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Investigating intestinal parasitic infections with emphasis on molecular identification of *Strongyloides stercoralis* and *Trichostrongylus colubriformis* in north of Iran

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ABSTRACT

Currently, parasitic infections are one of the important health problems in the world, especially in developing countries. This study aims to investigate intestinal parasites with an emphasis on molecular identification through the analysis of mitochondrial *COX1* and *ITS2* gene sequences of *Strongyloides stercoralis* (*S. stercoralis*) and *Trichostrongylus* spp. in north of Iran. Five hundred forty stool samples were collected from medical diagnostic laboratories affiliated with Mazandaran University of Medical Sciences in Sari city, north of Iran. First, all the samples were examined using direct smear, formalin-ether sedimentation, and trichrome staining technique. Suspected samples of *Strongyloides* larvae were cultured in agar plate. Then, DNA was extracted from samples containing *Trichostrongylus* spp. eggs and *Strongyloides* larvae. To amplify DNA, PCR was performed and the samples with a sharp band in electrophoresis were sequenced by Sanger method. Overall, the prevalence of parasitic infections in the study population was 5.4%. The highest and the lowest level of infection was observed with *Trichostrongylus* spp. and *S. stercoralis* at 3% and 0.2%, respectively. No traces of live *Strongyloides* larvae were seen in the culture medium of the agar plate. The six isolates obtained from the amplification of the *ITS2* gene of *Trichostrongylus* spp. were sequenced, all of which were *Trichostrongylus colubriformis*. The sequencing results of *COX1* gene indicated *S. stercoralis*. In the present study, the prevalence of intestinal parasitic infections in north of Iran has relatively decreased that its main reason can be due to the coronavirus epidemic and compliance with health principles. However, the prevalence of *Trichostrongylus* parasite was relatively high that it requires special attention to apply appropriate control and treatment strategies in this field.

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1. Introduction

Parasitic infections are considered one of the most common health and economic problems in the world, especially in developing countries (Mohammadzadeh et al., 2018). Intestinal parasites are transmitted through the fecal-oral route (Kucik et al., 2004). Infections caused by intestinal parasites have usually chronic courses and in misdiagnosis cases may be asymptomatic for a long time and transfer the contamination to a healthy person. Complications of the infections are severe digestive disorders, anemia, growth problems in children, aggression, weight loss, abdominal pain, and physical and mental injuries (Greigert et al., 2018; Keystone et al., 1980; Limoncu et al., 2005; Miller et al., 2003). *Strongyloides stercoralis* (*S. stercoralis*) is a human intestinal nematode with global distribution, especially in tropical and subtropical regions (Repetto et al., 2016). The life cycle of this parasite is complex and has three stages: skin, lung, and digestive. The infection occurs when larvae enter the human skin (Schär et al., 2013). Common symptoms of the disease are indigestion, digestive symptoms, Loeffler's syndrome, respiratory symptoms, peripheral eosinophilia, nausea, vomiting, diarrhea, secondary bacterial infections, and meningitis (Schär et al., 2013). Disseminated strongyloidiasis often occurs in immunocompromised patients (Knopp et al., 2014) due to autoinfection (Buonfrate et al., 2017). In hyper infection syndrome, a fatal disease occurs with extensive tissue invasion, causing a mortality rate of 15 to 87% (Formenti et al., 2019). Currently, strongyloidiasis is the most forgotten tropical disease in the world (Buonfrate et al., 2018) and hyper infection syndrome is considered an emerging infectious disease in some areas. Approximately 613.9 million people around the world are infected with *S. stercoralis* (Buonfrate et al., 2020).

Another nematode that is often found in the digestive system of humans and animals feeding on plants is *Trichostrongylus* spp. (Sharifdini et al., 2017a). It is found 30 species of *Trichostrongylus* in ruminants and 11 species in humans all over the world, of which 9 species have been identified in people living in Iran (Gholami et al., 2015; Pandi et al., 2021). Human infections mainly occur by eating water and vegetables contaminated with third-stage larvae (filariform) or occasionally through skin penetration by the larvae (Gholami et al., 2015; Mizani et al., 2017; Sharifdini et al., 2017a). An infected person with a low parasite load usually does not show any symptoms, but in severe infections occur symptoms such as anorexia, nausea, weakness, anemia, stomach pain, abdominal bloating, severe diarrhea, epigastric involvement, high fever, and convulsion (Ashrafi et al., 2020; Phosuk et al., 2013).

Mazandaran province (north of Iran) has favorable weather conditions and sufficient humidity for the growth and transmission of intestinal parasites (Gholami et al., 2015; Sharifdini et al., 2017b) and due to the increase in the consumption of immunosuppressive drugs, especially corticosteroids, there is a risk of developing dangerous forms of this disease in infected people. Therefore, conducting a study using high-sensitivity methods seems to be a vital necessity for diagnosing strongyloidiasis in asymptomatic people in Mazandaran province. According to the mentioned above and the importance of parasitic infections, especially *S. stercoralis* in this region, this study was conducted to investigate intestinal parasitic infections with emphasis on molecular identification of *S. stercoralis* and *Trichostrongylus colubriformis* (*T. colubriformis*) through the analysis of mitochondrial gene sequences *COX1* and *ITS2* in people referred to diagnostic laboratories in north of Iran (Sari city).

2. Materials and methods

2.1. Study approval and study area

This study was approved by the ethics committee of Mazandaran University of Medical Sciences with the code of ethics IR.MAZUMS.REC.1400.290.

Sari city is the provincial capital of Mazandaran province, located in the north of Iran. The approximate population of Sari is 261,293 people, and the average annual relative humidity and temperature are 85.83% and 17 °C, respectively.

2.2. Sample collection and parasitological methods

Five hundred forty stool samples were collected from diagnostic laboratories affiliated with Mazandaran University of Medical Sciences in Sari city in 2022. The samples were concentrated by the formalin-ether method after being transferred to the parasitology research laboratory. The sediment of all the concentrated samples was examined first with a 10× lens and then with a 40× lens.

2.3. Nutrient agar culture

In the culture method, 3–4 g of fresh stool sample was placed on a nutrient agar plate for 3–4 days at a temperature of 28–30 °C and the plates were examined with a stereomicroscope to identify the larvae. To collect the larvae, the surface of the agar plate was washed with physiological serum and centrifuged at 1000 ×*g* for 2 min. The sediment was fixed in formalin 10% for identification. The positive samples of *Strongyloides* spp. and *Trichostrongylus* spp. were stored in ethanol 70% at 4 °C for molecular examinations.

2.4. DNA extraction

Before DNA extraction, all samples were washed three time with Phosphate-buffered saline (PBS) buffer to remove residual alcohol. To break the cell wall and release DNA, the samples containing *Trichostrongylus* eggs and *Strongyloides* larvae were freeze-thaw and placed in liquid nitrogen and then Bain-Marie at 100 °C (This step was repeated three times and each time for two sets of 3 min).

Finally, DNA extraction was performed using the instructions of the Favorogen kit (stool DNA isolation Mini kit). The amount of DNA optical absorption (OD) was measured using a nanodrop device and the extracted DNAs were stored at −20 °C.

2.5. Molecular studies of Trichostrongylus

The *ITS2* region of *Trichostrongylus* was amplified using forward (NC1: 5-ACGTCTGGTTCAGGTTGTT-3) and reverse primers (NC2: 5-TTAGTTTCTTTTCCTCC).

The final reaction volume was 25 µl, which includes 12.5 µl Master Mix, 5 µl genomic DNA, and 1.5 µl of each of the forward and reverse primers (15 pmol).

The PCR temperature protocol was as follows: initial denaturation (94 °C for 10 min), followed by 35 cycles of denaturation (94 °C for 30 s), annealing (55 °C for 30 s), and extension (72 °C for 30 s) and a final extension (72 °C for 5 min).

2.6. Molecular studies of S. stercoralis

The *COX1* region of *S. stercoralis* was first amplified using the external primers COXF (5'-TGGTTTGGGTACTAGTTG-3′) and COXR (5'-GATGAGCTCAAACTACACA-3′). The PCR reaction mixture included 12.5 µl Master Mix, 5 µl genomic DNA, and 1 µl of each of the forward and reverse primers (10 pmol). The PCR program was executed as follows: initial denaturation (95 °C for 6 min), followed by 35 cycles of denaturation (95 °C for 45 s), annealing (55 °C for 60 s), and extension (72 °C for 62 s) and a final extension (72 °C for 6 min).

The second step of PCR was conducted using internal primers CNF (5′- TTCTAGTGTTGATTTGGC T-3′) and CNR (5′-TTACCAC-CAAAACTAGGATC-3′) to amplify the 261 bp region.

In this step, the amount of 1.4 µl diluted DNA (in the ratio of 1:9), 12.5 µl Master Mix, and 1.5 µl of each of the primers (15 pmol) were added to the reaction mixture. The optimal temperature and time program for nested PCR were as follows: initial denaturation at 95 °C for 2 min, then 32 cycles including denaturation at 94 °C for 30 s, annealing at 60 °C for 60 s, and extension of 72 °C for 35 s and a final extension of 72 °C for 10 min.

After completing the thermocycler temperature protocol, the PCR products of *Trichostrongylus* and *S. stercoralis* were run separately on 1.5% agarose gel and electrophoresis was performed for 40 min at voltage 90. Finally, the bands were visualized and captured using the Gel Doc system.

2.7. Sequencing and phylogenetic analysis

To sequence, PCR products of *Trichostrongylus* and *Strongyloides* were sent to Bioneer Korea company. Multiple and unread sequences were edited by the Clustal W method and Chromas software.

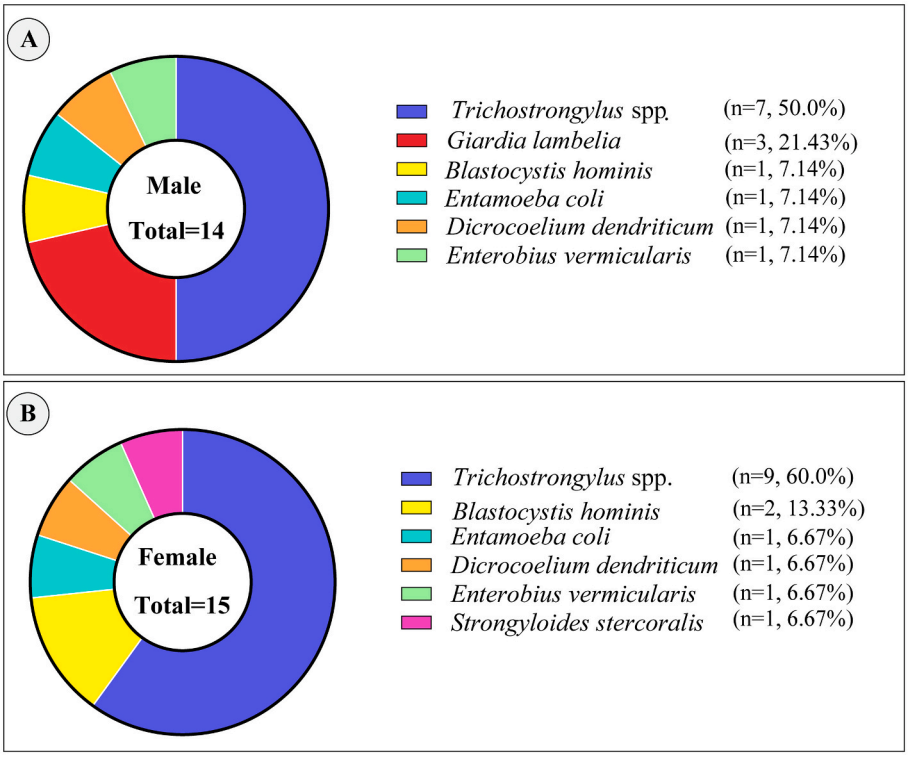

Fig. 1. The prevalence rate of parasitic infection according to gender, A) men B) women.

Analysis of sequencing results was performed using the National Center for Biotechnology Information Programs and Databases (http://www.ncbi.nlm.nih.gov/). Using this database and the nucleotide BLAST, the obtained sequences were compared with the sequences available in GenBank. Finally, the phylogenetic tree was drawn using MEGA-X software based on the maximum likelihood analysis method with Tamura-Nei model using 1000 bootstrap replicates. Interspecies sequences were compared with each other.

3. Results

3.1. Results of parasitological methods

Out of 540 samples, 251 were women (46.5%) and 289 were men (53.5%). The age of the participants was from 1 year to 90 years. The prevalence of parasitic infection in the study population using formalin-ether was 5.4% (29 out of 540). The highest rate of infection was observed with *Trichostrongylus* spp. (3%, 16 out of 540), followed by *Giardia lambelia* (0.6%, 3 out of 540), *Blastocystis hominis* (0.6%, 3 out of 540), *Entamoeba coli* (0.4%, 2 out of 540), *Dicrocoelium dendriticum* (0.4%, 2 out of 540), *Enterobius vermicularis* (0.4%, 2 out of 540), and *Strongyloides* spp. (0.2%, one out of 540). The infection rate in women was 6% and in men 4.8%. The frequency of infection with different parasites by gender is presented separately in Fig. 1.

3.2. Results of nutrient agar culture

Larvae were observed in one fecal sample, and no traces of live *Strongyloides* larvae were seen in its agar plate culture medium.

3.3. Results of molecular investigation

In PCR investigation, six samples of the *Trichostrongylus* DNA were showed a specific band with a molecular weight of approximately 330 bp of *ITS2* gene (Fig. 2).

A stool sample containing larvae of *Strongyloides* was found in the parasitological examinations and the nested-PCR was performed to amplify a fragment 261 bp of *COX1* gene. After the electrophoresis of the PCR product, a specific band with a molecular weight of 261 bp was observed (Fig. 3).

3.4. Sequencing and phylogenetic analysis

Six isolates obtained from the amplification of the *ITS2* gene of *Trichostrongylus*, and one isolate from the amplification of the *COX1* gene of *S. stercoralis* were sequenced. The sequence of these isolates was compared and checked with the valid sequences registered in GenBank. All six isolates related to *Trichostrongylus* had 99.9 similarity with *T. colubriformis*. The sequences of the isolates were registered in GenBank under the accession numbers ON479715.1-20.1. The sequencing results of the only isolate obtained from the amplification of the *COX1* gene indicated *S. stercoralis*. This sequence was deposited in GenBank under the accession number

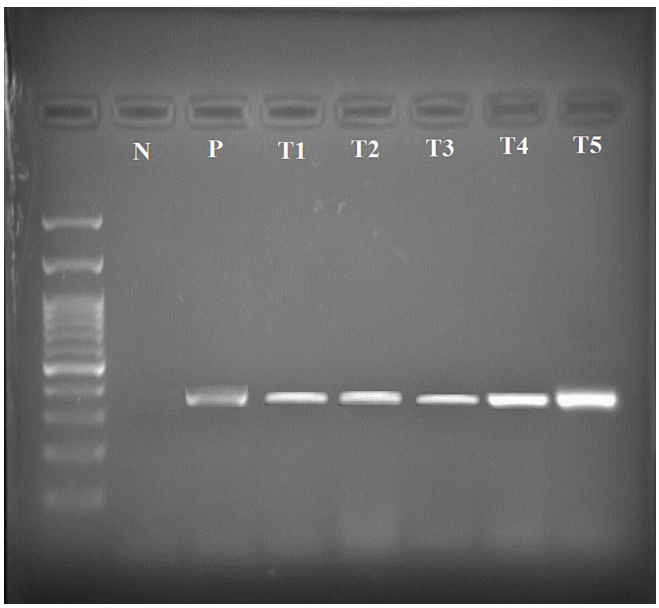

Fig. 2. The electrophoresis results of *ITS2* gene amplification for stool samples containing *Trichostrongylus* eggs, N: negative control, P: positive control, T1–5: five positive samples of *Trichostrongylus* with a fragment size of 330 bp and a DNA ladder of 100 bp.

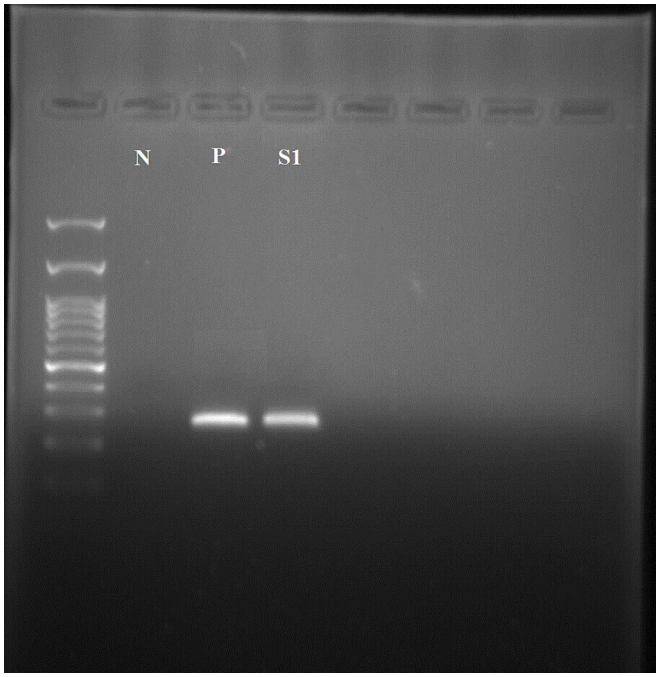

Fig. 3. The electrophoresis results of *COX1* gene amplification for stool sample containing *S. stercoralis* larvae, N: negative control, P: positive control, S1: positive sample of *S. stercoralis* with a fragment size of 261 bp and a DNA ladder of 100 bp.

ON461372.1, which had 100% similarity with the sequences registered in GenBank.

To show the relationship between the common and new haplotypes of the *COX1* gene of *S. stercoralis* and the *ITS2* gene of *T. colubriformis* in stool samples and compare them with the similar and standard haplotypes registered in Gene Bank, the phylogenetic tree was drawn as Maximum-likelihood with the Tamura-Nei model using 1000 bootstrap replicates (Figs. 4 and 5).

4. Discussion

Parasitic infections are considered one of the most common health and economic problems in the world (Mohammadzadeh et al., 2018). Our studies showed that 5.4% people were infected with intestinal parasites, of which 3% and 0.2% were infected with *Trichostrongylus* and *S. stercoralis,* respectively. It is important to mention that the sampling was done at the height of the coronavirus epidemic. In this time period, health protocols such as the use of alcohol disinfectants and constant hand washing with soap and water were followed to prevent the transmission of the virus. One of the drugs used during the corona pandemic was ivermectin, (Kamal et al., 2022) which is one of the common drugs in the treatment of intestinal parasites (Heukelbach et al., 2004). Therefore, this situation can be the reason for the decrease in the frequency of intestinal parasites in this time period. On the other hand, isolation during the epidemic of this virus and as a result less exposure to parasites has been effective in reducing the rate of infection with intestinal parasites.

The number of sampling times also can play a significant role in estimating the prevalence rate of intestinal parasitic infections and it is underestimated with a single stool sampling. Due to the difficulties in increasing the number of sampling times, few studies are able to do it, as we were not able to do it.

One of the endemic areas of *Trichostrongylus* in the world is the northern region of Iran due to the climatic conditions (Mizani et al., 2017). In our study, an almost high prevalence of trichostrongyliasis was observed and out of 16 stool samples that were positive by microscopic examination, six samples were confirmed by PCR. Sequencing results showed that all six samples were *T. colubriformis*. These results were similar to the findings of Gholami et al. (Sharifdini et al., 2017b).

Various species of *Trichostrongylus* have been reported from different regions of Iran, including *T. colubriformis*, *T. vitrinus*, *T. axei*, *T. capricola*, *T. probolurus*, *T. longispicularis*, *T. orientalis*, *T. lerouxi*, *T. skrjabini*, and *T. hamatus* (Ghatee et al., 2020; Hosseinnezhad et al., 2021; Jadidoleslami et al., 2022; Sharifdini et al., 2022).

According to previous studies, one of the most common species of *Trichostrongylus* is *T. colubriformis* (Rayan et al., 2012) and our study confirmed the previous findings.

In recent years, the prevalence of geohelminths, hookworms, and *Ascaris* has decreased due to the improvement of the health-social situation, the increase in personal and social health knowledge, the correct disposal of human and animal feces, and the knowledge about the transmission of the parasites. However, the prevalence of *Strongyloides* and *Trichostrongylus* has increased in some regions, especially in the northern regions (Repetto et al., 2016).

The prevalence of *Trichostrongylus* in different regions of Iran is estimated from 0.4 to 18%. In Gilan province (north of Iran), 42% of

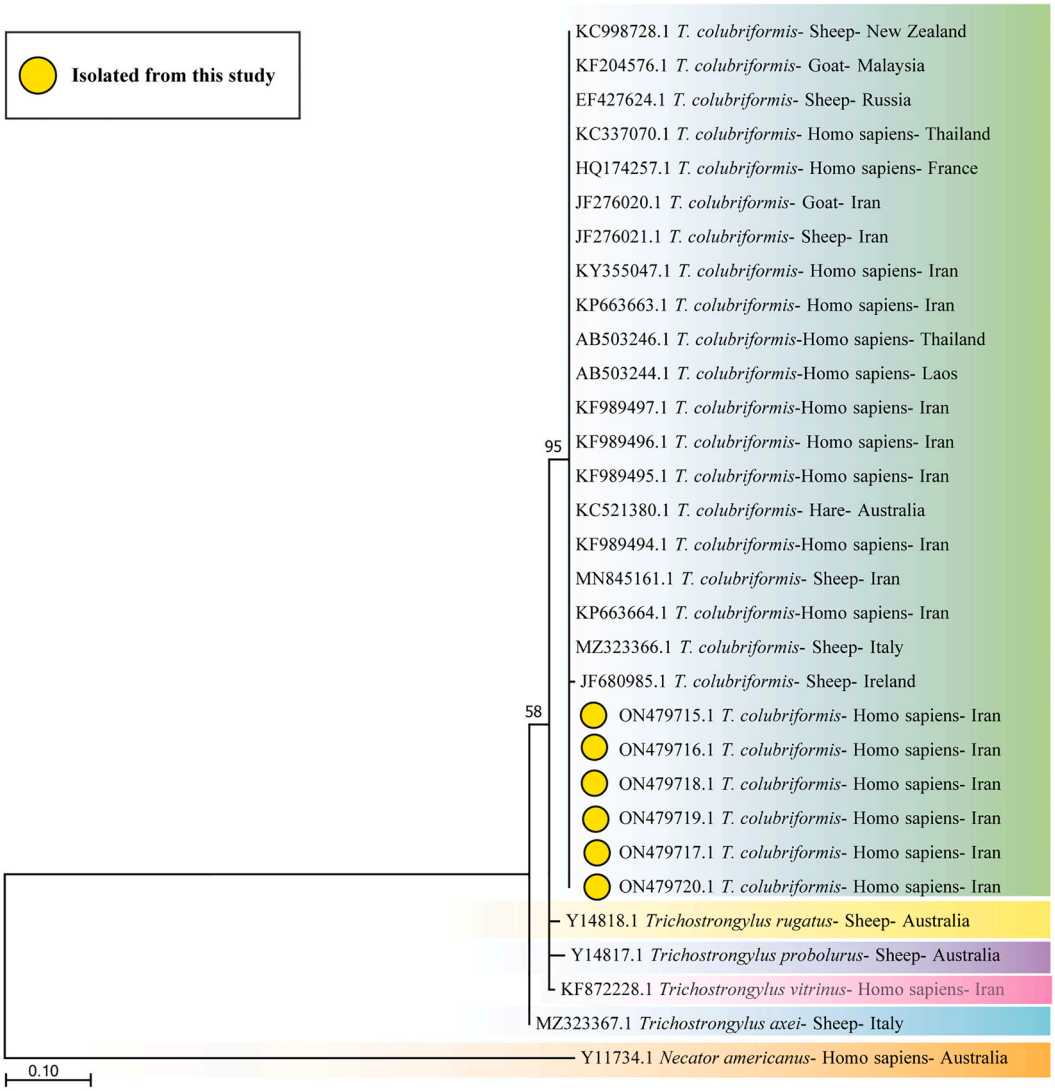

Fig. 4. Maximum likelihood phylogenetic tree was drawn by MEGA-X software to show the position of the studied species of *Trichostrongylus* based on allelic differences. *Necator americanus* is considered as outgroup.

people who had hypereosinophilia were infected with *S. stercoralis* (Ashrafi et al., 2020).

Sharifdini et al., 2018examined 155 stool samples in Khuzestan province (southwest of Iran) using formalin-ether, nutrient agar, and nested PCR, that the prevalence of *Strongyloides* was estimated at 9.7% (Sharifdini et al., 2018). Shokri et al. (2012) examined 133 stool samples in an institution for the mentally retarded in the south of the country using direct smear, formalin-ether, and trichrome and Ziehl-Neelsen staining that the prevalence of *S. stercoralis* was calculated at 17.3% (Shokri et al., 2012). In a study conducted by Ahmadi et al., 2015 on 341 stool samples from the rehabilitation center of Mazandaran province using formalin-ether, agar plate, direct smear, and trichrome staining, the prevalence of *S. stercoralis* was calculated at 2.1% (Ahmadi et al., 2015).

Kia et al. (2007) examined 900 stool samples from rural areas of Mazandaran province by direct smear, formalin-ether, and agar plate. They calculated the prevalence of *S. stercoralis* at 4.9% (Kia et al., 2007).

Gorgani-Firouzjaee et al. (2018) analyzed 120 stool samples by PCR method in Babol city (Mazandaran province). The prevalence rate of *S. stercoralis* in the studied population was 30% (Gorgani-Firouzjaee et al., 2018).

Common diagnosis of strongyloidiasis relies on parasitological methods detecting larvae in fresh stool samples, the most important of which are Bearmann or Rugai and agar plate methods (Inês et al., 2011). The agar plate method has great importance because can detect a high percentage of positive cases of strongyloidiasis in low parasite burden (Arakaki et al., 1990; Koga et al., 1992; Dreyer et al., 1996; Hirata et al., 2007).

The sensitivity and specificity of the PCR technique for identifying *Strongyloides* are around 93.8% and 86.5%, which causes the infection rate to be underestimated. An explanation for the low specificity of PCR is the irregular and low larvae exit from infected

Fig. 5. Maximum likelihood phylogenetic tree depicting the position of the investigated *S. stercoralis* species based on allelic differences. The tree was generated using MEGA-X software, with *Necator americanus* considered as the outgroup.

individuals or the small volume of stool samples (Buonfrate et al., 2018). In individuals with immune system deficiency, load of infection and larvae excretion may be low, in misdiagnosis, the infection may appear in an uncontrollable disseminated form, so a correct diagnostic technique with strong sensitivity is required for identification (Moghaddassani et al., 2011).

It should also be noted that the stool contents may contain bacterial proteases, nucleases, cell debris, and bile acids that can inhibit DNA amplification by the PCR technique. Although PCR is recognized as a confirmatory test, it is not recommended for screening. Therefore, the PCR technique is suggested along with other diagnostic methods to identify intestinal parasites.

5. Conclusion

Overall, our findings indicated that the prevalence of intestinal parasites has decreased to some extent, likely due to the COVID-19 pandemic and the implementation of health protocols such as quarantine measures and the use of anti-parasitic medications. However, the prevalence of the *Trichostrongylus* parasite was relatively high. The use of molecular techniques to identify chronic *Strongyloides* infection and the accurate identification of *Trichostrongylus*, which leads to high risks, can be useful, affordable, and reliable. Health education about the method of transmission, hygienic washing of vegetables, hygienic disposal of feces, and wearing suitable shoes in agricultural fields are mandatory to reduce the spread rate.

Declaration of Competing Interest

The authors declare that they have no competing interests.

Acknowledgments

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