et al., 2013; Geurden et al., 2015). A recent questionnaire conducted in SK, Canada, found that nearly 80% of dairy producers in that province administered an anthelmintic product to their replacement heifers annually (Scott et al., 2019). Of those that used anthelmintic products, 97% used a macrocyclic lactone product for their replacement heifers, and 73% used a blanket, whole-herd treatment method (Scott et al., 2019). Therefore, the predominance of *C. oncophora* relative to nematode species other than *O. ostertagi* may also be a reflection of current parasite control practices. In Canadian beef cattle, a recent study found *C. oncophora* and *O. ostertagi* to be the most prevalent GIN species, and producer-applied macrocyclic lactone products had reduced efficacy against *C. oncophora* as well as *O. ostertagi* on some farms (Avramenko et al., 2017). No published studies have evaluated anthelmintic resistance in the Canadian dairy industry, but the reports of *Cooperia* spp. and *O. ostertagi* resistance in other Canadian industries and around the world are concerning in light of the nemabiome assay results here.

In conclusion, this is the first study in Canadian dairy heifers that combines conventional epidemiologic approaches to assess GIN prevalence and FEC with a novel deep-amplicon sequencing assay to determine species diversity in heifers across the nation. Although the risk of cattle infection with GIN is complex and not all risk factors could be evaluated in this study, it provides a conservative assessment of the minimum prevalence and FEC intensity and a current understanding of

predominant GIN species in these heifers. This helps to narrow the knowledge gap that existed in this population, and acknowledging that the prevalence and FEC have likely been somewhat underestimated, the results still suggest that the overall risk of GIN infection in Canadian replacement dairy heifers is likely low but differs in some regions. The study also highlighted that more comprehensive investigations are needed that better take into account regional and farm-level environmental and management conditions and that an assessment of the anthelmintic efficacy of frequently used drugs is warranted to more accurately interpret the risk of GIN infection in Canadian dairy heifers across the nation.

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