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Evaluation of larval culture and conventional PCR methods for the detection of *Strongylus vulgaris* in equines of Iran

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Abstract: *Strongylus vulgaris* (*S. vulgaris*) is known as the equine's most pathogenic nematode due to the extraintestinal migration in mesenteric arterial vessels. Since a few kinds of *S. vulgaris* can threat the animals' health and working efficiency, high precision and early detection of strongylid nematodes is essential. Traditionally, the larval coproculture method was used frequently for identification of strongylid species. The technique of larval culture is costly, time consuming, and does not have sufficient accuracy and reliability. Here, we critically evaluated the polymerase chain reaction (PCR) with larval culture methods for the detection of *S. vulgaris* eggs in fecal samples. To this aim, fresh fecal samples were obtained from 42 horses and 71 donkeys in different provinces of Iran. All samples containing strongyle form eggs were individually cultured and third–stage (L_3) *S. vulgaris* larvae were harvested from Baermann apparatus. The collected strongyle form eggs were individually kept in ethanol for molecular study. The L_3 strongyle larvae were identified by morphological characteristics. The genomic DNA of strongyle eggs extracted and the second internal transcribed spacer (ITS-2) region was amplified using PCR technique. Then, the phylogenetic tree was drawn based on sequenced products. The results showed that the shedding eggs of strongylid nematodes were observed in 85%, 27.58%, and 50% of the donkeys in Chaharmahal and Bakhtiari, Isfahan, and Khuzestan provinces and 12% of the horses in the Khuzestan province. Also, *S. vulgaris* was identified in 10 (8.85%) and 11 (9.73%) of the donkeys by larval culture and PCR methods, respectively. Phylogenetic analysis showed the Iranian *S. vulgaris* isolates found in clusters in other countries, and isolates of this parasite have recorded. Molecular findings of the PCR method are closer to the realistic values of *S. vulgaris* infection and detect a higher rate of positive samples. So, it can be applied for large scale screening programs of equine population.

Key words: PCR, *Strongylus vulgaris*, larval culture, equine

1. Introduction

Working equines, a source of income in the fields of transportation and agriculture, play an important role in rural life in many regions around the world. Lack of attention to the health of these animals has led to a high prevalence of gastrointestinal parasitic diseases, which are often neglected. More than 50 species of strongylid nematodes belonging mainly to the Strongylidae (large *Strongyles*) and the Cyathostominae (small *Strongyles*) subfamilies have infected horses and other equines [1–4]. Today, strongylosis is the most common parasitic disease in equines.

Prophylactic treatment of the last 50 years with the frequent administration of antiparasitic medicines caused anthelmintic resistance and parasite survival [5–9]. A few numbers of *S. vulgaris* can threat the animals' health and working efficiency despite of its serious economic losses on ranchers [10,11]. Infection with large strongyles can lead to serious clinical complications in horses and other equines. *S. vulgaris* is considered as the most pathogenic helminth in horses and other equines [12]. After migrating to the cranial mesenteric artery, L_3 of *S. vulgaris* remain there for several months, molt to fifth–stage larvae, and return to the large intestinal lumen. During this process, they can produce verminous endarteritis, aneurysms, intestinal ischemia, and infarctions [13–15]. They can manifest with symptoms such as colic, hind leg lameness, and lesions in the heart, liver, and kidneys [16, 17]. Therefore, early detection of these limited pathogenic parasites can significantly affect treatment, prevent the dispersion of parasitic eggs in pastures, and contribute to parasite control programs.

The application of selective anthelmintic therapy (SAT) in German horse farms has been successful and effective [9,18]. This procedure has been accomplished by focusing on the control of strongyle egg shedding to reduce anthelminthic resistance and minimize the risk of parasitic infection, especially in foals and yearlings

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[19,20]. Therefore, the accurate identification of strongyle infection is important in antistrongyle spectrum therapy.

Most studies of strongyle species in Iran are limited to the northwestern areas, where the prevalence rate of strongylosis ranged from 10.3% to 96.66% in horses and donkeys of different regions [21–24]. The detection of strongyle species is traditionally based on the microscopic evaluation of collected larvae from coproculture. The larval culture process takes 7 days for cyathostomins and 10–14 days for large strongyles [25]. Moreover, the identification of different L_3 strongyles species using morphological characteristics is not easy and requires highly competent and experienced personnel [26–28]; it is costly as well, and there is a risk of false negative results.

Genomic DNA technology is used as a highly accurate and sensitive tool to identify various helminth species in each developmental stage. It is a more time saving and less laborious technique. Due to the abundance of ribosomal DNA (rDNA) in these organisms and the low genetic diversity in intraspecies of strongyles, molecular techniques were applied to identify the eggs and larval stages of *S. vulgaris*. ITS-2 segment (the second internal transcribed spacer r DNA) is a reliable rDNA region to differentiate closely related species with the same morphological characteristics [25]. All studies in Iran are based on morphological features and no molecular studies have been reported on *S. vulgaris* in this region. This is the first effort to evaluate the genomic sequence of the ITS-2 region to identify the molecular characteristics and phylogenetic position of *S. vulgaris* in equines of southern and central provinces of Iran.

2. Materials and methods:

2.1. Fecal samples

From December 2017 to May 2018, fresh fecal samples of 71 donkeys (26 females and 45 males) and 42 horses (19 females and 23 males) located in Isfahan, Chaharmahal and Bakhtiari and Khuzestan provinces were individually packed airtight in plastic bags and transported to the parasitology laboratory in the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz. Some information about the equines, including age, gender, and housing condition (the type of animal feed) were recorded for each sample. The animals were 1 month to 45 years of age, and most of the animals free grazed in pastures, except for 22 horses raised in farms.

Fecal egg counts

Strongyle fecal egg count was carried out using modified McMaster (Clayton Lane) [29] with Sheather's solution. Positive strongyle samples were selected for culture and molecular studies. Some strongyle eggs of each sample were isolated through a flotation technique and kept in 70% ethanol for molecular survey.

Larval cultures

To detect *S. vulgaris* larvae, all strongyle eggs isolated from the samples were individually cultured via the following methods: 10–50 g of feces were mixed with equal amounts of vermiculate (1% of bicarbonate solution) and incubated under the appropriate temperature (24–26 °C) for 14 days. The humidity of fecal samples was maintained by daily sprinkling of tap water and ventilating, so that the soft and moist texture of the samples was consistently preserved. After the incubation period is over, the samples were Baermannized and the L_3 strongyle larvae were harvested after 24 h of sedimentation in the Baermann apparatus [30]. The first 10 mL of the sediment were collected from each sample. Then, the centrifuged samples were gradually identified under the microscope at 400× magnification based on the morphological characteristics described by Bowman and Russell [31, 32]. The samples containing *S. vulgaris* larvae were recorded. Some morphological identified L_3 *S. vulgaris* were placed in 70% ethanol as positive control for future use.

Egg isolation and DNA extraction

The tubes containing strongyle eggs were centrifuged with distilled water to deethanol from the samples. The genomic DNA of the isolated eggs was extracted via a method similar to that described by Harmon et al. [33]. To destroy the chitinous layer and leave the contents of the strongyle eggs, a number of metal beads were added in a tube and vortexed for 5 min, and then the metal beads were removed by a magnet. Then, DNA was extracted using the DNA extraction kit (DNP Kit, CinnaGen, Tehran, Iran) according to the manufacturer's instructions. The extracted DNA was eluted in 50 µL solvent buffer and kept at – 20 °C for molecular study.

PCR

The second internal transcribed spacer (ITS-2) was amplified using the primers previously applied by Nielsen et al. [34]. The DNA sequence of the forward primer was 5-GTATACATTAAATAGTGTCCCCATTCTAG-3 and the reverse primer was: 5-GCAAATATCATTAGATTTGATTCTTCCG-3. The 25 µL reaction volume consisted of 7 µL of template DNA, 2 µL (1 pM) of each primer, 12.5 µL Master Mix and 1.5 mM $MgCl_2$ (RED Ampliqon, Ampliqon A/S, Odense, Denmark). Polymerase chain reactions were performed on a thermal cycler (Hangzhou Bioer Technology Co. Ltd., Hangzhou, Zhejiang, China). After an initial activation at 95 °C for 2 min, to activate Taq DNA polymerase enzyme, 40 cycles were run with the following protocol: denaturation step at 95 °C for 20 s, annealing step at 57 °C for 30 s and an extension step at 70 °C for 30 s. Also, negative and positive controls were run for each PCR. After loading the PCR products on 1% agarose gel containing safe stain at 90 V, samples were visualized under UV light via transilluminator (Kiagen Advanced Biotech Co., Tahran, Iran).

DNA sequencing

Since accessing the adult worm was unlikely, a number of morphological identified L_3 larvae *S. vulgaris* amplified by PCR and sent to a valid commercial sequencing service (Microsynth AG, Balgach, Switzerland) for morphologic confirmation. After reading the sequences, the data were analyzed using BioEdit V. 7.0.5.3 software, the basic local alignment search tool (BLAST) program, and NCBI databases (National Center for Biotechnology Information, Bethesda, MD, USA). The alignment sequences were analyzed using Molecular Evolutionary Genetics Analysis (MEGA) software, version 10, using the default parameters for the molecular phylogenetic study of the species.

Phylogenetic analysis

The evolutionary history was inferred by using the maximum likelihood method and Tamura's 3-parameter model [35]. The tree with the highest log likelihood (–819.24) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree(s) for the heuristic search were obtained automatically by applying neighbor joining, and BIONJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach and selecting the topology with superior log likelihood value. A discrete gamma distribution was used to model evolutionary rate differences among sites [5 categories (+ G, parameter = 0.8113)]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 15 nucleotide sequences, and there were 151 positions in the final dataset. Evolutionary analyses were conducted using MEGA X [36].

Statistical analysis

The relationships between *S. vulgaris* infection and age, gender, and housing conditions of animals were evaluated using SPSS version 16 (SPSS Inc., Chicago, IL, USA). The paired proportions (QuickCalcs, GraphPad Sofware Inc., San Diego, CA, USA) and Kappa values of McNemar's test were employed to compare the statistical differences between the frequency rates obtained by PCR and larval culture. A P-value of less than 0.05 was considered significant.

3. Results

Of the 113 equine samples evaluated via Sheather's flotation solutions, 38 cases (33.62%) were infected with strongyle eggs. The highest rate of strongyle infection was observed in Chaharmahal and Bakhtiari (85.71%), Khuzestan (30.23%) and Isfahan (14.28%). In total, 34 donkeys (47.89%) and 4 horses (9.52%) were diagnosed with strongylosis. Also, strongyle egg shedding was observed in 85%, 27.58%, and 50% of the donkeys in Chaharmahal and Bakhtiari, Isfahan, and Khuzestan provinces respectively, and 12% of the horses in the Khuzestan province. These differences between the strongyle infection frequency and studied animals from different provinces were statistically significant ($P < 0.05$).

In addition, the results showed that the infection rate of female animals (35.56%) was slightly higher than male animals (32.35%), although this difference was not significant ($P > 0.05$). The highest rate of infection was observed in animals with 1–5 years of age (45.16%), but the difference between them was not significant ($P > 0.05$). The results showed that *S. vulgaris* was more common in the donkeys from Chaharmahal and Bakhtiari province with free grazing in pastures, male gender, and age 5–10 years (90.9%).

EPG was found to range from 0 to 1155 strongyle eggs. Moreover, of the 38 samples containing strongyle eggs, *S. vulgaris* was identified in 10 animals (8.85%) by larval culture and in 11 animals (9.73%) by PCR. McNemar's test showed no significant relationship between the larval culture and PCR methods ($P < 0.05$). The morphological identification of L_3 *S. vulgaris* was performed based on the length of the larvae and the number of intestinal cells of different strongyle species. The body of L_3 *S. vulgaris* is slender and stretched and has 21–32 dark intestinal cells [31,32] (Figure 1). The PCR results showed the sharp band about 175 bp in size observed in all the samples was identified as *S. vulgaris* (Figure 2).

In the present study, 2 ITS-2 sequences of *S. vulgaris* isolated from donkeys were deposited in the GenBank (Accession no. MT258561.1 and MT258562.1). The interpretation of the sequencing results showed that the 2 obtained sequences had more than 94% similarity with a sequence of *S. vulgaris* in donkeys from Egypt (GenBank accession no. LC500237.1) and isolated from German horses (GenBank accession numbers: KT250609 and KT250611) [37]. Multiple sequence alignments compression of the amplificons of partial ITS-2 rDNA region showed much more polymorphism in MT258561.1 (Sv1) sequence than in other *S. vulgaris* (Figure 3). ITS-2 rDNA phylogeny analysis of 2 individual sequences showed the Iranian *S. vulgaris* isolates have high similarity to the other registered isolates in GenBank from around the world. The Iranian *S. vulgaris* species found in the same clade with other large strongyles (Strongylinae) and as sister clade of subfamily Cyathostominae (small strongyles). The Sv1 isolate (MT258561) was clustered in a separate branch next to the other *S. vulgaris* with high support (bootstrap 100%) whereas Sv2 isolate of *S. vulgaris* of Iranian donkey (MT258562.1) was monophyletic with the Egyptian isolate (GenBank accession no. LC500237.1) and as sister clade of Turkish (GenBank accession no. MF48225.1), Chinese (GenBank accession

Figure 1. The L_3 larvae of *S. vulgaris* cultured from infected equine faeces of different regions of Iran (400x).

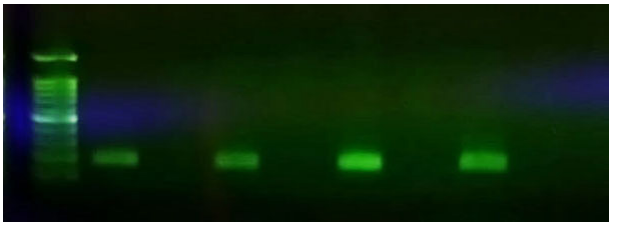

Figure 2. Agarose-gel electrophoresis of the single PCR products amplified ITS-2 region of *S. vulgaris*, Lane S119, S123 and S129: *S. vulgaris,* Lane S48, S24, and S121: Strongylid nematodes, Lane PC and NC: Positive and negative controls, respectively, Lane Marker: Ladder 100 bp.

no. KP693439.1), Australian (GenBank accession no. X77863.1), and German (GenBank accession no. KT250617.1) in equines (Figure 4).

4. Discussion

Strongylosis involves equines all over the world and imposes rancher economic losses and animal health hazards. In this study, we found strongyle infection in 34 (47.89%) and 4 (9.52%) of the studied donkeys and horses, respectively, in different regions of Iran. Of these, *S. vulgaris* was identified in 10 (8.85%) donkeys by larval culture and in 11 (9.73%) by the PCR method. The frequency of strongylosis in donkeys was in line with the results obtained by Hosseini et al. [23] by macroscopically observing adult parasites in the gastrointestinal tract of equines from different regions of Iran. They found that 46.7% of donkeys suffered from strongylosis, and *S. vulgaris* was identified in 33.3% of them [23]. Also, Shabazi et al. [24] reported that 34% of the club and rural horses of Ardabil province were infected with strongyle spp. The findings of Tavassoli et al. study (2010) by larval culture in the Northwest of Iran showed that 72.9% of working horses shed strongyle eggs, 6.5% of which were *S. vulgaris* [21]. Also, in their other study (2016), 26.66% of donkeys were infected with *S. vulgaris* [22]. In Tavassoli's studies [21, 22], the rate of *S. vulgaris* infection in donkeys was significantly higher than in horses, mainly due to insufficient attention to the donkey`s health. Also, the most important cause of this decreased *S. vulgaris* involvement could be the bioclimatic condition of different regions of Iran. Northwest regions of Iran are suitable for the long–term persistence and survival of L_3 strongyle species compared with the central and southwest of Iran. This parasite can be found worldwide. There have been various

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                                10        20        30        40        50        60        70
                                ....|....|....|....|....|....|....|....|....|....|....|....|....|
S._vulgaris-MF489225.1_         CCCCATTCTAGAAAAGAA--TAT-ATTGCAACATGTATATTAT---TCAATAA---------TA--TACA
S._vulgaris-KP693439.1          ..................--...-..................---.......---------..--....
S._vulgaris-KT250617.1          ..................--...-..................---.G.....---------..--....
S._vulgaris-X77863.1            ..................--...-..................---.G.....---------..--....
S._vulgaris-LC500237.1          ..T...............--...-..................---.AT....---------..--....
S._vulgaris-MT258562.1          ..................--...-..................ACA.AT....---------..--....
S._vulgaris-MT258561.1          ..................G-...-..................-CA.AT....---------..--....

                                        110       120       130       140
                                ....|....|....|....|....|....|....|....|....
S._vulgaris-MF489225.1_         ACTTAATTGTTTCGCGACTTATTAACA-ATTTAGTAGAGCCTGT
S._vulgaris-KP693439.1          ............................-.............
S._vulgaris-KT250617.1          ............................-.............
S._vulgaris-X77863.1            ............................-.............
S._vulgaris-LC500237.1          ............AC..............-.............
S._vulgaris-MT258562.1          ............AC..............-.............
S._vulgaris-MT258561.1          ............AC..............-.........C.....
```

Figure 3. Alignment of partial ITS-2 rDNA sequences from *S. vulgaris*. Dots indicate identity with the *S. vulgaris* sequence. Since all isolates belonging to the same species had an identical ITS-2 rDNA sequence, MT258561.1 (Sv1) sequence showed more nucleotide polymorphism in some positions.

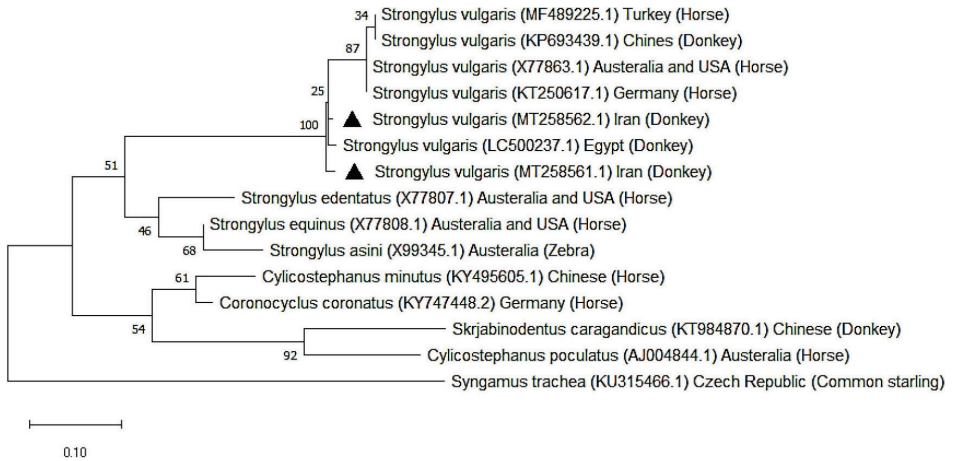

Figure 4. Phylogenetic tree of *S. vulgaris* isolates from Iranian donkeys with other strongylid nematodes based on partial ITS2 sequences and constructed using the Tamura 3-parameter (T92) model with Gamma distribution (+G) in MEGA software version 10. GenBank sequences of *S. vulgaris* included with *Syngamus trachea* as out group. Numerals above the branches indicate bootstrap values (%) from 1000 replicates. The scale bar indicates the proportion of sites changes along each branch.

studies reporting the infection rate of strongyle spp. ranging from 4.92% to 89% in different parts of Oceania [38], Europe [39, 40], and Africa [41, 42]. In Sweden, the prevalence of *S. vulgaris* in horses was reported at 28% by real time PCR [43]. Differences in habitats from where equines originate have led to this variated infection rates. Most previous Iranian reports on strongyle spp. infection were obtained using the larval culture method. According to the present study, the frequency of *S. vulgaris* infection by PCR (9.73%) was higher than the result obtained by the larval culture (8.85%) method. These findings are in concordance with many studies that have compared molecular method with the larval culture. Studies of horse fecal samples in Denmark showed that the *S. vulgaris* infection rate detected by conventional PCR (12.1%) was significantly higher than that obtained by cultural methods (4.5%) [44]. Also, in studies on farm horses of Denmark, the results of the real time PCR were higher and more realistic than larval culture methods 12.21% versus 12.06% [13] and in German horses 1.9% versus 1.1% [37], respectively.

The larval culture of L_3 strongyle is the most common choice used among veterinarians [45] but the losing of positive samples during the larval culture process in Baermann tube prevents the detection of small numbers of this parasite in large volumes of feces. Also, the developmental stages of larvae may be halted due to inappropriate culture conditions or various contaminations. Since large strongyles often comprise less than 5% of the total egg output [46, 47], the PCR is a suitable option for detecting the low rate of this potential pathogen species.

The findings of this study are in accordance with other studies and showed the superiority of the PCR method to the larval culture. Also, it was noted that the PCR method was the most accurate, sensitive and feasible tool to detect *S. vulgaris* helminth by saving time. It can be applied for large-scale screening programs of equine population, whereas other methods such as reverse line blot (RLB) hybridization [48] and PCR–ELISA methods [49] are used to recognize numerous parasites such as Cyathostomin species simultaneously. Phylogenetic analysis did not show a significant difference between positive samples in the present study and other *S. vulgaris* records. The fact that an Sv1 sample was placed in a separate branch from the *S. vulgaris* cluster is probably due to the presence of more nucleotide differences in this sample. There are limited studies in phylogenetic study of *S. vulgaris* to compare this conserved region with other genomic DNA regions. ITS-2 rDNA region is a small part of the conserved regions of parasite genome. Obviously, further studies to find more significant molecular markers to differentiate the *Strongylus* species, are needed. The present study are the first molecular investigations performed on equines strongylosis infection in southwestern Iran.

In conclusion, polymerase chain reaction is a more reliable and feasible diagnostic method for the detection of *S. vulgaris* as compared to the traditional larval culture method. The frequency rate obtained using the molecular method is closer to the real values and can be applied to large scale screening programs of equine population.

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Conflict of interest
The authors declare that they have no competing interests.

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