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Metagenomic Studies for the Detection of *Parelaphostrongylus odocoilei*, *Ostertagia leptospicularis* and *Eimeria ahsata* in Northern Cyprus Sheep for the First Time

J.T. Olaifa[1], E. Ozgor[2]

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ABSTRACT

Background: Gastrointestinal parasitism is considered one of the major health problems of ruminants in developing and undeveloped countries in the world. Despite the universal knowledge of these parasites, little is known about their prevalence and distribution in Northern Cyprus. This study is aimed at the microscopic and metagenomic study of gastrointestinal parasites across Cyprus sheep farms.

Methods: A total of 200 sheep faecal samples were randomly collected across sheep farms in suburban regions of Northern Cyprus during 2021-22. Samples were analyzed using the centrifugal floatation technique. Data were subjected to chi-square test analysis on SPSS to determine the relationship between age, sex of animals and prevalence of gastrointestinal nematodes. Samples were randomly selected to extract DNA and subjected to metagenomic analysis after which DNA has been extracted from the samples.

Result: Our investigations in the Northern Cyprus sheep farms allowed us to identify three species of gastrointestinal parasites namely *Parelaphostrongylus odocoilei*, *Ostertagia leptospicularis* and *Eimeria ahsata* which are new to this region. The GINs recovered are *Haemonchus*, *Moniezia* and *Toxocara* among others. A high prevalence (85%) of gastrointestinal parasites was recorded. Results showed a considerable significant relationship (P<0.05) between the age of animals and parasite prevalence.

Key words: Cyprus, *Eimeria*, GINs, prevalence, sheep.

INTRODUCTION

The cost of managing diseases caused by gastrointestinal nematodes in ruminant animal production is a major pain for farmers globally (Francis and Šlapeta, 2022). The losses incurred are due to disease manifestation, low productivity and a higher susceptibility to other infections (Kamal *et al.,* 2021; Khan *et al.,* 2021). GIT parasitism can alter milk's nourishing characteristics, such as protein, lessened fat and lactose composition. Livestock animals can become diseased with diverse parasites and disseminate them to other animals around them (Khan *et al.,* 2021). Many protozoans are usually found in ruminant animals such as sheep and goats (Isah, 2019). Though microscopy has been a conventional technique of choice for parasitological studies, it permits the identification of the parasites typically to genus level, since the eggs of interrelated species are repeatedly indistinguishable (Chessa *et al.,* 2020), immunological, hybridization and molecular methods were established and utilized for a better taxonomic identification (Jaeger and Iñiguez, 2014; Chessa *et al.,* 2020).

Molecular parasitological examinations are primarily centred on PCR amplification and Sanger sequencing of short barcoding loci as 18S rDNA, primarily making use of primers for a precise parasite taxon. The latest established next-generation sequencing (NGS) permits the identification of many taxonomic groups simultaneously by direct shotgun sequencing of DNA hauled out of the samples (metagenomics) or by PCR-centred metabarcoding of target genes (Srivathsan*et al.,* 2016), this technique has been applied on human samples i (Güler and Süer, 2021; Chihi *et al.,* 2022) and swine samples i (Wylezich *et al.,* 2019), but has never been applied to parasitology on any ruminants in Northern Cyprus. The infection of gastrointestinal parasites is influenced by many factors such as the climatic condition, the host nature, farming operations, the functional state of the host and the epidemiology of the parasite of interest. Owing to the significant challenges with drug resistance, there is an apparent necessity and substantial global interest in the advance of enhanced methods of controlling GIT parasites for which epidemiology of parasites is a prerequisite.

Hence, the present study is aimed to investigate the prevalence of GIT parasites in 200 faecal samples collected

[1]Departmentof Bioengineering, Cyprus International University, Haspolat, Lefkosa, North Cyprus.
[2]Department of Molecular Biology and Genetics, Cyprus International University, Haspolat, Lefkosa, North Cyprus.

Corresponding Author: J.T. Olaifa, Departmentof Bioengineering, Cyprus International University, Haspolat, Lefkosa, North Cyprus. Email: joshuatunde17@gmail.com

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across the sheep farms of Northern Cyprus utilizing microscopy and metagenomic analysis. Moreover, three gastrointestinal parasites of economic importance were discovered through metagenomic analysis for the first time in Northern Cyprus.

MATERIALS AND METHODS

Ethical approval for the present study was obtained from the Ethics Committee of Cyprus International University (020-7952). All samples were collected across Northern Cyprus sheep farms between September 2021 and February 2022. The farms raise sheep and goats largely for milk and meat production. Microscopic studies were carried out in the microbiology laboratory of Cyprus International University.

A total of 200 faecal samples were collected from 200 sheep considering age and sex of animals. The samples were collected and kept in airtight containers. Upon arrival at the laboratory, each sample was prepared using the centrifugal floatation technique (Zajac, 2002) The samples were recovered and kept at 4°C until they were checked for the presence or absence of nematodes and *Eimeria* oocysts.

Processed samples were identified up to genus level based on morphological description (Shorb, 1940; Christie and Jackson 1982).

Further, QIAamp PowerFecal Pro DNA Kit (Qiagen, Hilden, Germany) was used for the extraction and purification of the genomic DNA from positive samples. The primer pair used for the construction of amplicon libraries targeted a region of nearly 1400 bp covering the V1-V9 region of the 18SrRNA gene. Oxford Nanopore Technologies Nanopore barcode DNA sequences of the generated library were added to the 5' end of the target-specific primer pairs. The target-specific primer-connector sequences specific to 18SrRNA are TTTCTGTTGGTGCTGATATTGC-GRGTTTGA TYHTGGCTCAG -3′ for the forward primer and 5′-ACTTG CCTGTCGCTCTCTATCTTC-TACCTTG TTAYGACTT-3′ for the reverse primer. The taxonomic annotations were performed by comparing the created OTUs according to the RDP 18SrRNA database and results were obtained by correlating the OTUs detected as the same genus. Pie charts were created with various statistical analyzes using Minitab and R programs according to the organisms to which the OTUs matched, their quantitative values and the metadata data of the samples.

Statistical analysis for prevalence was carried out employing SPSS software for Windows version 22.0 (SPSS Inc., Chicago, USA). Chi-square test was carried out to determine the relationship between variables and the relationship between variables was assumed to be real when the probability of finding the observed difference by chance was less than 5% ($P<0.05$). The complete prevalence was estimated as a percentage of the number of sheep infested in the entire number of sheep investigated.

RESULTS AND DISCUSSION

Prevalence of parasite

Out of the 200 samples analysed for the presence of gastrointestinal parasites from the farms, 75 are male and 125 are females. At least one parasite was present in 160 of the analyzed samples amounting to 80% parasite prevalence. Thus, eggs of gastrointestinal nematodes (GIN) recovered from the samples as eggs in the genus of *Moniezia, Cooperia, Oesophagostomum, Parelaphostrongylus, Trichurus, Heamonchus, Nematodirus, Ostertagia, Toxocara* and other strongyles (Fig 1). The differentiation of srongyle eggs were done morphologically with the aid of microscopic using aforementioned keys. *Eimeria* oocysts were spotted in 65 of the total number of samples analyzed. It was also observed that eggs of GIN in the genera of *Heamonchus* were the most encountered (80) accounting for 40% prevalence (Table 1). Our results of high prevalence are similar to those of Kulišić *et al.* (2013) and Mphahlele *et al*. (2021) and gastrointestinal parasite infection has a direct effect on the health and productivity of animals, thereby causing a reduction in output and, consequently, a decrease in the owner's and community's income (Mathewos*et al.,* 2022). The predominance of *Haemonchus* over other GIT parasites has also been reported by Tariq *et al*. (2008) and Wani *et al*. (2011) in Kashmir Valley. The major reason for this prevalence can be connected to the fact that *Haemonchus* females are principally fertile egg layers (laying about 10,000 eggs a day) (Tramboo *et al.,* 2015). The high prevalence of GIT parasites observed in the current study could be as a result of the accessibility of appropriate climatic conditions such as fairly high temperatures and rainfall that support the lengthy survival and development of an infective larval stage of most nematodes (Shearer and Ezenwa, 2020; Mphahlele*et al.,* 2021).

Effect of age on gastrointestinal parasites prevalence

The results showed that younger animals are more susceptible to gastrointestinal parasites infection than the older animals (Table 2), animals under the age of 2 years generally gave a result of above 90% prevalence in terms of infection and this shows that there is a statistically significant relationship between the age of animals and prevalence ($P<0.05$). The higher prevalence in younger sheep is in agreement with the results of Abebe *et al*. (2018)

Table 1: Prevalence of GIT nematodes and *Eimeria* in Northern Cyprus sheep farms.

Nematode genera	Number infected	Prevalence (%)
Haemonchus	80	40
Nematodirus	40	20
Oesophagosuturom	38	19
Moniezia	70	35
Toxocara	40	20
Trichurus	42	21
Parelaphostrongylus	30	15
Cooperia	20	10
Ostertagia	17	8.5
Other *strongyles*	32	16
Eimeria oocyst	65	32.5

who reported significantly higher GIT parasites prevalence in younger animals in Ethiopia but disagree with that of Tramboo *et al*. (2015) as they established an overall higher prevalence in older animals than younger ones. The high prevalence of parasites in younger animals indicates a high degree of susceptibility, unlike the adults which acquire immunity to some of these GIT parasites.

Poly-parasitism of gastrointestinal parasites

Out of the 200 examined sheep, 95 (47.5%) were cross infected with two parasites, 10 of the analyzed samples (5%) were infected with three gastrointestinal parasites and 60 (30%) were infected with one gastrointestinal parasite while 40 (20%) were negative for the presence of GI parasites. The present study has indicated the presence of mixed infection with two or more GIT parasite genera in sheep and this is in agreement with the findings of other researchers (Asif *et al.,* 2008; Samuel *et al.,* 2016). Polyparasitism has been proposed to be a vital source of morbidity and loss of production in sheep (Samuel *et al.,* 2016). Additionally, the incidence of interaction and compromisation of the immune system of the host by polyparasitism has been detailed to intensify their vulnerability to other diseases or parasites (Wang *et al.,* 2006). Therefore, polyparasitism is a significant issue in sheep production in the present study area.

Effect of animal sex on prevalence

In terms of animal sex, observations revealed that all the parasites encountered were found in the faecal samples of the male animals except for *Oesophagosuturom* while three of the 12 encountered parasites namely Ostertagia, *Trichurus* and *Oesophagostomum* were not found in any of the female animals across the farms. Though, the interpretations were

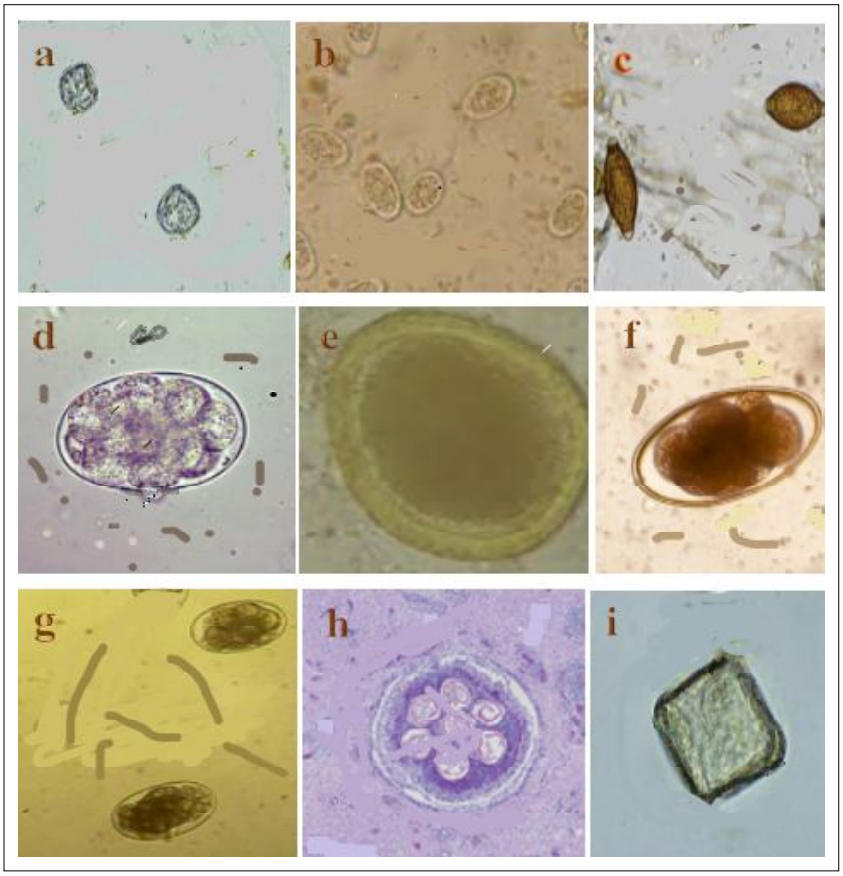

Fig 1: Images of parasite genera found in the microscopy study (a) *Monieza* (b) *Eimeria oocysts* (c) *Trichurus* (d) *Oesophagostomum* (e) *Ostertagia* (f) *Nematodirus* (g) *Heamonchus* (h) *Parelaphostrongylus* (i) *Monieza.*

Table 2: Age wise prevalence of gastrointestinal parasites among Northern Cyprus sheep.

Age	Number examined	Number positive	Prevalence	χ^2	P-value
under 1 year	60	58	96.70%		
1-2 years	60	56	93.30%	36.7	0.00
Above 2 years	80	59	60%		

Significant at P 0.05; Non- significant at P>0.05.

not substantial. The effect of sex on the vulnerability of sheep to infections could be ascribed to innate tendency and distinctive proneness due to hormonal regulation. The total number of samples collected from male animals tested during the study was 75 and 58 were positive for at least one endoparasite while 102 out of 125 samples taken from the female animals yielded a positive result accounting for 81.6% prevalence therefore a higher prevalence of major gastrointestinal parasite infection was observed in female animals though the p-value (0.33) suggests that the relationship between the sex and prevalence is not statistically significant (P>0.05) (Table 3). This is a result of the same exposure of both sexes and they are as well from the same agroecology. The influence of sex on the susceptibility of sheep to infections could be ascribed to innate tendency and distinctive proneness as a result of hormonal regulation. The existing gender-wise remarks are in agreement with the finding of Tariq*et al*. (2008) and Gauly *et al*. (2006) who described the occurrence of gastrointestinal parasite infection in sheep as higher but not statistically significant in females than in their male counterparts.

Microbiota composition according to genus level

In this study, the gut microbiota community composition was analysed, Fig 2 shows the complete composition and abundance of Eukaryota domain presents in the feacal samples. At the genus level, the top three predominant populations in the samples analysed were Blastocystis, Mucor, Ichthyobodo and Entemoeba. In terms of the composition, Blastocystsis which has the highest abundance with 33.5% followed by Mucor (14%), Ichthyobodo (13%), Entamoeba (8%), Parelaphostrongylus (4%), Spironucleus (3.5%) and Ripella (0.6%) with the least abundance among the families. Furthermore, it was observed that four species

Table 3: Prevalence of GIT parasites according to the animal sex.

Sex	Number examined	Number positive	prevalence	χ^2	P-value
Male	75	58	77.30%		
				0.182	0.33
Female	125	102	81.60%		

Significant at P 0.05; Non- significant at P>0.05.

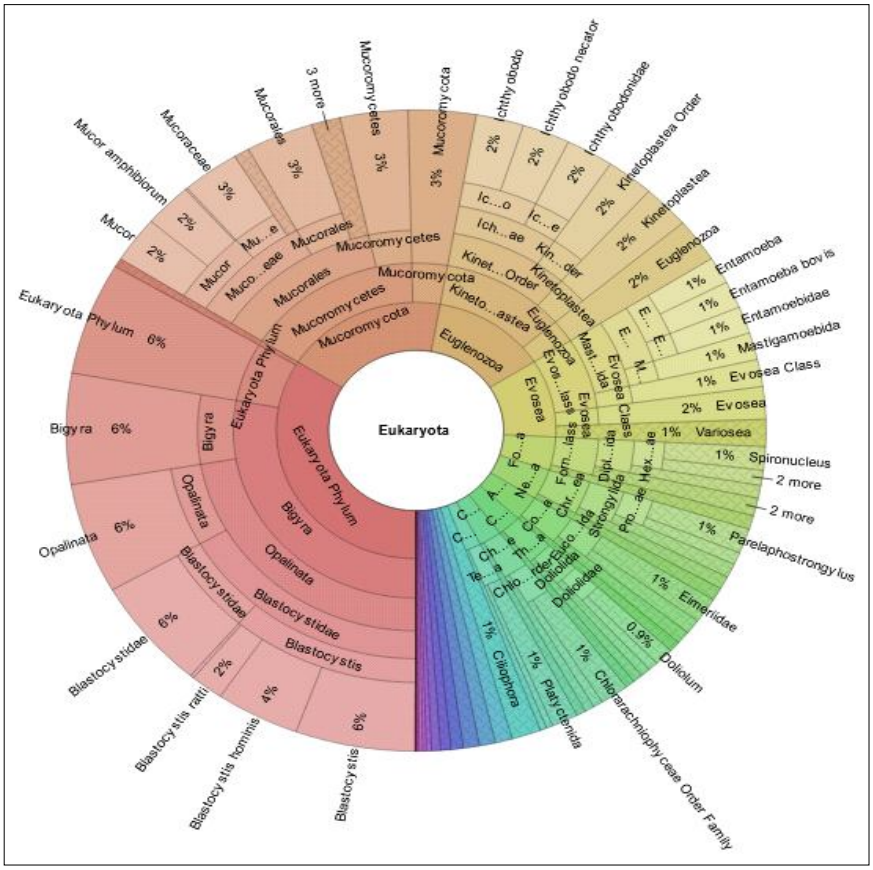

Fig 2: Composition and abundance of domain Eukaryota in Cyprus sheep faecal sample.

were present in the Blastocystis genus namely *Blastocystis hominis, Blastocystis ratti, Blastocystis python* and *Blastocystis cycluri*. Three species present in Mucor genus namely *Mucor amphibiorum, Mucor mucedo* and *Mucor indicus* while the other families were represented with just a species (Table 4).

Metagenomic analysis detects three species of GIT parasite

The results obtained from the present study of GIT parasites with a metagenomic tool detailed the identification of three parasites which are new to the study area. The GIT parasites detected from the analysis are *Parelaphostrongylus odocoilei*, *Ostertagia leptospicularis* and *Eimeria ahsata*.. The krona graphs presented showed the percentage of each parasite species identified. Fig 3-6 revealed the species identified for the first time in the study area through the use of metagenomic analysis. Eggs in the genus of the three new species of parasite discovered were also identified in the microscopy analysis which shows some level of congruency between the two techniques. However, we could not identify species from other genera of parasite identified using microscopy analysis, this could be a result of the very low number of samples analysed in the metagenomic study as compared to microscopy analysis.

The high prevalence of protostrongylid infections in Dall's sheep in Subarctic and Arctic North America was first reported by Jenkins *et al*. (2006). The findings of Jenkins *et al*. (2005b) further suggest that adding to muscular and respiratory pathology, *P. odocoilei* also has the likelihood of causing neurological disease in thinhorn sheep (Jenkins *et al*. 2005b). *Ostertagia leptospicularis* which is not only found in sheep but also other ruminants such as cattle and goats is a gastrointestinal nematode of the genus *Ostertagia*, it develops in the abomasal glands of ruminants. The *Ostertagia*specieshave been connected to pathophy siological changes resulting from abomasal parasitism which include a reduced acidity of the abomasal contents, which is associated with a decreased peptic digestion and bacteriostatic activity (Hertzberg *et al.,* 2000). The presence and prevalence of *Eimeria ashata* have been reported by Olmos *et al*. (2020) in Argentina, Souza *et al*. (2015) in Brazil and Reeg *et al*. (2005) in Germany. This is the first time the presence of *Eimeria ashata* in a ruminant animal is reported. Coccidiosis has been reported as a globally important clinical and subclinical parasitic infection in ruminant animals. Coccidiosis is caused by coccidian parasites of the genus *Eimeria* which add to enteric disease, particularly in young or stressed animals under poor farm management thereby leading to high mortality in young ruminant animals (Yusof and Isa, 2016). Furthermore, investigations in many countries such as Brazil (Souza *et al.,* 2015) and Egypt (Al-Alfy*et al.,* 2020) have indicated that coccidiosis has a huge economic significance to the

Table 4: Taxonomy of species detected by metagenomic analysis.

Taxon	Taxonomy	Sample barcode	Mean
Nematoda	Phylum	3274	1637
Chromadorea	Class	3274	1637
Strongylida	Order	3274	1637
Protostrongylidae	Family	3165	1582.5
Parelaphostrongylus	Genus	3165	1582.5
Parelaphostrongylus odocoilei	Species	3165	1582.5
Haemonchidae	Family	109	54.5
Ostertagia	Genus	109	54.5
Ostertagia leptospicularis	Species	109	54.5
Apicomplexa	Phylum	2695	1524
Conoidasida	Class	2695	1524
Eucoccidiorida	Order	2563	1458
Eimeriidae	Family	2168	1084
Eimeria	Genus	2168	1084
Eimeria ahsata	Species	2168	1084

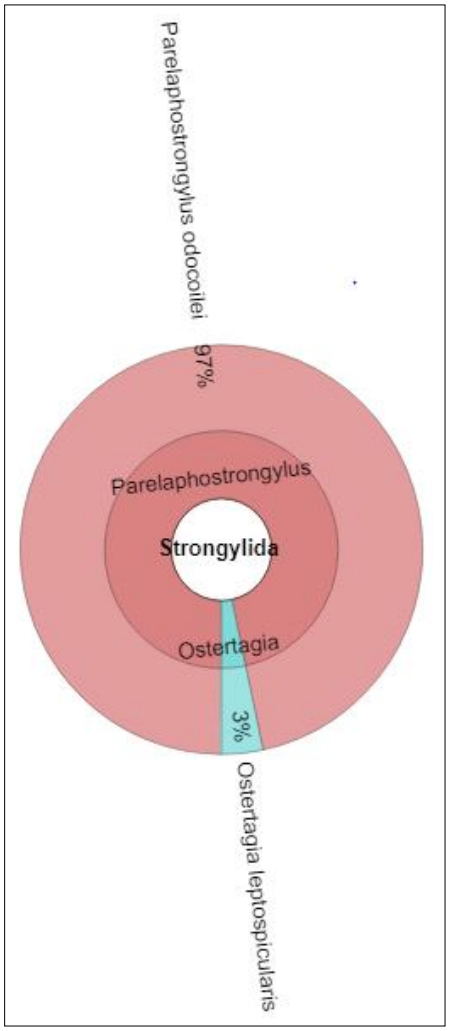

Fig 3: Composition and abundance of species found in Strongylida order.

production of livestock owing to the clinical disease (diarrhoea) and sub-clinical disease (poor weight gain) caused by the parasite kept in large numbers under differs management methods.

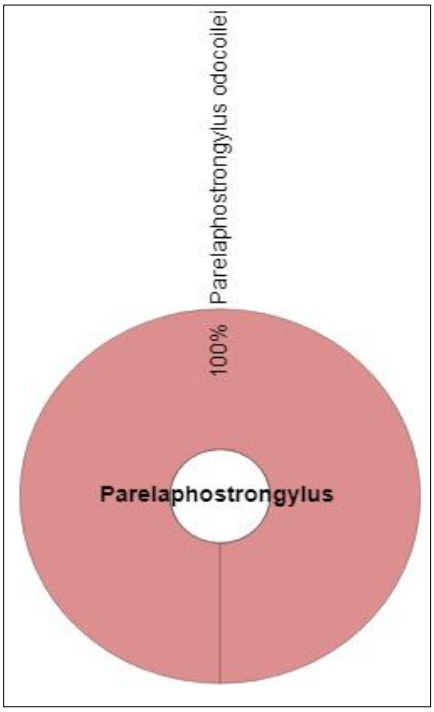

Fig 4: Krona chart showing the only parasite discovered in the Parelaphostrongylus genus.

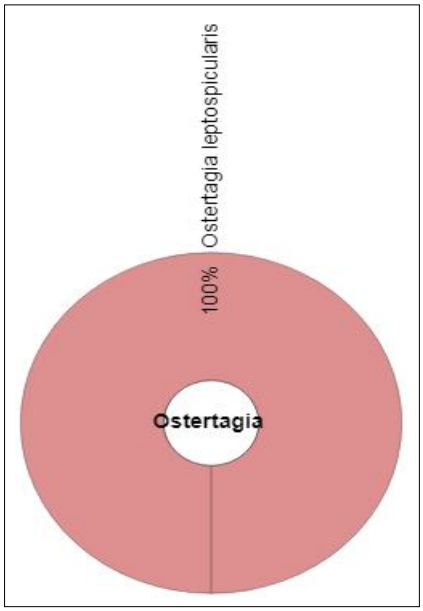

Fig 5: Krona chart showing the only parasite discovered in the Ostertagia genus.

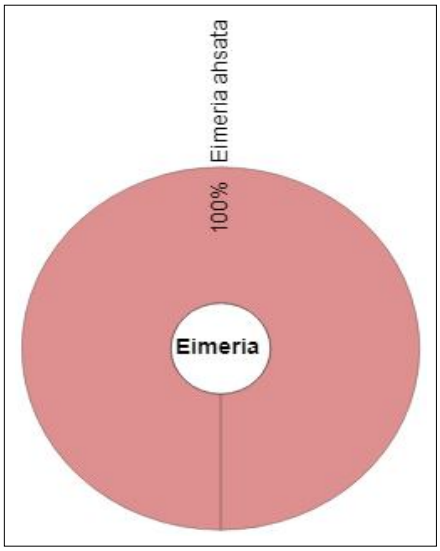

Fig 6: Krona chart showing the only parasite discovered in the Eimeria genus.

CONCLUSION

This study indicated that both GINs and *Eimeria* infections were leading in sheep farms in Cyprus. Younger animals were more susceptible to infection with GIT parasites. The combination of microscopic identification and metagenomic technique resulted in a more complete parasitological identification and could be recommended as a new approach in parasitological studies. The parasitic weight constituted a pronounced financial influence and limitation to the sheep venture in attaining improved and viable productions. Consequently, these results recommended a need for well-harmonized, healthy regulation of sheep farms by scientists and the spreading of awareness on GINs and *Eimeria* to breeders and farmers to lessen the incidence of infections.

Conflict of interest: None.

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