Experimental Parasitology 254 (2023) 108625

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Molecular identification and phylogenetic analysis of gastrointestinal nematodes in different populations of Kazakh sheep

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ARTICLE INFO

Keywords:
Kazakh sheep
Gastrointestinal nematodes
Molecular epidemiology
Phylogenesis
The second internal transcribed spacer of ribosomal DNA

ABSTRACT

Gastrointestinal nematode (GIN) infection in sheep has been recognized globally as a major problem challenging animal health and production. The objective of this study is to use a molecular diagnosis of the prevalence for gastrointestinal nematode (GIN) dominant species of Kazakh sheep and its hybrid (Kazakh × Texel). The internal transcribed spacer 2 (ITS-2) sequences of ribosomal DNA (rDNA) were used as the target sequence. In the study, three dominant species of nematodes, namely *Haemonchus contortus*, *Trichostrongylus* spp., and *Teladorsagia (Ostertagia) circumcincta* from the Kazakh sheep and the F1 and F2 generations of Texel × Kazakh sheep hybrids were subjected to molecular identification and phylogenetic analysis. The fecal and single larva genomic DNA were extracted and amplified by PCR using specific primers to determine the infection rate of the three nematode species. In addition, the PCR products were sequenced and analyzed using bioinformatics methods to construct a phylogenetic tree. The results showed that all the three species had their ITS-2 specific amplified. According to the sequence homology analysis of PCR products, the results showed a high homology (above 98.5% homology) with *H. contortus*, *Trichostrongylus* spp., *T. circumcincta* ITS-2 sequences in GenBank. Phylogenetic analysis showed that the ITS-2 sequences of the three species were on the same branch as the ITS-2 sequences of the same species in NCBI. And on different branches from those of the ITS-2 sequences of different families, genera and species. Sequences carried out on three species from different samples showed a close relationship and little genetic difference in phylogenetic tree. The infection rates based on fecal DNA were 35.59, 25.55, and 11.24% for *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta*, respectively. While the infection rates based on larva DNA, were 24.07, 18.89, and 13.26% for *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta*, respectively. The seasonal prevalence of the three dominant species in spring was significantly higher than that in autumn and winter. And there was no significant difference between Kazakh, F1 and F2 sheep considering the infection rate of the studied three species of nematodes. This study provides valuable molecular approaches for epidemiological surveillance and for assisting in the control of Nematodirus infection in sheep.

1. Introduction

The chronic wasting disease (CWD), caused by the gastrointestinal nematode (GIN) infection has been recognized globally as a major problem challenging animal health, welfare, and productivity of the grazing livestock. Therefore, GIN mediated weight loss and death of the infected animals, together with the costs of deworming drugs have caused tremendous financial losses to farming enterprises and farmers (Martins et al., 2022). The losses could go as high as of 100% in Xinjiang grazing cattle and sheep, of which 30% were severely infected with a mortality rate of 2%. This led to an annual economic loss of approximately 120 million yuan (Xun FQ, 2010), something around U$ 17 million/year. Various epidemiological surveys have shown that the degree of GIN infection rates, intensities, and type in all breeds of sheep are

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https://doi.org/10.1016/j.exppara.2023.108625
Received 9 December 2022; Received in revised form 26 August 2023; Accepted 25 September 2023
Available online 26 September 2023

varied. In particular, the composition of *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta* in GIN were seen generally higher worldwide compared to the other species (Baihaqi et al., 2019; Domke et al., 2013; Zajac and Garza, 2020). Our previous study also demonstrated that these three species were the dominant among GIN in the study area, Zhaosu County, Yili, China (Yan et al., 2021). GIN usually occurs as a mixed infection-community, and the distribution of infectious species and their numbers vary with the season, weather conditions (especially precipitation and temperature), ruminant species, and individual differences (Waller, 2006). There are significant differences in fecundity and pathogenicity among the species, which contributing to differences in infectivity. Accurate diagnosis of GIN infection is the core of epidemiological survey and disease control. Traditional parasitological studies, which are still routinely used, can be time-consuming to perform (Bowman, 2009). For instance, using coproscopic methods, it is not always possible to identify or distinguish individual eggs or larvae of different species of strongylid nematodes with a degree of certainty. Apart the method of larval culture is laborious, takes at least one week duration to perform and even then, can be inaccurate (Dobson et al., 2002). Using this technique, the microscopic identification of L3s up to genus or species level requires experience but may still be unreliable. So, to identify the species of strongylid nematodes accurately, there occurs an urgent need to establish molecular techniques.

The first and second internal transcribed spacers (ITS-1 and ITS-2) of ribosomal DNA (rDNA) have proved to be particularly useful sources of species- and/or genus-specific markers (Roeber et al., 2013). They generally show moderate levels of sequence variability between species but relatively low levels of variability within species (Bisset et al., 2014). A number of studies have utilized ITS-1 and ITS-2 sequence information in PCR-based assays which are designed to detect the presence of particular nematode species or genera in domestic or feral livestock (e.g. Roeber et al., 2011; Sweeny et al., 2012; Höglund et al., 2013). In addition, knowledge on the genetic variation within and between GIN species of different animal populations and regions can help learnings on the transmission of GIN and developing control strategies. Studies in Italy (Cerutti et al., 2010) and Brazil (Brasil et al., 2012) reported that *H. contortus* in domestic and wild animals showed high genetic variation and relatively low host specificity. Genetic surveys of *H. contortus* population were conducted worldwide, involving Australia, Brazil, Malaysia, the USA and some countries of Europe (Troell et al., 2006; Hunt et al., 2008). However, those studies neither provide accurate identification of individual larvae of strongylid species which frequently infect sheep in mixed sheep grazing population, nor reveal phylogenetic relationships between or within species of GIN. The present study is based on the previous epidemiological survey results in Zhaosu County, Xinjiang (Yan et al., 2021). In addition, combined with the feeding practices on pasture, we collected fecal samples and hatched larvae as test materials and used the ITS-2 sequences of rDNA as genetic markers. Later on, molecular identification and phylogenetic analysis of *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta* were also conducted on Kazakh sheep and the F1 and F2 generations of Kazakh × Texel sheep hybrids. Therefore, the objectives of this study were to adopt the specific molecular-diagnostic method of GIN and to apply that in fecal examination. So as to make the diagnosis of GIN infection more accurate, overcoming the limitations of the traditional fecal examination techniques. And thus, provides more accurate diagnostic data for the prevention and treatment of GIN infection.

2. Material and methods

2.1. Ethics statement

All animals utilized in this research were prospectively approved and granted a formal waiver of ethics approval by the Animal Welfare Committee of Shihezi University (Xinjiang, China) with the ethical code: A2022-160-01. All animal procedures were carried out in accordance with The Care and Use Guidelines of Experimental Animals established by the Shihezi University, China.

2.2. Sample collection

Rectal fecal samples were collected from the Kazakh ewe sheep (adult, 4–5 years old, Fig. 1), the F1 ewe sheep (F1 generation of Kazakh ewe × Texel ram, 3–4 years old), and the F2 ewe sheep (F2 generation of F1 ewe × Texel ram, 2 years old) in April (spring), July (summer), September (autumn), and December (winter) of 2020 in Ili Kazakh Autonomous Prefecture. The total number of samples collected for the present study were 916 (Table 1). Each fecal sample weighed 20–30 g, which was put in a clean zip bag after collection. The sheep data such as breed, gender, age, unique chip number were recorded. After collection, the fecal samples were transported to the laboratory and stored in the refrigerator at 4 °C. During spring and autumn, deworming was carried out within 1–2 days after the sampling was completed. For spring sample, intramuscular injection of ivermectin (0.04 ml/kg) (from Shaanxi Yangtze River Animal Pharmaceutical Co. LTD) and closantel sodium (0.1–0.2 ml/kg) (from Harbin Yinshan Technology Animal Husbandry Co. LTD) was administered in April. But for autumn sample, intramuscular injection of ivermectin (0.04 ml/kg) and oral albendazole (0.1–0.15 ml/kg) (from Hanzhong New Tianyuan Animal Pharmaceutical Co. LTD) were administered in September. The interval between sampling and deworming was three months.

2.3. Reagents and instruments

The modified McMaster egg counting chamber was obtained from the Shanghai Institute of Veterinary Medicine, Chinese Academy of Agricultural Sciences. The fecal DNA kit (spin-column type) and 2 × Taq PCR Master Mix were purchased from Tiangen Biotech (Beijing) Co., Ltd. The 10 × Taq PCR buffer with KCl was purchased from Thermo Fisher Scientific, etc. All other instruments used for the present investigation were as follows: optical microscope (Motic, model: SK200); gradient thermal cycler (Eppendorf, Germany, model: Mastercycler pro); microcentrifuge (Eppendorf, German, model: Eppendorf 5424); nucleic acid and protein quantification instrument (Eppendorf, Germany, model: BioSpectrometer basic); multifunctional gel imaging system (ProteinSimple, the United States of America, model: AlphaImager HP); and UV meter (Beijing Liuyi Biotechnology Co., LTD., model: WD-9403 F).

2.4. Primer synthesis

The specific primers for rDNA ITS-2 sequences of the three species of nematodes reported by Bott et al. (2009) were synthesized by Sangon Biotech (Shanghai) Co. Ltd. (Table 2).

2.5. Experimental methods

2.5.1. Conventional fecal analysis and egg counting

To screen a total of 916 collected fecal samples, egg counting was done by floatation method (Yan et al., 2021). Saturated NaCl (specific gravity 1.2 kg/m^3) was used as the flotation solution to check the infecting GIN species in the fecal samples. The eggs were trapped on to a cover slip which was then subjected to microscopic examination. The digital microscope and microscopic image acquisition and analysis system were used to observe the morphology, structure, color, and size of the eggs. Subsequently, the images of eggs were captured and saved. Identification of eggs was confirmed only at the genus level in accordance with the bibliographic map. The eggs of the parasites were counted using the modified McMaster method (Maff, 1986). The eggs per gram of feces (EPG) in these two counting chambers were counted under a microscope and the average of the two counting chambers values were multiplied by 200 to obtain EPG.

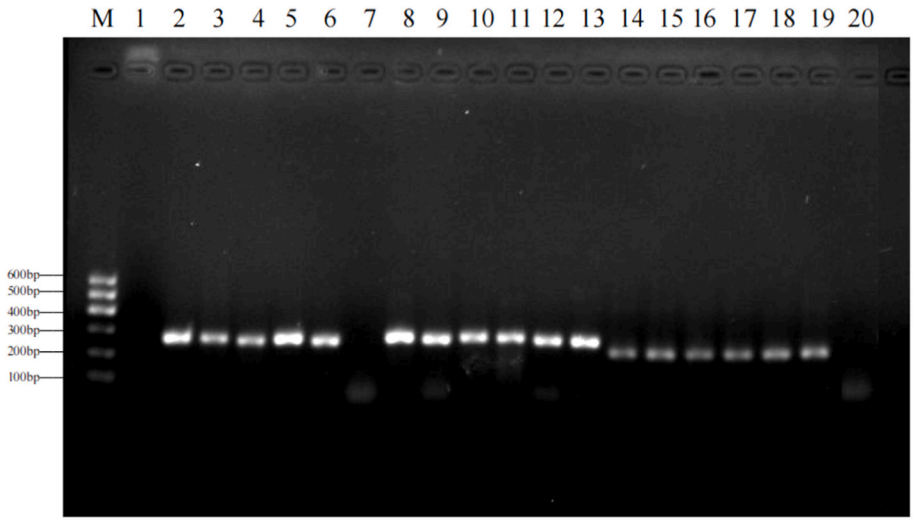

Fig. 1. PCR amplification products of *H. contortus, Trichostrongylus* spp., *T. circumcincta* ITS-2 sequences.
Note: M: 100 bp DNA Mark; 1: Negative of *H. contortus* primer; 2-4: Larval DNA, PCR product of *H. contortus* primer; 5-6: Fecal DNA, PCR product of *H. contortus* primer; 7: Negative of *Trichostrongylus* spp. primer; 8-10: Larval DNA, PCR product of *Trichostrongylus* spp. primer; 11-13: Fecal DNA, PCR product of *Trichostrongylus* spp. primer; 14-16: Larval DNA, PCR product of *T. circumcincta* primer; 17-19: Fecal DNA, PCR product of *T. circumcincta* primer, 20: Negative of *T. circumcincta* primer.

Table 1
Sample collection in the four seasons (number of samples).

Population	Spring	Summer	Autumn	Winter	Seasonal total
Kazakh	137	100	126	102	465
F1	122	50	80	79	331
F2	44	5	41	30	120
Grand total	303	155	247	211	916

Table 2
rDNA ITS-2 specific primers of three parasites species.

Species	Primer sequence	Amplification length (bp)
H. contortus	Forward: CAAATGGCATTTGTCTTTTAG Reverse: TTAGTTTCTTTTCCTCCGCT	265
T. circumcincta	Forward: TATGCAACATGACGTACGACGG Reverse: TTAGTTTCTTTTCCTCCGCT	218
Trichostrongylus spp.	Forward: TATGCAACATGACGTACGACGG Reverse: TTAGTTTCTTTTCCTCCGCT	267–268

2.5.2. Fecal DNA extraction

DNA extraction of 916 fecal samples was performed according to manufacturer's instruction. An amount of 2 μL of the extracted DNA was used to measure the concentration with a BioSpectrometer.

2.5.3. Incubation and isolation of L3 larva

Incubation of 20 fecal samples to collect and isolate the larvae were done via random selection from different Kazakh sheep population in each season. The total number of samples was 140. From each fecal sample, 3 g was put in a mortar, and Coir soil was added to that and then ground to powder. The powder was put into a 50 ml plastic cup with holes. The above steps were practiced to make the fecal sample fluffy and preventing mildew. At this stage, the samples were placed in an electric thermostatic incubator for continuous cultivation at 25 °C for 7 days, during which the appropriate humidity was maintained.

After hatching, L3 larvae were isolated using Baermann's Technique (Zajac and Conboy, 2012). In the process, a 15 cm latex tube was fitted at the end of a sterile glass funnel carefully placed on a funnel-stand. The lower end of the latex tube, at a distance of 5 cm, did hold a stopcock. Then the cultured feces were fixed with gauze and neutral filter paper and was placed in the funnel. At this stage, 40 °C warm water was added to the funnel and the feces were submerged. After standing for 5 h, the stopcock was opened to drain the liquid into a beaker. The collected liquid was filtered with qualitative filter paper. At this stage, the larvae remaining attached to the qualitative filter paper was rinsed with 9% sodium chloride spraying. The filtrate-wash was collected and put into a 2 ml centrifuge tube for picking out the larvae under the microscope. After the incubation of each fecal sample, at least 20 L3 individuals were randomly selected for PCR. Samples containing <20 L3 individuals were also selected.

2.5.4. Genomic DNA extraction of single L3 larva

The collected larvae were poured into a plate (5 cm dia) and placed on the loading platform of the microscope. Under the mirror, a single larva was absorbed with a sterile spear. The single larva was rinsed and transferred to 8 μL ddH_2O, 1 μL 10 × Taq Buffer with KCl, and 15 mM $MgCl_2$, followed by flash freezing in liquid nitrogen for 1 min, and then heated at 85 °C for 2 min. The sample was then supplemented with 1 μL proteinase K (20 mg/μL), incubated at 56 °C for 15 min, 95 °C for 10 min, and stored at −20 °C.

2.5.5. PCR identification and analysis

Using the DNA of fecal samples and the DNA of single L3 larvae as templates, ITS-2 sequences of the three species (*H. contortus*, *Trichostrongylus* spp., and *T. circumcincta*) were amplified. The PCR reaction had a total volume of 20.0 μL, including Reaction-mix (Taq 2.5 U/μL, dNTP Mixture 2.5 mM each) 10.0 μL, fecal/larva DNA 2.0 μL, ddH_2O 7.0 μL, forward primer (10 μM) 0.5 μL, and reverse primer (10 μM) 0.5 μL. The PCR reaction program was as follows: 94 °C for 5 min, a total of 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and then 72 °C for 7 min. Samples without genomic DNA were included as negative controls for each PCR (Bott et al., 2009). The PCR products were subjected to electrophoresis on 2% agarose gel at 5 V/cm distance of the electrodes for 30 min, photographed and documented by a gel imaging system. The samples of PCR identification with positive PCR results were sent to Sangon Biotech (Shanghai) Co. Ltd. for sequencing. The sequences, thus obtained were subjected to BLAST homology analysis with the

sequences of these three species as published in Genbank to verify their identity. DNAMAN 8.0 was used to compare and analyze the sequencing results, which were further subjected to similarity analysis with the three species-sequences (MN845169.1, KP663663.1, and JQ989274.1) as published in Genbank.

2.5.6. Construction of the phylogenetic tree

Phylogenetic analyses of ITS-2 sequences were performed using maximum likelihood (ML). The analysis was based using 25 sequences of each of the three species obtained in this study along with relevant sequences deposited in the GenBank. To study the evolutionary relationships with other species, *Nematodirus oiratianus* (AJ239112.1), *Marshallagia marshallli* (AJ400715.1), *Cooperia oncophora* (AJ544389.1), and *Oesophagostomum dentatum* (AJ889571.1) of sequences were out group, sequences were aligned using Mafft. ML tree was constructed using IQ-TREE version 1.6.12, using automated model selection and bootstrapped using 1000 ultrafast bootstraps, *H. contortus* Best-fit model: TPM2u + F + R3, *T. circumcincta* Best-fit model: TIM3e + G4, *Trichostrongylus* spp. Best-fit model: TVMe + R2 chosen according to BIC.

2.5.7. Data calculation and statistical analysis

The infection rate was calculated using the following expressions: Infection rate = (number of infected samples/total number of samples) × 100%. The statistical analysis was conducted using IBM® SPSS® Statistics, version 20.0., with the statistical significance level set at $p < 0.05$. The independent sample *t*-test and one-way analysis of variance were performed to test the differences between seasons, populations, and methods.

3. Results

3.1. PCR amplification of ITS-2 sequences of the dominant species of GIN in sheep

The ITS-2 sequences of *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta* were amplified respectively using the fecal genomic DNA of Kazakh sheep and the F1- and F2-generations of the hybrid sheep and the DNA from the lysate of hatched larva as templates. The PCR products were subjected to 2% agarose gel electrophoresis, showing specific single bands at 200-300 bp (Fig. 1). To test the specificity of the three pairs of primers, the DNA template of a single larva was used to check whether there was crossover among the three pairs of primers, and the results revealed no non-specific amplification for any pair of primers. For example, *H. contortus* primer was used to detect *Trichostrongylus* spp. and *T. circumcincta* positive samples, but no bands were amplified (Fig. 2).

3.2. ITS-2 sequences analysis of the dominant species of GIN in sheep

The resulted sequences of the three species were analyzed and compared online by BLAST. It was found that these sequences were highly (all >98.5%) homologous to the ITS-2 sequences of *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta* in GenBank. Each sequence was edited and clipped with BioEdit 7.0, removing both ends of the sequence. DNAMAN 8.0 was used to conduct intraspecific comparison between the obtained six sequences of each species and the ITS-2 sequences of the three species in GenBank, and the results showed small intraspecific differences. In detail, they shared 99.31% similarity with the ITS-2 sequence of *H. contortus* in GenBank (accession number MN845169.1), as shown in Fig. 3-N; shared 99.12% similarity with the ITS-2 sequence of *Trichostrongylus colubriformis* in GenBank (accession number KP663663.1), as shown in Fig. 3-M; and shared 98.76% similarity with the ITS-2 sequence of *T. circumcincta* in GenBank (accession number JQ989274.1), as shown in Fig. 3-O.

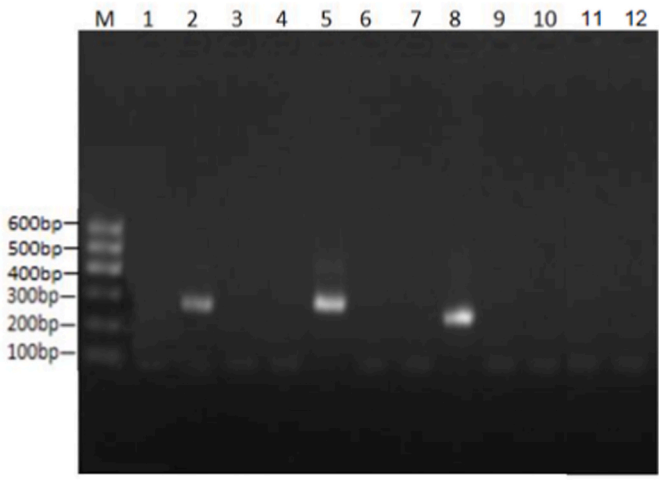

Fig. 2. *H. contortus*, *Trichostrongylus* spp., *T. circumcincta* ITS-2 sequence primer specificity detection.
Note: M: 100 bp DNA Mark; 1: Negative of *H. contortus* primer; 2-4: PCR product of *H. contortus* primer, respectively are larval DNA of *H. contortus*, larval DNA of *Trichostrongylus* spp., larval DNA of *T. circumcincta*. 5-7: PCR product of *Trichostrongylus* spp. primer, respectively are larval DNA of *Trichostrongylus* spp., larval DNA of *H. contortus*, larval DNA of *T. circumcincta*.; 8-10: PCR product of *T. circumcincta* primer, respectively are larval DNA of *H. contortus*, larval DNA of *Trichostrongylus* spp.; 11: Negative of *Trichostrongylus* spp. primer; 12: Negative of *T. circumcincta* primer.

3.3. Cluster analysis of ITS-2 sequences of the three dominant species of GIN

To determine the genetic relationships of *H. contortus*, *Trichostrongylus* spp., *T. circumcincta* from the Kazakh sheep, F1 and F2 generation, a phylogenetic tree of 25 ITS-2 sequences of *H. contortus*, *Trichostrongylus* spp., *T. circumcincta* was constructed separately by the ML method. Furthermore, the phylogenetic analysis showed that the 25 obtained *H. contortus* ITS-2 sequences were closely related to the *H. contortus* ITS-2 sequences from NCBI, sharing the same branch, high homology between them was observed. They were also remotely related to the ITS-2 sequence of other genera of *Trichostrongylidae*, locating in different branches (Fig. 4). The obtained *Trichostrongylus* spp. ITS-2 sequences in this study were closely related to the ITS-2 sequences of *Trichostrongylus* spp. from NCBI, locating in the same branch and closer to *T. colubriformis*. They were also remotely related to the ITS-2 sequences of other genera of *Trichostrongylidae*, on different branches (Fig. 5). In the present study, the obtained *T. circumcincta* ITS-2 sequences were closely related to the ITS-2 sequences of *Teladorsagia circumcincta* and *Ostertagia trifurcata* from NCBI, locating in the same branch. *Ostertagia* and *Teladorsagia* were closely related, belonging to the same genus. They were also remotely related to the ITS-2 sequences of other genera of *Trichostrongylidae*, in different branches (Fig. 6). The results indicated that the adopted three pairs of ITS-2 primers could accurately separate *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta*. In addition, the three intraspecific sequences from different samples showed close relationship with little genetic difference in phylogenetic tree.

3.4. Infection of the three dominant species

3.4.1. Detection of infection with the three dominant species in fecal DNA

A total of 916 fecal samples from the four seasons were subjected to molecular identification. Among them, the number of samples showed infections with *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta* were 326, 234, and 103, respectively with corresponding infection rates of 35.59, 25.55, and 11.24%. The above mentioned three species also

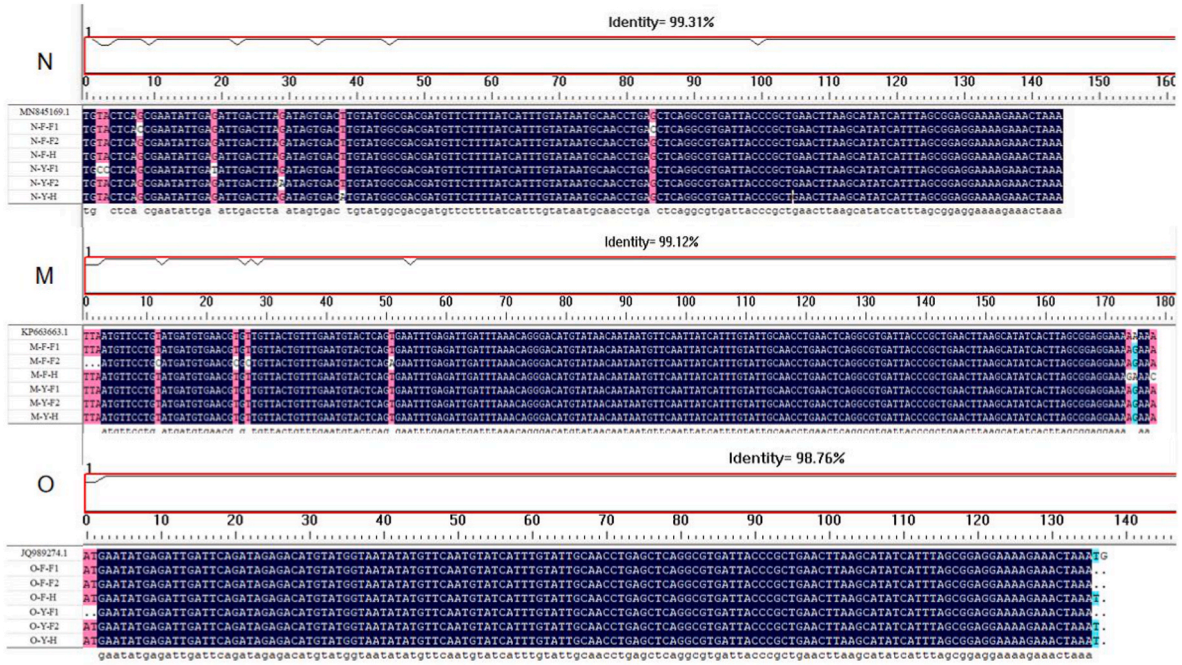

Fig. 3. Homology analysis of ITS-2 sequences.
Note: N: *H. contortus* ITS-2 sequence homology analysis; M: *Trichostrongylus* spp. ITS-2 sequence homology analysis; O: *T. circumcincta* ITS-2 sequence homology analysis. F: ITS-2 sequence obtained from fecal DNA; Y: ITS-2 sequence obtained from larval DNA; h: Kazakh sheep; F1: hybrid F1 sheep; F2: hybrid F2 sheep.

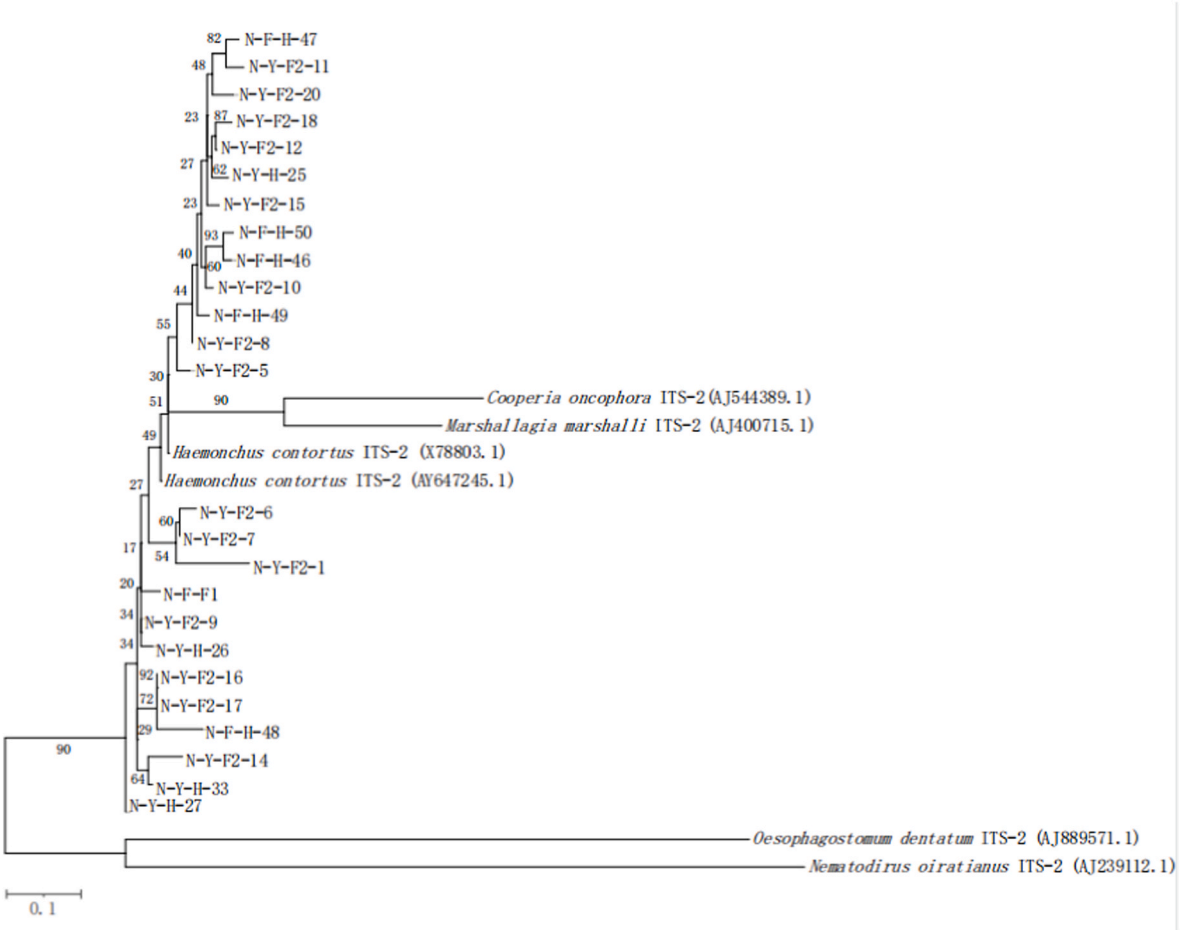

Fig. 4. Maximum likelihood tree of the ITS-2 sequences from *H. contortus* isolated in this study and selected data base entries.

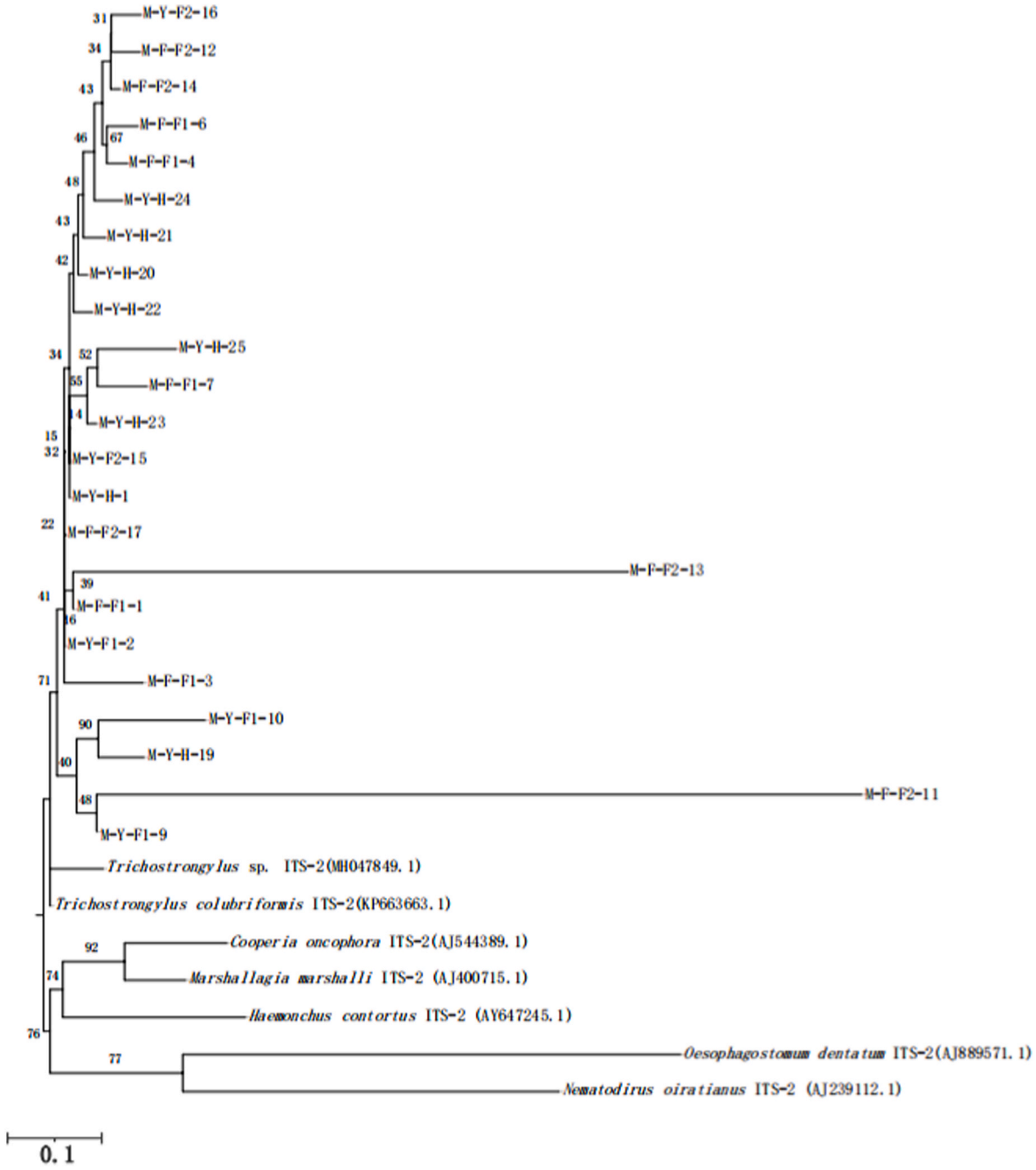

Fig. 5. Maximum likelihood tree of the ITS-2 sequences from *Trichostrongylus* spp. isolated in this study and selected data base entries.

showed mixed pattern of infections, wherein a number of 477 samples showed infection with one or two or three dominant species, with a mixed infection rate of 52.07%. The mixed infection rate of dominant species was significantly higher in spring, closer to each other in summer and autumn, and lower in winter ($P < 0.05$). There is no significant difference between Kazakh, F1 and F2 sheep in mixed infection rate of dominant species ($P < 0.05$) (Table 3).

The mixed infection rate of three species of nematodes was 75.98%, which was higher than that of fecal molecular identification. Over different seasons, the infection rate was 80.20, 75.48, 77.73, and 68.25% in spring, summer, autumn, and winter, respectively. The infection rate was also high in spring but closer rates were observed in summer and autumn. The lowest of the infection rates was recorded in winter ($P < 0.05$). According to egg counts, there were 696 fecal samples with an EPG at least 100, but the consistency with fecal DNA infection rate was 50.98%.

3.4.2. Detection of infection of the three dominant species in larva-DNA

In this study, a total of 2081 larvae were incubated from 140 faecal samples, and molecular detection of three dominant species was carried out. The result showed that infections with *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta* were 501, 393, and 276, respectively with corresponding infection rates of 24.07, 18.89, and 13.26%. The infection rate in spring was significantly higher than that in autumn and winter, and the difference was not significant in spring and summer ($P < 0.05$). There was no significant difference between Kazakh, F1 and F2 sheep in the infection rate of three species ($P > 0.05$) (Table 4). Similarly, no significant difference in the infection rate between fecal DNA and incubated larvae DNA in 140 fecal samples from different seasons ($P > 0.05$) was also observed (Table 5).

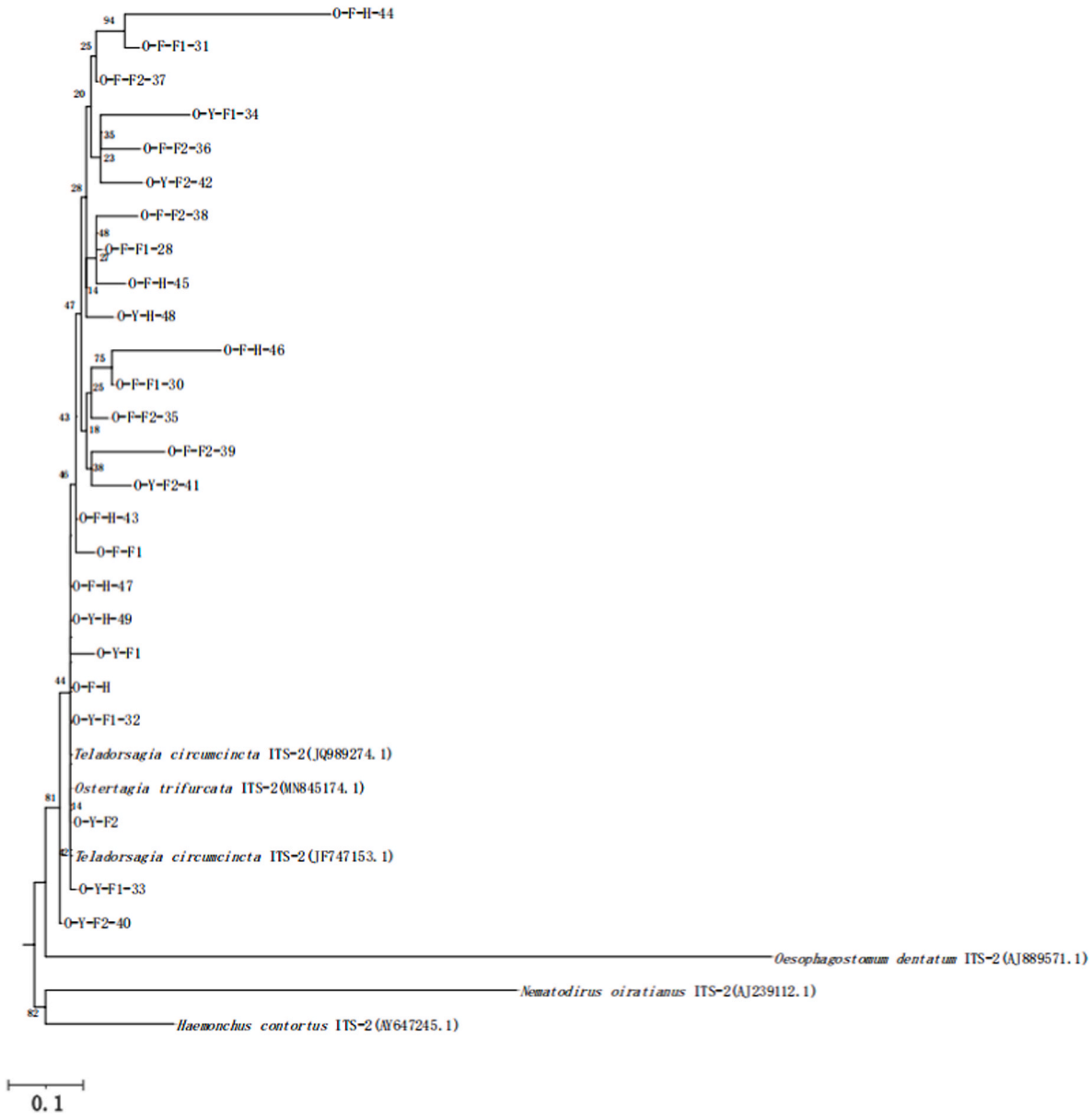

Fig. 6. Maximum likelihood tree of the ITS-2 sequences from *T. circumcincta* isolated in this study and selected data base entries.

Table 3
Mixed infection rate (%) of dominant species in different sheep populations for various seasons.

Population	Spring	Summer	Autumn	Winter	Overall
Kazakh	79.56	53.33	49.21	33.33	55.60^{A}
F1	77.05	34.00	40.00	20.25	48.04^{A}
F2	88.64	26.67	34.15	26.67	50.00^{A}
Overall	79.87^{A}	44.52^{B}	43.72^{B}	10.43^{B}	52.07

Note: Data in the same line, without the same uppercase superscripts (A-D) indicate a highly significant difference ($P < 0.05$), data in the same column, without the same uppercase superscripts (A-D) indicate a highly significant difference ($P < 0.05$).

Among 140 fecal samples collected by traditional method, the mixed infection rate of the three dominant species was 92.14%, and that of larva PCR was 89.29%. In the traditional fecal detection method, 11 samples did not show the infection of 3 dominant species, and 5 samples could be detected by PCR after larvae hatching.

Table 4
Mixed infection rate (%) of dominant species in different sheep populations for various seasons.

Population	Spring	Summer	Autumn	Winter	Overall
Kazakh	67.52	32.35	52.53	16.39	38.15^{A}
F1	63.69	65.29	34.55	46.15	59.92^{A}
F2	54.09	43.48	38.57	40.68	46.58^{A}
Overall	61.77^{Aa}	40.21^{Ba}	33.33^{B}	32.70^{B}	43.68

Note: Data in the same line, without the same uppercase superscripts (A-D) indicate a highly significant difference ($P < 0.05$), the difference was no significant between the data labelled with the same small letters ($P > 0.05$).

4. Discussion

Despite the high prevalence of mixed GIN infections in small ruminants, and the diversity in pathogenicity and anthelmintic susceptibility among infecting species (Borkowski et al., 2020), accurate identification of different species and understanding of the parasitic epidemiology are the basis for developing sustainable parasite control measures. Although

Table 5
Comparison of larval and fecal infection rate (%) in different seasons.

species	Spring		Summer		Autumn		Winter		Overall	
	Larval	Fecal	Larval	Fecal	Larval	Fecal	Larval	Fecal	Larval	Fecal
H. contortus	29.13	75.58	24.04	14.84	20.41	21.05	15.72	10.43	24.07^{A}	35.59^{A}
Trichostrongylus spp.	22.73	32.01	16.17	13.55	16.67	18.18	30.82	23.22	18.89^{B}	25.55^{B}
T. circumcincta	9.30	15.84	15.73	21.29	16.33	4.45	1.89	5.21	13.26^{C}	11.24^{C}

Note: Data in the same line, without the same uppercase superscripts (A-D) indicate a highly significant difference ($P < 0.05$).

fecal examination methods have been traditionally used to diagnose the infections by GIN (Amarante et al., 2014), for example, *Trichuris* and *Nematodirus* which are easily identified according to their shapes and sizes. However, most species of Strongyle nematode eggs are similar in size and shape, and usually cannot be accurately identified at the genus level, such as *Haemonchus*, *Trichostrongylus*, *Teladorsagia*, *Cooperia*, *Bunostomum*, etc. To achieve genus-level identification/differentiation, L3 can be produced from eggs in the feces using the method of larval culture (Schnieder, 2006). This method is time-consuming (taking 1–2 weeks for eggs to hatch and larvae to develop to L3s, depending on conditions) and requires an experienced microscopist to identify and distinguish L3s (Gasser et al., 2008).

DNA molecular diagnostic technology has excellent specificity and sensitivity, and it is often used for the specific identification of GINs in livestock (Santos et al., 2020; Gasser, 2006; Zarlenga et al., 2001). The most popular molecular markers are cytochrome *c* oxidase subunit I (cox1) of mitochondrial DNA (mtDNA), NADH dehydrogenase subunit 4 (Nad4) and the first and second internal transcribed spacers (ITS1 and ITS2, respectively) of ribosomal DNA (rDNA). Most studies consistently demonstrated that the ITS regions of rDNA could serve as reliable genetic markers for the specific identification of *Strongyloides* nematodes in domestic animals (Gasser, 2006; Zarlenga et al., 2001; Chilton, 2004; Wimmer et al., 2004). The results of other studies have shown that the intraspecies sequence variation of ITS-1 and ITS-2 (usually <1.5%) was much smaller than interspecies variation (Huby-Chilton et al., 2006). In this study, the sequence similarity analysis of the three nematode species revealed intraspecific differences between 0.88 and 1.24%. By amplification and sequence analysis of the ITS-2 regions of parasite eggs or larva DNA (Santos et al., 2020) identified 12 species of seven genera, including *Chabertia*, *Cooperia*, *Haemonchus*, *Oesophagostomum*, *Ostertagia*, *Teladorsagia*, and *Trichostrongylus*. Elmahalawy et al. (2018) used Droplet Digital™ PCR with designed ITS-2 primer/probe combinations for *Haemonchus*, *Trichostrongylus,* and *Teladorsagia* and achieved effective results, distinguishing the most important sheep GINs in Sweden. In the present study, three pairs of ITS-2 primers targeting *H. contortus, Trichostrongylus* spp., and *T. circumcincta* were selected to amplify the fecal DNA and single larva DNA of naturally infected Kazakh sheep. As a result, single clear bands were obtained. In addition, no PCR amplification was detected using single larva DNA templates that belonged to different nematode species from the primers. It also provided evidence for the specificity of PCR. Through phylogenetic tree analysis, sequences of the same genus were clustered in one branch, while sequences of different genera were present in different branches, showing clear interspecific grouping. The ITS-2 sequences from different samples that located in the same branch of the same species cannot be effectively distinguished, therefore such intraspecies conserved genes with large interspecies differences could be used as ideal molecular markers for the taxonomic identification and evolutionary genetic research of various GIN species. In this study, *Bunostomum trigoncephalum*, *Oesophagostomum*, *Nematodirus*, and *Marshallagia* were all attempted to amplify respectively, but not all species could be detected. Presumably, it is because of the difference in infection levels of nematode species from different samples.

Heavy parasitic infections can result in mortality; however, the reduction in herd productivity is the main consequence of verminosis (Amarante et al., 2014). In addition, indiscriminate and misguided use of anthelmintics is frequent, uneconomical and can lead to drug resistance (Vieira et al., 2014). Therefore, the ecological and integrated parasite management need a development to minimize the parasite population below the disease threshold level. For this, it is necessary to study all risk factors associated with parasite prevalence in a defined geographic region (Sorathiya et al., 2017). The free-living stages of sheep GINs are strongly affected by climate. Thus, extreme heat and cold are detrimental to development and survival, while within tolerable limits, increasing temperatures generally accelerate development but increase mortality. Moisture is needed for development and translation of larvae from feces to pasture where rainfall acts as a limiting factor for transmission, and these factors underpin seasonal patterns of infection in sheep (Morgan and Van Dijk, 2012). The present study revealed that the infection rate of three species in spring was significantly higher than that in autumn and winter of fecal DNA and hatching larval DNA. The results indicated also that the seasonal temperature and humidity had significant effects on the epidemiology of infection rate of the three species in Zhaosu and Nilka counties, which was consistent with other areas. Just like the occurrence of most prevalent *Haemonchus* spp., EPG data indicates that the peak of egg production was in the spring on Santana do Livramento, West Frontier of Rio Grande so Sul (Osório et al., 2021). In a semiarid region of northeastern Brazil, there is also a seasonal pattern of GIN infection prevalence in sheep. The rainfall variation between the months showed a significant relationship with the number of nematodes recovered at autopsy. The species *H. contortus* occurred throughout the rainy season. It was the most prevalent and abundant species found in the tracer lambs and varied in intensity among the animals (de Souza Mde et al., 2013). A large number of studies have shown that the resistance to parasites varies among sheep populations, including Merino (Woolaston and Windon, 2001), Romney (Morris et al., 2000), Scottish Blackface (Riggio et al., 2013), and Soay (Smith et al., 1999), providing a possibility for breeding varieties that are resistant. This study has revealed no significant difference between Kazakh, and F1 and F2 sheep hybrids in infection rate of three species, which might be closely related to the common environment in which they lived. However, it does not mean that there is no difference in the intensity of infection among different groups.

In the presently studied method, no significant difference in the infection rate between fecal DNA and incubated larvae DNA was observed. So, two different DNA templates were used for specific amplification of three species, and the results showed the reliability of the primers. The mixed infection rate was 92.14% (129/140) by conventional fecal test, which was higher than 89.29% (125/140) by larva molecular identification. Because of the similar size and shape of the eggs of Trichostrongylidae, different species of nematodes may not be accurately distinguished when examining the eggs following the saturated saline solution flotation method. Different genera of nematodes may be mixed up too. For example, the egg size of *H. contortus* and *Oesophagostomum* spp. are 70-81 × 39-55, and 70-90 × 34–45 μm, respectively and both of them are oval-shaped (Kong, 2016; Zajac and Conboy, 2012). Hence, the saturated saline solution flotation method may mistake the eggs of other species of nematodes as one of the three dominant species, thereby increasing the triple infection rate. In contrast, the primers for molecular identification were species-specific. The sequences of the resulted PCR products only identified three nematode species and will not be mixed up with other species. In addition,

11 samples of the traditional fecal detection method did not detect the infection of 3 dominant species, but 5 samples of the larvae were incubated and the infection could be detected by PCR, which further provided an important basis for the diagnosis. Although, the three species could be detected specifically, there was no quantitative detection of the species. Harmon et al. (2007) developed a quantitative, real-time PCR, based on ITS-2, for the quantification of *H. contortus* eggs. And more importantly, nemabiome metabarcoding was a developed "microbiome-style" approach which involves short-read next generation sequencing of ITS-2 rDNA amplicons for nematode species identification and relative quantitation (Avramenko et al., 2015). It was initially developed for the relative quantitation of cattle GIN species from larval L3 coproculture, but has more recently been validated for use on eggs and L1 larvae isolated from sheep fecal samples (Redman et al., 2019). Application of ITS-2 rDNA nemabiome metabarcoding to fecal samples from ewes from over 90 flocks across western Canada revealed high GIN infection intensities in many flocks with *H. contortus* being the most abundant species (Queiroz et al.,). While simplicity of this study can be utilized "on the field" by the farmer, this method might need to be optimized for each specific worm species.

5. Conclusion

In this study, the ITS-2 sequences of rDNA were used to perform molecular identification on fecal samples and hatched larvae of Kazakh sheep and the F1- and F2-generations of Kazakh × Texel sheep hybrids. Infections with three dominant species, *H. contortus*, *Trichostrongylus* spp., and *T. circumcincta* were successfully identified. At the same time, the seasonal prevalence of the three dominant species in spring was found significantly higher than that in autumn and winter. And there was no significant difference between Kazakh, F1 and F2 sheep in the infection rate of three species. Therefore, molecular detection can further supplement the traditional fecal detection methods, improve the accuracy of species detection, and lay a foundation for understanding the molecular epidemiology of GIN.

Authors' contributions

Conceived and designed the experiments: Xiaofei Yan, Mingjun Liu, Sangang He.

Performed the experiments: Xiaofei Yan, Yiyong Liu, Haifeng Deng, Bing Han.

Analyzed the data: Xiaofei Yan, Ning Zhang, Yuqi Wang.

Contributed reagents/materials/analysis tools: Mingjun Liu, Sangang He, Yiyong Liu, Haifeng Deng.

Wrote the paper: Xiaofei Yan, Mingjun Liu.

Ethics approval

All animal procedures were approved by the Animal Welfare Committee of Shihezi University (Xinjiang, China) with the ethical code: A2022-160-01. Experiments were conducted in accordance with animal ethics guidelines and approved protocols.

Consent to participate and consent for publication

All authors read and approved the final manuscript, and consent for publication.

Declaration of competing interest

The authors declare no competing financial interest.

Data availability

The data that has been used is confidential.

Acknowledgments

This work was supported by the Scientific Research Project of University of Xinjiang Autonomous Region (No. XJED2020Y049); the National innovation and entrepreneurship Training Program for college students of China (No.2020KJ0018), the KeyLab funding of Xinjiang Ugrus Autonomous region: identification of genes with resistance to sheep gastrointestinal nemotode infection by genome-wide association study, the Tianshan Innvation Team (No. 2018D14004), and the Regional Collaborative Innovation Project of Xinjiang Ugrus Autonomous Region (Shanghai Cooperation Organization Science and Technology Partnership and INTERNATIONAL COOPERATION Program). Cooperative Research and Application of Genomic Selection Technology for sheep Meat Traits (No. 2020E01004).

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