Research in Molecular Medicine

Molecular Identification of *Trichostrongylus* Species Among Small Ruminants in Mazandaran Province, Iran

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ABSTRACT

Background: *Trichostrongylus* is an intestinal parasite that is highly prevalent in humans and livestock worldwide. There is limited information about the prevalence and epidemiology of *Trichostrongylus* species among the infected livestock in Mazandaran Province, northern Iran. This study aimed to identify *Trichostrongylus* spp. among small ruminants using morphometric and molecular methods.

Materials and Methods: Small intestinal organs of sheep and goats, slaughtered in Mazandaran Province, were examined for infectivity with *Trichostrongylus* parasites. Primary species identification was conducted based on the morphological characterization of the male worms. The internal transcribed spacer (ITS) II regions of the ribosomal DNA of the worm tissues were amplified using the polymerase chain reaction (PCR) assay and then the product was subjected to sequencing. Subsequently, the PCR products of the ITS II region were subjected to digestion by HinfI and DraI restriction enzymes using the PCR-restriction fragment length polymorphism (RFLP).

Results: Of 180 samples, 98 (54.44%) were confirmed positive for *Trichostrongylus* based on the conventional PCR. The digestion of the PCR products with HinfI and DraI facilitated the identification of three *Trichostrongylus* species, namely *Trichostrongylus colubriformis* (35%, 90.81%), *Trichostrongylus axei* (4%, 4.08%), and *Trichostrongylus vitrinus* (5%, 5.1%). Both morphometric and RFLP techniques resulted in the differentiation of the three *Trichostrongylus* species.

Conclusion: The present study was the 1st attempt in the last 30 years for the identification of Trichostrongylus species in small ruminants in Mazandaran Province. The findings of this study can be helpful for epidemiological and ecological studies, the establishment of effective control programs, and the management of gastrointestinal parasites in Mazandaran Province.

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Introduction

Trichostrongyliasis, caused by any of the several Trichostrongylus species, is a major parasitic disease in a variety of herbivores [1, 2]. Trichostrongyliasis in livestock and humans has been reported from various geographical regions as 18.13% in India, 41.53% in Indonesia, 36.9% in Thailand, 75.8% in Malaysia, 36.8% in China, 44.4% in Thailand, 9.8% in Iran, 14.7% in Egypt, 69.2% in Nigeria, 90% in France, 4% in the UK, 1.2% in Brazil, 2% in the Caribbean island, and 90% in Australia [3]. Trichostrongylus species are

Important parasites of livestock accounting for economic losses, such as the decreased amount of meat, wool, and milk production, increased mortality rate, as well as weight loss in ruminants [4]. Trichostrongylus spp. have a direct and simple life cycle, including the parasitic stage in which parasites spend part of their life inside the host and in the free-living stage, they spend part of life outside the host. Human and livestock infections occur incidentally via ingestion of larvae from contaminated food and water or rarely by penetrating through the skin [5].

Human infections are usually light and asymptomatic, but heavy infections may be accompanied by skin rash, abdominal pain, diarrhea, nausea, mild anemia, flatulence, dizziness, systemic poisoning, leukocytosis, and eosinophilia [5].

Eggs and larvae develop in areas with high humidity, shade, and vegetation. Temperature and moisture are two factors that can control the development and survival of the free-living stage of this nematode genus [6]. Domesticated animals can be infected by several *Trichostrongylus* spp., which exert various pathological effects [7]. Therefore, the accurate and reliable identification of *Trichostrongylus* spp. is important for the implementation of epidemiological studies and the establishment of effective programs and strategies for controlling the parasite. The conventional morphological methods cannot be used for the identification of female parasites, larvae, and eggs, while these methods are common and reliable for the identification of male *Trichostrongylus* [8]. The traditional coprological diagnostic techniques of nematode species are problematic [9] because of morphometric similarity between species and genus (i.e. *Trichostrongylus* and *Teladorsagia*, *Chabertia ovina,* and *Oesophagostomum venulosum*).

The application of molecular techniques, such as polymerase chain reaction (PCR), has facilitated the accurate detection and differentiation of parasite species. These reliable techniques are sensitive, specific, and highly precise in the identification of strongylid nematodes up to the species level [10].

Limited information has been reported about the molecular identification and epidemiology of *Trichostrongylus* spp. in the animal population in the northern region of Iran among the infected livestock in Mazandaran Province, Iran, since 1975. With this background in mind, the present study aimed to identify *Trichostrongylus* spp. among small ruminants grazing in Mazandaran Province, using morphometric and molecular methods.

Materials and Methods

Study area

The study was conducted in Mazandaran Province, near the Caspian Sea (North of Iran). These areas have suitable geographic and natural climatic conditions for livestock production, with an average temperature of 17°C and annual rainfall of 650-1300 mm.

Parasites preparation

The small intestines of 180 ruminants, including 119 sheep and 61 goats, were collected from 10 local abattoirs located in various parts of Mazandaran Province. All animal hosts were examined for infection with *Trichostrongylus* parasites. The small intestine contents were washed through a 100-mesh sieve and observed under a stereomicroscope. Every worm recovered from the contents was cleaned with normal saline, and then cleared in lactophenol for genus identification. The *Trichostrongylus* genus was detected based on the V shape of the excretory pore from the anterior end of the worms. Parasite species identification was done based on the copulatory bursa and morphometric identification keys [11]. Then, the samples were washed in distilled water 3 times and preserved in ethanol 70% until further use.

DNA extraction and PCR assay

One *Trichostrongylus* male worm isolated from each host was processed for DNA extraction. The worms were washed with distilled water to remove ethanol. Briefly, each worm was crushed between two microscopic slides for 1 minute with lysis buffer.

Genomic DNA was extracted using Dyna Bio DNA extraction kit (Takapouzist Co, Iran) according to the manufacturer's instructions. The DNA was stored at -20°C for further application.

The ribosomal DNA ITS (internal transcribed spacer) II region was amplified by forward (NC1: 5'-ACGTCTGGTTCAGGGTTGTT-3') and reverse (NC2: 5'-TTAGTTTCTTTTCCTCCGCT-3') primers [12]. The PCR reactions were performed in a final reaction volume of 20 µL containing 1 µL template DNA (20 ng), 0.25 µL of each dNTP (0.1 mM each), 0.6 µL $MgCl_2$ (1.5 mM), 0.25 µL of each primer (25 pmol), 0.4 U *Taq* DNA Polymerase (2U), 15.25 µL double-distilled water (DDW), and 2 µL 1X PCR reaction buffer. The reaction mixtures were carried out in a thermocycler (MWG, Germany) under the following condition: denaturation at 94°C for 5 minutes, followed by 35 cycles of 1-minute denaturation at 94°C, annealing for 1 minutes at 52°C, extension for 1 minute at 72°C, and final extension at 72°C for 5 minutes. The DNA of *T. colubriformis* (NCBI accession no KX417708) and 1 µL of distilled water were respectively used as the positive and negative controls. The specificity of the *Trichostrongylus*-specific PCR assay was determined by amplification of DNA templates of *Haemonchus contortus*. About 10 µL of the PCR products were run on a 1.5% agarose gel at 100 V for 1 hour. Gels were stained with ethidium bromide (0.1 µg/mL) and visualized on a transilluminator (Syngene, UK). A 100-bp DNA ladder (Thermo Fisher Scientific) was run to estimate the size of DNA in each gel.

Sequencing and restriction fragment length polymorphism (RFLP) assay

At the next step, 20 µL of 14 PCR products of each *Trichostrongylus* species were subjected to sequencing, using forward (NC1) and reverse (NC2) primers. One sequence of each species was subjected to in silico cutting with almost all the known restriction enzymes using online Web Cutter software. Subsequently, the predicted restriction profiles were determined for each of the species with two enzymes, namely HinfI and DraI (Jena Bioscience, Germany). The baseline characteristics of the restriction enzymes used in this study are summarized in Table 1.

The PCR-RFLP was performed in a final volume of 15 µL, containing 5 µL of PCR products, 1 unit of restriction enzymes (i.e. Hinf I and DraI), 2 µL of the 10X supplied reaction buffer, and 7 µL distilled water. The reaction mixture was incubated at 37°C for 1-3 hour. The restriction fragments were separated on 2.2% agarose gel in TBE buffer, stained with ethidium bromide, and then photographed.

Results

Morphological and morphometrical examinations

A total of 180 small intestines were collected from the 119 sheep (66%) and 61 goats (33%) in the abattoir inspection. Among the examined samples, 98 hosts (54.44%), including 56 sheep (47.05%) and 42 goats (68.85%) were microscopically positive for identified *Trichostrongylus* parasites. The rest of the 82 hosts were either not infected or infected with other intestinal nematodes excluded from the study. Parasite species identification was based on spicules and gubernaculum in the copulatory bursa of the male worms (Figure 1). All 98 positive samples for the *Trichostrongylus* genus were kept in 70% ethanol for PCR assay. Based on the morphometric method, three species, including 35 *T. colubriformis* (35.71%), 4 *T. vitrinus* (4.08%), and 4 *T. axei* (4.08%) were identified. *T. colubriformis* was the most common species in sheep and goat small intestines in this study, followed by *T. vitrinus* and *T. axei*. *Trichostrongylus* species were more predominant in goat intestines than in sheep. Results showed a high prevalence of *Trichostrongylus* infection in goats (68.85%) compared to sheep (47.05%).

Table 1. Restriction enzymes used for differentiation of *T. colubriformis*, *T. vitrinus*, and *T. axei*

Species	DraI		HinfI	
	FragmentSize After Digestion (bp)	Recognition Site	Fragment Size After Digestion (bp)	Re Recognition Site
T. colubriformis	110, 218	TTTAAA	238, 90	GANTC
T. axei	110, 218	TTTAAA	-----	
T. vitrinus	145, 185	TTTAAA	-----	

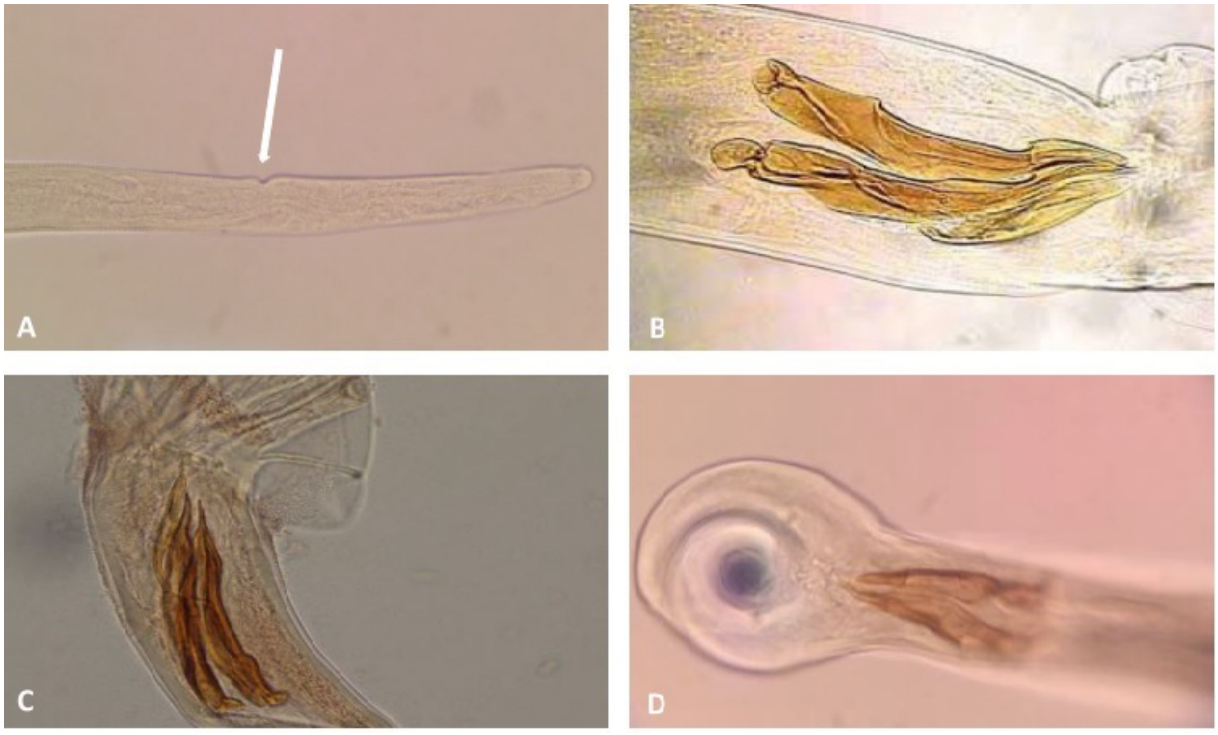

Figure 1. (A) Anterior end and V shape excretory pore of adult Trichostrongylus genus, (B) Digital pictures of spicules and gubernaculum of Trichostrongylus colubriformis, (C) Trichostrongylus vitrinus, (D) Trichostrongylus axei

Molecular findings

A 328-bp band of the ITS II region was successfully amplified from each of the male worms (Figure 2). Based on a few nucleotide differences between *Trichostrongylus* spp., the PCR- restriction fragment length polymorphism (RFLP) patterns of *T. colubriformis*, *T. vitrinus*, and *T. axei* were obtained after the endonuclease digestion of the PCR products with HinfI and DraI restriction enzymes (Figure 3). After the RFLP assay, DraI produced two different patterns for *T. axei/T. colubriformis* (218, 110 bp) and *T. vitrinus* (185, 145 bp). HinfI had one cutting site for *T. colubriformis*, producing two fragments of approximately 238 bp and 90 bp. On the other hand, it had no cutting sites for the other two species.

The sequences of the study were compared with different sequences available in the GenBank database, using the Basic Local Alignment Search Tool. The results showed 98%-100% homology between our samples and those that originated from other regions available in GenBank (Table 2). The results of the microscopy and PCR-RFLP techniques in terms of the number of *Trichostrongylus*-positive samples in the sheep and goats are presented in Table 3.

Discussion

Gastrointestinal nematode infections (GIN) have remained the most prevalent and important topics affecting small ruminants [13]. Small ruminants, such as sheep and goats, are susceptible to GIN, such as *Trichostrongylus* infection. This is because these animals graze in

Table 2. List of *Trichostrongylus* species sequences of ruminants used in this study

Species	GenBank Accession No.	Identity (%)
T. colubriformis	KU891930.1, AB908960.1, JF276021.1, KF204576.1, MG770112.1, AB908959.1	99-100
T. axei	AY439026.1, KC998727.1, KF880746.1, KF204573.1, KJ755059.1, KC998725.1, KC998724.1	99
T. vitrines	KJ755061.1, KF880745.1, KC998732.1, JF276025.1, KC998733.1, JF680986.1	98-100

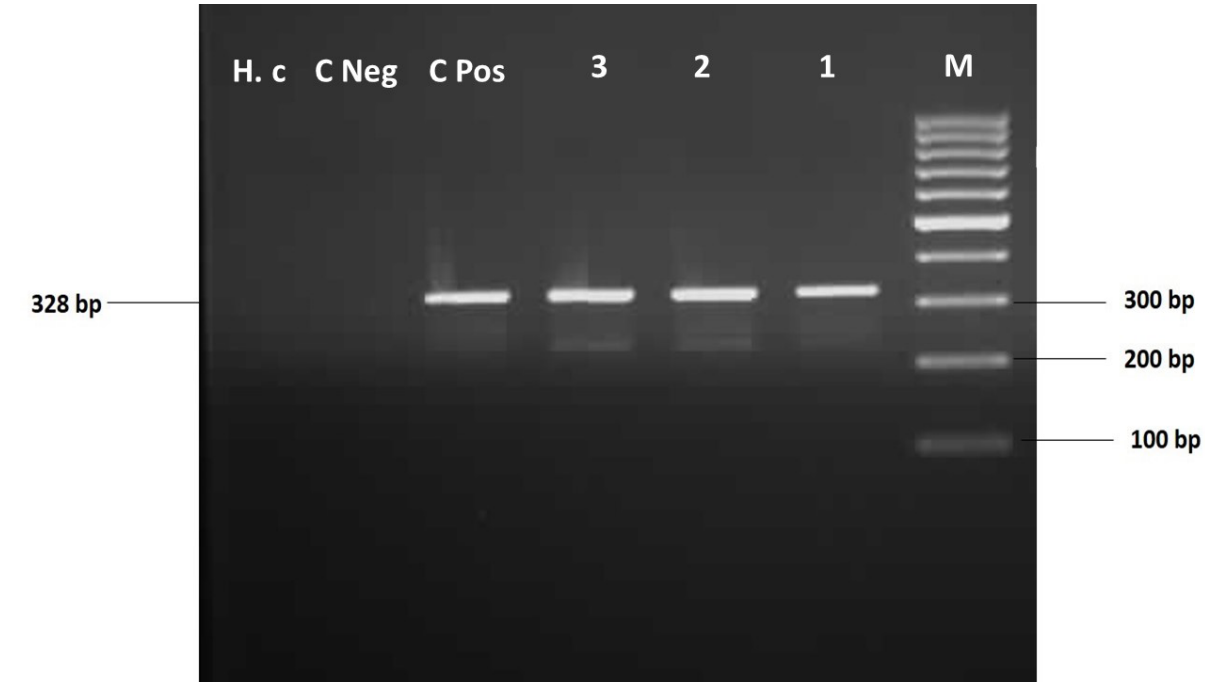

Figure 2. Example of agarose gel (1.5%) electrophoresis of internal transcribed spacer (ITS) II–PCR products (10 µL/well) of genus *Trichostrongylus* (Lanes 1–3)

Lane C Pos: Positive control; Lane C Neg: Negative control; Lane *H. c: Haemonchus contortus* to specify PCR assay; Lane M: 100-bp DNA ladder.

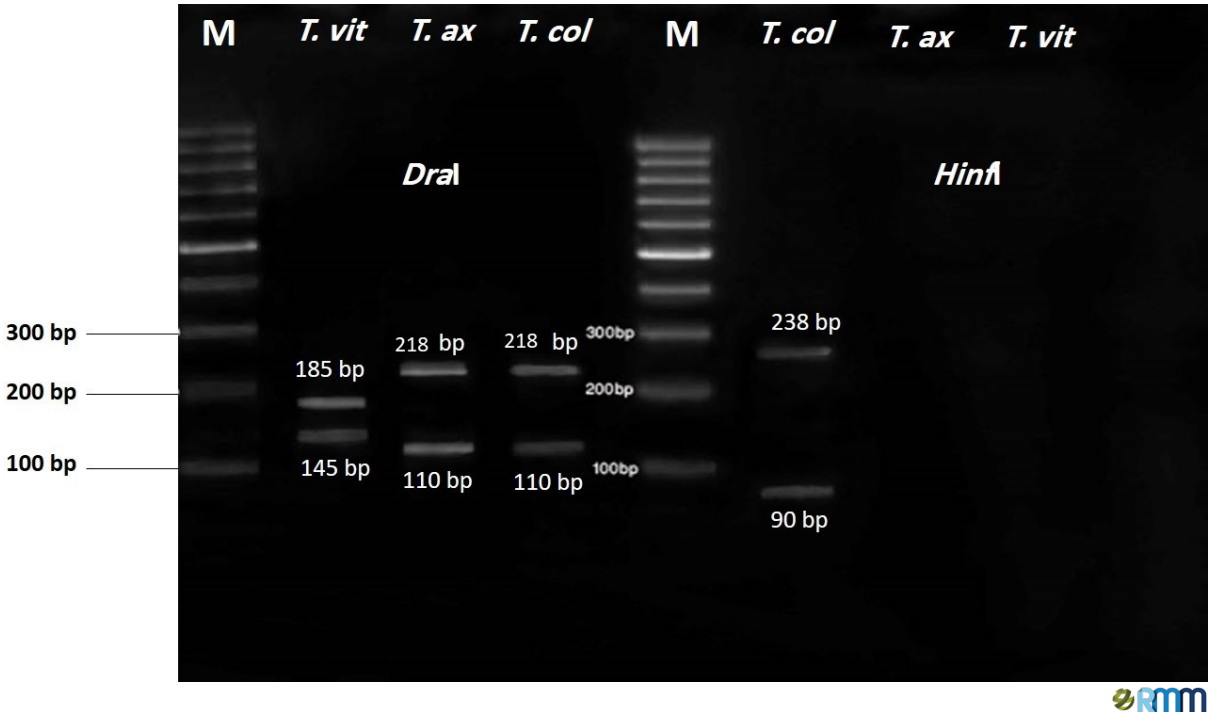

Figure 3. Agarose Gel (2.2%) electrophoresis of PCR-restriction fragment length polymorphism (RFLP) (15 µL/well) of *Trichostrongylus* species

The pattern of PCR products after digestion with DraI: Lane *T. vit: T. vitrinus*; Lane *T. ax: T. axei*; Lane *T. col: T. colubriformis* (left). The pattern of PCR products after digestion with HinfI: Lane *T. col: T. colubriformis*; lane *T. ax: T. axei*, Lane *T. vit: T. vitrinus* (right); Lane M: 100-bp DNA ladder.

Table 3. *Trichostrongylus* species in ruminant small intestine samples (microscopically positive) determined by PCR assay

Methods	*T. colubriformis*			*T. axei*			*T. vitrinus*		
	Total	Positive	%	Total	Positive	%	Total	Positive	%
Morphometric*	98	35	35.71	98	4	4.08	98	4	4.08
PCR-RFLP	98	89	90.81	98	4	4.08	98	5	5.1

*55 Trichostrongylus isolated were not detectable based on the morphometric method under light microscopy.
PCR-RFLP: PCR-restriction fragment length polymorphism.

pastures contaminated with parasites, resulting in their re-infection or continuous infection [14]. The Trichostrongylid nematodes of small ruminants are among the main veterinary health problems because of their widespread distribution, high prevalence, severe pathological effect, and economic loss [15].

The intensity of GIN in sheep and goats is mainly influenced by parasite species available in the gastrointestinal tract, the general health and immunological status of the host, environmental factors, and so on. These factors are all essential in designing useful strategies for controlling these nematodes [14]. Nonetheless, conventional diagnostic techniques, such as morphological methods, fail to fully provide such information on eggs or larvae [10]. The diagnosis of *Trichostrongylus* species is routinely performed by the detection of eggs or the production of 3rd-stage larvae by culturing fecal samples. However, the eggs are not morphologically differentiable at the level of species, and culturing process is very time-consuming and sometimes both methods need identification keys [16]. The PCR method as a molecular technique helped us to develop reliable parasite-species identification for molecular epidemiology. For the discrimination of *Trichostrongylus* or other nematode species, the ITS I and II, small and large subunit regions of ribosomal DNA, and cytochrome c oxidase subunit I are valuable genetic markers. Several various PCR-based assays have been described to detect parasite species or genera in domestic animals [17]. Restriction enzymes are powerful and simple tools for the identification of parasite species based on differences in their genomes.

In our study, the PCR-RFLP was used for differentiation among *Trichostrongylus* species obtained from the samples that were microscopically positive for worm, based on the patterns generated by the effects of endonucleases on ITS II region in ruminants in Mazandaran Province. DraI restriction enzyme produced similar profiles for both *T. colubriformis* and *T. axei*, whereas it generated different patterns for *T. vitrinus*. Based on the digestion profile of HinfI on the ITS II region, *T. colubriformis* was differentiated from the other two species by its unique restriction patterns.

The results of the morphometric and molecular methods were consistent, confirming *T. colubriformis* as the most frequent species in the sheep and goats' small intestines, while *T. vitrinus* and *T. axei* were found to be less common in the region under investigation. In Iran, the prevalence rates of *Trichostrongylus orientalis*, *T. colubriformis*, *T. vitrinus*, *T. axei*, *Trichostrongylus capricola*, *Trichostrongylus probolurus*, *T. leroxi*, *Trichostrongylus longispicularis*, and *Trichostrongylus skrjabini* have been reported in different animal hosts, such as sheep, goats, cattle, camels, and buffaloes, based on the morphometric and molecular methods [16, 18, 19]. According to the literature, *T. colubriformis* has also been confirmed as the definitive species in humans in Mazandaran Province by molecular evidence. Moreover, in the current study, the ITS II sequences obtained from the sheep, goats, and human [20] samples showed 100% homology, and they were identical. Therefore, *T. colubriformis* was considered to be the main zoonotic species in the area under investigation, in which the climatic conditions facilitated the establishment of the life cycle of soil-transmitted helminths.

The cohabitation of humans with farm animals in Mazandaran Province establishes the ground for animal-to-animal, animal-to-human, human-to-human, and human-to-animal transmissions. Moreover, ruminants, such as sheep and goats, can be the main sources of the contamination of the vegetables and water in this region [16, 21]. *Trichostrongylus* genus, such as *T. colubriformis* and *T. orientalis,* were reported to be common species in ruminants in Mazandaran Province in the 1970s [21]. From 1975 onwards, there have been no data documenting the identification of *Trichostrongylus* species among small ruminants in Mazandaran Province. Generally, our results identified *T. colubriformis* as the most common species among the small intestine of sheep and goats in this region of Iran. One of the main reasons for the higher occurrence of this species can be the high zoo-

notic potential and prevalence of this parasite in small ruminants and humans.

Conclusion

Considering the current growing trend of using animal residues as fertilizer in organic farms and the high prevalence of *Trichostrongylus* parasites, human infections may increase in the future. The present study was the 1st attempt in the last 30 years, targeted toward the morphometric and molecular identification of *Trichostrongylus* species among small ruminants in Mazandaran Province. The findings of this study could be helpful for the in-depth conceptualization of the epidemiological and ecological studies, implementation of effective control programs, achievement of better disease surveillance, and management of gastrointestinal parasites in Mazandaran Province.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Research and Technology, Islamic Azad University, Science and Research Branch, Tehran, Iran (IR.IAU.SRB.REC.4/P/33).

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Authors contribution's

Conceptualization: Naser Hoghooghi-Rad, Shirzad Gholami, and Ali Bakooie Katrimi; Methodology: Ali Bakooie Katrimi, Naser Hoghooghi-Rad, Shirzad Gholami, Azadeh Mizani, Afsaneh Amouei, Shahrokh Ranjbar-Bahadori, and Ali Eslami; Investigation: Ali Bakooie Katrimi, Azadeh Mizani, Afsaneh Amouei, Mehdi Mehralinezhad Shiadeh, Bahram Laktarashi, Saeid Salehi, Tooran Nayeri Chegini, Zahra Hosseininejad, Amir Hossein Pourmand, Mehdi Sharif, Ahmad Daryani and Shahabeddin Sarvi; Writing--original draft: Ali Bakooie Katrimi and Azadeh Mizani; Writing--review & editing: Shirzad Gholami, Naser Hoghooghi-Rad, Afsaneh Amouei, Shahrokh Ranjbar-Bahadori, Ali Eslami, Mehdi Mehralinezhad Shiadeh, Bahram Laktarashi, Saeid Salehi, Tooran Nayeri Chegini, Zahra Hosseininejad, Amir Hossein Pourmand, Mehdi Sharif, Ahmad Daryani and Shahabeddin Sarvi; Funding acquisition: Naser Hoghooghi-Rad and Shirzad Gholami; Supervision: Shirzad Gholami.

Conflict of interest

The authors declare no conflict of interest.

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