Sheep Nemabiome Diversity and Its Response to Anthelmintic Treatment in Swedish Sheep Herds

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Research

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Abstract

Background: A novel way to study the species composition and diversity of nematode parasites in livestock is to perform deep sequencing on composite samples containing a mixture of different species. Herein we describe for the first-time the nematode community structure (nemabiome) inhabiting Swedish sheep and how these are/were affected by host age and recent anthelmintic treatments.

Methods: A total of 158 larval cultures were collected (n=35 in 2007 and n=126 in 2014-2016) from groups of sheep on 61 commercial farms in the south-central part of country were most animals are grazed. Among the samples, 2 x 44 (56%) were paired collected from the same groups pre- and post-treatment with macrocyclic lactones, benzimidazoles or levamisole. The sequences were analyzed for their nemabiome using the PacBio platform followed by bioinformatic sequence analysis with SCATA. Species richness and diversity were calculated and analyzed in R.

Results: Nematode ITS2 sequences were found in all samples except two, despite that the fecal egg counts were below the McMaster threshold in 20 samples. Sequencing yielded on average 1,011 sequences per sample. Totally 26 operational taxonomical units (OTU) among which 18 (69%) had ≥99 % identity to sequences in the NCBI database were recognized. The OTUs found represented nematode species among which 10 are commonly associated with sheep. Multiple species were identified in all pre anthelmintic treatment samples. No effects on nematode diversity were found in relation to host age. On the other hand, recent anthelmintic treatment lowered species richness, especially after use of IVM and ABZ. Interestingly, despite zero egg count after use of levamisole, these samples still contained nematode DNA and especially H. contortus.

Conclusions: Our findings provide evidence for that nemabiome analysis combined with diversity index analysis provide a sensitive and objective methodology in the study of the efficacy of anthelmintic treatment.

Introduction

Infections with gastro-intestinal nematode (GIN) parasites is globally well-known as a major veterinary problem which contributes to decline the productivity in the global sheep industry [1]. Grazing sheep in Sweden are typically infected by a wide range of GIN with a majority belonging to superfamily Trichostrongyloidea within order Strongylida [2], among which some species such as Haemonchus contortus is considered more pathogenic than others [3]. Accordingly, parasite identification is fundamental for the improvement of sustainable parasite control strategies. The relative abundance of the different species present in sheep is driven by a wide range of factors. These include external factors such as climate and managerial factors affecting the exposure to the free living stages [4], and not at least selection reinforced by use of anthelmintic compounds [5]. On top of this parasites are influenced by host immunity affected by previous exposure [6], presence of other animals that may act as reservoirs [7]

Traditional parasitological diagnostic techniques based on microscopical examination of fecal eggs counts (FEC) and larval cultures can provide rough measures about the nematode species or genus composition in sheep [8]. However, a disadvantage with these diagnostic tools is that they rely on trained expertise that is rare to found today, but it is also getting clearer that they may have major constraints in terms of both sensitivity and specificity [9]. Thus, there is a need to utilize improved diagnostics for the investigation of complex nematode communities. With access to diagnostic instruments that rapidly could rank strongylid nematodes to their relative contribution to mixed infections would represent a major advantage particularly in terms of the understanding of how selection by anthelmintics shape nematode communities. This need is getting important given the increasing evidence for anthelmintic resistance (AR) and request for evidence-based use of anthelmintic drugs in parasite control [10]. Although effective parasite control in general can be achieved through a combination of grazing management strategies and treatment with anthelmintics, this practice is under threat due to the increasing spread of resistance to these veterinary drugs [11].

For some time, the molecular identification of parasitic nematodes largely relies on amplification of the internal transcribed spacer (ITS) regions located between the 18S and 5.8S subunits of the ribosome encoding genes [12]. Today there are several DNA-based tests available for genotyping of GIN that offers the potential to detect, identify and quantify especially strongyle nematode parasites in ruminants (Hunt and Lello, 2012; Elmahalawy et al., 2018; Roeber et al., 2011). However, these different technologies in general suffer from limitations in terms of the number genera and/or species that can be detected since they are restricted to the few parasites that are considered to be of particular interest and to which primer probe sets have been designed. Clearly the advent of deep amplicon sequencing using next-generation sequencing (NGS) platforms has generated new prospects and simplified the sequencing of gene amplicons in the study of microbial communities that usually exist as mixed infections within their hosts. Like for any microbe NGS
allows for the characterization of complex nematode communities and it open up new possibilities to identify community components even at low relative abundances at an unprecedented depth with minimal cost and labor. Recently the term "nemabiome" was created to describe the community structure of nematodes in ruminant livestock [16]. By using an Illumina based deep amplicon next generation pooled sequencing approach targeting ITS-2 rDNA locus of the nematode rRNA genes, a pipeline was developed which so far has been used to study the entire nemabiome in beef cattle [16], bison [17] and dairy cattle [18] in Canada and the US, as well as in UK sheep [19]. Similarly, the nemabiome of equines were recently studied by a slightly different methodology [20]. Combined these studies have revealed detailed insights of the nematode diversity composition in each of these hosts using a truly non-invasive diagnostic approach.

In this paper we describe the species composition and diversity of GIN by studying the nemabiome in 158 sheep larval cultures collected on 61 commercial farms distributed across Sweden. The results were analyzed by investigate how community structure was affected; i) by host age, ii) in response to anthelmintic treatments, and iii) its long term temporal effects. This was conducted by using data generated on the Pacific Biosciences (PacBio) platform, which for other microbial communities has shown to produce less bias compared to other deep sequencing technologies [21]. The OTUs we refer to were distinguished using SCATA (https://scata.mykopat.slu.se/), which is a specifically designed analysis framework for the evaluation of sequenced tagged amplicons derived from eukaryotic microbial communities.

Material And Methods

Sample collection

The sampling was carried out either as part of random investigation or from commercial sheep farms suffering from recurrent problems with GIN. Totally 158 samples were obtained from 19 farms during 2007 (n=35), and on 42 farms between 2014 and 2016 (n=126), (Fig 1). On each sampling occasion approximately two tablespoons of fresh faeces were collected from each of 10 or 15 randomly selected animals (either ewes or lambs) in the flock. This was done by the farmer or a veterinarian at the Farm and Animal Health in Sweden. The individual samples were placed in marked zip-locked plastic bags where after the air was pressed out before sealing and then sent over night by national post to the diagnostic laboratory (Vidilab AB).

On 44 out of the sampling occasions paired pre- and post-treatment samples were obtained, i.e. 88 samples. Among these 26 groups were treated according to the recommended dose with the macrocyclic lactone ivermectin (IVM), 13 with the benzimidazole albendazole (ABZ), and 5 with the imidazothiazol derivative levamisole (LEV). On 10 farms the efficacy of several anthelmintics were tested in parallel using different groups of animals in the flock. The post-treatment collection was done from the same animals between 7 to 10 days after worming.

Parasitological investigation and DNA extraction

Upon arrival to the laboratory faeces were investigated for gastrointestinal nematode (GIN) strongyle eggs. The number of nematode eggs were first counted using a modified McMaster technology based on 3 g and with a minimum diagnostic sensitivity of 50 nematode eggs per gram faeces (EPG) as described previously [22].

There after collected subsamples of approximately 2 gram from the animals in each group were pooled in separate plastic containers. After blending the pooled fresh faeces with Vermiculite, the eggs were cultured for between approximately 10 days in a moisten chamber at ≈20 °C. Infective third-stage larvae (L3) were harvested using the Petri-dish method, concentrated in a Falcon tube and collected into an Eppendorf-tube before storage in a freezer at approximately -18 °C [3]. After approximately 10 days, third-stage infective larvae were harvested overnight using the microplate dish method. DNA was then extracted from the larval cultures (one per farm and sampling occasion) using the Nucleospin® DNA tissue kit (Macherey-Nagel, Düren, Germany) following the manufacturers guidelines.

Library preparations and sequencing

Nematode parasite ITS-2 rDNA fragments were amplified using the NC1-NC2 primer pair [23] tagged with an 8 bp long barcode [24] on the 5’ side of each primer. PCR was conducted in 50µl reactions consisting of 0.2 µM dNTPs, 0.75 µM MgCl2, 1µM of each of NC1-NC2 primer pair, 2.5µl DNA template and 1.25 U DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The PCR cycling consisted of an initial denaturation for 3 min at 95 °C followed by 20-34 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C and finally 7 min at 72 °C. The number of PCR cycles for each sample was determined depending on template concentration. Each sample was purified using Agencourt AMPure beads (Beckman Coulter, IN, USA) and the purified PCR product was measured fluorometrically (Qubit
Flourometer). The same amount of PCR product from each sample was pooled into four composite samples. Sequence ligation to each composite sample and sequencing on PacBio SMRT cells were performed by SciLifeLab, Uppsala, Sweden.

**Bioinformatics**

The raw sequence reads were processed using the SCATA bioinformatics pipeline (http://scata.mykopat.slu.se/). Sequences with an average quality score below 20 or below 10 at any single position were removed. The primer and tag sequences were then removed from the remaining reads. SCATA using blast as a search engine to cluster sequences based on similarity. During the clustering procedure, sequences were pairwise aligned with the minimum length set to 85% of the longest sequence. Pairwise alignments were scored using a scoring function with 1 in penalty for mismatch, 0 for gap opening and 1 for gap extension. Homopolymers were collapsed to 3 bp before alignment. Sequences were clustered into operational taxonomic units (OTUs) by single linkage clustering with a 98.5% sequence similarity required to be assigned to OTUs. 159,868 high quality reads were assembled into 26 OTUs, on average 1,011 reads per sample. OTU sequences were searched for similarity with reported sequences using NCBI BLAST and OTUs not representing nematodes were omitted. Of the 158 samples, all except two post-treatment samples produced nematode OTUs.

**Statistical analysis**

All statistical analyses on community structure were performed in R v4.0.2 [25]. Species richness was calculated by summing up the number of helminth species per individual. To investigate helminth community diversity and if it is dominated by a few species, the inverse Simpson's diversity index was calculated using Vegan package [26]. Generalized linear mixed models (GLMM) with a repeated measure design was used to assess the effects on the nemabiome composition from anthelmintic treatment (Fig 4G-L), using respective diversity indices as response variable, treatment as fixed factor and farm as a random structure. GLMM was calculated using the MCMCglmm package [27]. Run parameters are presented in Table 2. Post-treatment samples for two IVM farms was omitted due to successful treatment (Fig. 4J), hence no nematode DNA was to be analyzed. Data was visualized with ggplot2 [28].

**Results**

**Species diversity**

We identified 26 nematode species, based on OTU identification, from 158 sheep samples, of which 18 had a NCBI BLAST query cover of more than 95% (Table 1). Of these, the five most common species consisted of roughly 97% of the reads for both adult sheep and lambs (Fig. 2; Table 1). Both ewes and lambs were on average infected with the same number of species (Species richness, Fig. 2B) F$_{1,112}$=2.7123, P = 0.1024. However, the species composition showed that the nematode community was dominated by a few species, among ewes compared to lambs (Inverse Simpson's diversity index, Fig. 2C) F$_{1,115}$=7.015, p = 0.00925.
Table 1

Identification of species from OTUs with BLAST. The top five species represent 97% of the reads. Nematodes were identified in 156 (99%) out of 158 the samples. The most common species was *Haemonchus contortus*, which was identified in 135 (85%) of the positive samples. The ITS2 fragments varied in length from 266 to 512 bp.

| Best species match from BLAST search | Query cover | Identity | Number of samples | ITS2 OTU Sequence length (bp) |
|--------------------------------------|-------------|----------|-------------------|-------------------------------|
| *Chabertia ovina*                    | 100         | 100      | 48                | 283                           |
| *Haemonchus contortus*               | 100         | 100      | 135               | 281                           |
| *Teladorsagia circumcincta*          | 100         | 99       | 97                | 296                           |
| *Trichostrongylus vitrinus*          | 100         | 100      | 57                | 288                           |
| *Oesophagostomum venulosum*          | 99          | 100      | 32                | 308                           |
| *Bunostomum trigonocephalum*         | 100         | 100      | 3                 | 281                           |
| *Cooperia curticei*                  | 100         | 99       | 1                 | 292                           |
| *Cooperia oncophora*                 | 100         | 100      | 10                | 291                           |
| *Coronoclycus coronatus*             | 100         | 99       | 2                 | 281                           |
| *Coronoclycus labratus*              | 100         | 100      | 32                | 367                           |
| *Craterostomum acuticaudatum*        | 86          | 100      | 1                 | 370                           |
| *Cylicocyclus nassatus*              | 86          | 100      | 24                | 370                           |
| *Cylicocyclus ultrajectinus*         | 87          | 100      | 1                 | 402                           |
| *Cylicostephanus minutus*            | 81          | 100      | 5                 | 266                           |
| *Cystocaulus ocreatus*               | 100         | 99       | 5                 | 418                           |
| *Dictyocaulus viviparus*             | 97          | 97       | 1                 | 512                           |
| *Muellerius capillaryis*             | 100         | 100      | 6                 | 455                           |
| *Nematodirus spathiger*              | 100         | 99       | 9                 | 280                           |
| *Ostertagia leptospicularis*         | 100         | 99       | 5                 | 289                           |
| *Ostertagia ostertagi*               | 100         | 100      | 5                 | 288                           |
| *Protostrongylus hobmaier*           | 95          | 97       | 3                 | 400                           |
| *Protostrongylus rufescens*          | 90          | 99       | 3                 | 411                           |
| *Strongyloides fuelleborni fuelleborni* | 100     | 90       | 2                 | 360                           |
| *Trichostrongylus axei*              | 100         | 93       | 1                 | 290                           |
| *Trichostrongylus retortaformis*     | 100         | 98       | 2                 | 288                           |
| *Triodontophorus serratus*           | 86          | 100      | 1                 | 384                           |

Nemabiome composition

For lambs, we had samples from two time periods, 2007 and 2014–2016 while samples for adults only was collected between 2014–2016. Among the lambs, *Chabertia ovina* decreased and *H. contortus* increased in frequency with increasing age. The species richness in lambs between the two time periods remained the same ($F_{1.60}=1.896$, $p=0.1736$), but it was a higher dominance among a few species in 2014–2016 than in 2007, based on the difference in inverse Simpson's diversity index between the years according to a linear model ($F_{1.60}=5.086$, $p=0.0278$) (Fig. 3).

Influence of anthelmintic treatment

Treatment with IVM was most effective against *Oesophagostomum venulosum*, ABZ was effective against *C. ovina* and *Trichostrongylus vitrinus*, while levamisole showed effect against *Teladorsagia circumcincta* and also had good effect against *H. contortus* (Fig. 4A-C). Furthermore, the post-treatment EPG was lower for all anthelmintic substances (Fig. 4D-F), and especially for levamisole where no
nematode eggs were found after treatment. In contrast, eggs remained in 17/26 (65%) and 7/13 (54%) of the groups treated with IVM and ABZ, respectively. Post-treatment lowered the species richness on IVM or ABZ treated sheep, (Fig. 4D-E) while no change in species dominance was detected (Fig. 4J-K). For levamisole, the treatment was highly effective and the EPG in post-treated sheep was lower than 50 (the detection limit of the McMaster) for all farm samples (n = 5). Still, nemabiome data was generated and the low number parasites remaining notably changed the species composition although it did not notably affect species richness or species dominance (Fig. 4H-L). See Table 2 for detailed summary statistics.

**Table 2**

Output from MCMCglmm testing the impact of each of the three anthelmintic drug treatment (IVM = ivermectin, ABZ = albendazole, LEV = levamisole) on species richness and inverse Simpson's diversity index (see also Fig. 4G-L) for farms sampled before and after anthelmintic treatment.

| Pre- and post-treatment | Posterior mean | Lower 95% CI | Upper 95% CI | Effective sample size | pMCMC |
|-------------------------|----------------|--------------|--------------|-----------------------|-------|
| Species richness IVM    | 0.92261        | 0.01674      | 1.76027      | 10819                 | 0.0366* |
| Species richness ABZ    | 2.1461         | 1.0375       | 3.2327       | 10691                 | 0.0008*** |
| Species richness LEV    | 0.994394       | -1.699608    | 3.847177     | 9560                  | 0.4242 |
| Inverse Simpson IVM     | 0.20262        | -0.06626     | 0.47498      | 10000                 | 0.1320 |
| Inverse Simpson BZ      | 0.2544         | -0.08311     | 0.60872      | 10000                 | 0.1430 |
| Inverse Simpson LEV     | -0.2207        | -0.9139      | 0.4719       | 10000                 | 0.4858 |

1Statistically significant fixed effects are marked with * and ***

Prior settings: R (V = 2, nu = 0.2); G (V = 2, nu = 0.02)

Run settings: Burn-in = 50,000; Iterations = 100,000; Thinning interval = 10

**Discussion**

In this study we have for the first time investigated nemabiome in 158 samples from 61 sheep commercial farms in Sweden. The aims were to find out how nematode community structure (species composition and diversity) were influenced; i) by host age (in ewes and lambs), ii) on short-term following recent anthelmintic treatment with ABZ, IVM or LEV, and iii) on long-term in samples collected several years apart. For this we used DNA extracted from composite larval pools which then were analyzed using a PacBio NGS pipeline generating sequence data (amplicons) that were clustered into OTUs with a specifically designed analysis framework developed for eukaryotes. We identified five common OTUs representing 97% of the reads, among which *H. contortus* and *T. circumcincta* were the two dominating species. There was in general no significant differences in nemabiome post-treatment between ewes and lambs. In contrast, use of anthelmintic had a short-term dramatic effect, but not on nemabiome in post-treatment samples collected several years apart indicating there was no long-term effect.

Totally 26 OTUs were identified, of which five are well-known and globally distributed strongyle nematode species in sheep; i.e. *C. ovina*, *H. contortus*, *T. circumcincta*, *Trichostrongylus vitrinus*, *Oesophagostomum venulosum*. This group of big-five constituted of the majority of reads (97%). All of these had a match of ≥ 99 to 100 in query cover and identity according to BLAST searches and were therefore considered as valid species. We also recognized eight other species reported from sheep with the same confidence in most cases (Table 1). These were represented by four strongylids; *Bunostomum trigonocephalum*, *Cooperia curticei*, *Nematodirus spathiger* and *T. axel*, plus four metastrostrongylids (small lungworm); *Cystocaulus ocreatus*, *Muellerius capillaris*, *Protostrongylus hobmaieri* and *P. rufescens*. Among the small lungworms only *C. ocreatus* and *M. capillaris* had an identity of ≥ 99. However, combined these eight represented less than 2% of the total reads. Thus, both the epidemiological consequences and clinical impact of these minor species are probably of less importance for Swedish conditions. With the exception of *T. columbiformis*, which was absent in our data set, we recognized all eight major species found in UK sheep based on data generated with a similar approach [19]. However, the relative abundance of the distinctive species differed. In our study five species dominated and with *H. contortus* being more prevalent in Swedish compared to UK sheep. From this, it follows that there is a difference in nemabiome composition in sheep between different climatic conditions. Thus, microclimatic influences would be worthwhile to investigate especially in the light of the possible impact of climatic changes.

Besides, we identified 12 species usually not found in sheep. Among these, four species are mainly linked to cattle; *Cooperia oncophora*, *Dictyocaulus viviparus*, *Ostertagia leptospicularis*, and *O. ostertagi* [2]. However, we also identified several cyathostomins with best BLAST matches to *Coronoclycus coronatus*, *C. labratus*, *Craterostomum acuticaudatum*, *Cylilococcus nassatus*, *C. ultrajectinus*, *C. minutus*, and *Triodontophorus serratus*, as well as *Trichostrongylus seratus*, that normally are associated with equids. To the best of our knowledge it is unknown if transmission of nematodes from equids to sheep can occur. Still, at least the matches both for *Coronoclycus* spp. and *C. ocreatus* showed high query cover and identity (≥ 99–100), indicating that these findings are valid, whereas the rest had a poor match (81–87). In contrast, opportunities for cross-infections between cattle and sheep nematodes have been studied through experimental cross-infections [29]. It was shown that both *Cooperia* spp. and *Ostertagia* spp. can infect lambs, however there is varying degrees of host specificity with *C. oncophora* being more adapted to cattle than sheep, whereas *O. ostertagi* is more strongly adapted to cattle. However, as stated before by Herlich (1971), it cannot be precluded that small numbers of *O. ostertagi* may attain sexual maturity in sheep under
natural grazing conditions, which is in line with our findings. The presence of *Dictyocaulus viviparus* is more obscure. However, the query cover and identity were only 97% indicating that this OTU is likely another species; probably the lungworm of sheep, *D. filaria*. These species are similar but genetically distinct and host specific [31]. We also identified an OTU with best match to *Strongyloides fuelleborni* in two samples. This species is usually found in primates and occasionally also in humans [32], whereas *S. papillosus* infects ruminants [2]. This OTU had a query cover 100%, but only an identity match of 90% (Table 1). Like with *D. viviparus* this illustrates a difficulty with interpretation of data generated through nemabiome analysis. Since, cross infections of worms in this genus between unrelated hosts is uncommon, we probably dealt with *S. papillosus* [33]. This species has received less attention than those in humans from a sequencing point of view, and it is therefore not available in common databases.

Interestingly, we recognized only one species, *Trichostrongylus retortaeformis* (OTU 100/98), primarily associated with wildlife, which usually is found in lagomorphs [2]. As outlined above there are also several other species listed herein that are primarily parasites of sheep, that are generalist known to infect a wide range of wildlife ungulates in Europe [7]. Not at least roe-deer in Spain [34], Italy [35], Turkey [36], and France [37], is known to be infected with several of the species identified herein. Among these, particularly *H. contortus* is the most pathogenic nematode of sheep in Sweden [3] and is therefore of major interest. Although, roe-deer is abundant in Sweden, knowledge about its nemabiome and its role as a reservoir of livestock parasites is presently unknown. Thus, this needs to be studied in the future, especially with focus on *H. contortus*, not at least in face of climatic change and its propensity to develop resistance to anthelmintic drugs. Since it is known that there are both polymorphisms and cryptic species around, particularly among members of the members in superfamly Trichostrongyloidea that hybridize [38], we are convinced that nemabiome analysis is well suited for this.

Clearly, the culturing conditions for nematode eggs can have an impact the nemabiome composition. For example, it has been shown that fewer eggs of *T. circumcincta* develop to the third stage than for *T. colubriformis* [39]. Besides from the input material, the number of recognized OTUs are also influenced; i) by primer design and PCR conditions, ii) the choice of NGS platform and bioinformatics pipeline for OTU clustering and recognition, and not at least iii) available sequence information in the sequence databases. In this study we took advantage of the commonly used primers NC1 and NC2 and compared the obtained sequence with information in NCBI. In line with previous studies, these universal primers spanning the first (ITS1) and second (ITS2) internal transcribed spacers in the nuclear ribosomal DNA array not only amplify a wide range of livestock genera of nematodes of veterinary interest (i.e. *Bunostomum, Chabertia, Cooperia, Dictyocaulus, Haemonchus, Nematodirus, Oesophagostomum, Ostertagia, Protostrongylus, Teladorsagia*, and *Trichostrongylus*), but also provide suitable genetic markers for species delimitation (for a review see Gasser et al., 2008). In our study, all these genera were found as well as six additional genera (Table 1.). Still, it is unclear where to define the cut-off for discriminating between nematode species, as genetic isolation is generally used to define species boundaries rather than DNA differences [40]. Nevertheless, in agreement with previously studies on nemabiome in livestock and horses [16–20], a cut-off identity threshold of $\geq 99$ seems reasonable. However, as shown in our study, the query cover also needs to be considered. It was beyond the scope of the present study to investigate factors that may introduce sequencing bias. Still, when targeting the ITS region it has been shown that PacBio reflect the composition better than Illumina MiSeq for metabarcoding of fungal communities due to sequence length variation [41]. In this study we have shown that ITS2 for the different OTUs varied between 266 and 512 bp (Table 1.). However, to what extent nemabiome composition is affected by sequence length variation needs to be explored.

Although no major effects in relation to host age or long-term effects were observed when we compared the samples collected 2007 and 2014–2016 (Figs. 3 and 4), it is clear that use of anthelmintic drastically influenced the nemabiome composition (Fig. 4). In this context it is important to consider that the use of the tested drugs has changed during the last decades. In Sweden, the drug of first choice whenever *H. contortus* is present, has from the 1960’s until recently changed from ABZ to IVM [42]. However, with emerging evidence for double resistant *H. contortus* strains, this practice has changed. Today, LEV is increasingly used, but currently only on farms that neither respond adequately to IVM nor ABZ. Interestingly our data show that the identified nematode species responded differently to these drugs. Of particular practical interest is that both IVM and ABZ were unable to control *H. contortus* on several farms, whereas LEV still has a reasonable efficacy. However, despite there was always zero-egg counts post-treatment with LEV it is evident that in particular *H. contortus* survived at a low level. Likewise, *T. circumcincta*, which is the second most important nematode in Swedish sheep, survived treatment with either ivermectin or ABZ, but then to a lesser extent than *H. contortus*, whereas not at after LEV treatment. This is partly in contrasts to the situation reported several years ago, when resistance to LEV was widely prevalent among several trichostrongylid nematodes of sheep [43]. At large we believe the observed patterns reflect the current anthelmintic resistance situation in GIN of sheep in Sweden. Of major practical concern is whether LEV resistant *H. contortus* will appear with increasing use of this drug. In countries with more intense sheep production (i.e. New Zealand), LEV is considered as an old drug. However, in the past when this drug was more widely used in sheep, high levels of resistance was present, like for most other commonly used anthelmintics [44]. In the light of this, our observation showing low levels of surviving *H. contortus* is of great concern.
In conclusion, in our study, the nemabiome approach proved to be a powerful method of studying nematode community diversity in sheep and how it is influenced by factors such as host age and the most recent use of anthelmintic treatment. This approach has a high level of sensitivity and specificity, indicating that previous estimates of diversity of the sheep nemabiome may have been underestimated. More importantly it is also clear that the effects of recent anthelmintic treatment can be investigated with high precision and therefore provides more detailed information than generated by the more traditional parasitological methods such as egg counts and morphological identification of larvae.

**Declarations**

**Data availability**

The raw ITS2 reads are stored at the European Nucleotide Archive (ENA) under accession number XXXXXX.

**Ethics declaration**

Ethics approval and consent to participate

No ethical permissions were necessary for this study as the parasites were collected from samples sent in for routine veterinary diagnostics.

**Consent for publication**

Not applicable

**Availability of data and material**

The raw ITS2 reads are stored at the European Nucleotide Archive (ENA) under accession number XXXXXX.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**

Both authors designed the study. PH analyzed the data. JH and PH wrote the manuscript, reviewed it and have approved the final version. Funding was provided to JH.

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Figure 1
Map over sampling locations in Sweden. The map was created in QGIS 3.14 with Natural Earth vector data.

Figure 2

A) Nemabiome composition for adults and lambs. No difference in nematode species richness (B), but adults were dominated by a few species compared to lambs (C).
Figure 3

A) Nemambiome composition for lambs for two periods. B) Species richness and C) Inverse Simpson’s diversity index for each period.
Figure 4
Nemabiome composition (A-C), EPG (D-F) and diversity indices; Species richness (G-I). The shape of the violin plots in panel G indicate that anthelmintic treatment will have a larger effect for individuals with higher species richness prior to treatment. Inverse Simpson’s index (J-L) prior and after anthelmintic treatment, for ivermectin (n=25) in left column, albendazole (n=14) in middle column and levamisole (n=5) in right column.