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ORIGINAL ARTICLE

Molecular characterization and phylogenetic analysis of *Linguatula serrata* isolated from camels, sheep and goats in Iran

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Abstract

Linguatula serrata is an important zoonotic parasite with worldwide distribution. The objective of the present study was to investigate the molecular characterization and phylogenetic analysis of nymphal stage of *L. serrata* from camels, goats and sheep in Iran. The mesenteric lymph nodes were collected from various ruminants including goats, sheep and camels at Isfahan and Shiraz slaughterhouses and the nymphs were identified using morphological characteristics. After DNA extraction, the 18 S rRNA and Cox1 genes were amplified by polymerase chain reaction. The sequencing of the genes was conducted using specific primers and a capillary DNA analyzer. The comparison of amplified sequences with existing data confirmed the presence of *L. serrata* with 99.6–100% nucleotide sequence similarity. Based on 18 S rRNA and Cox1 sequences, two isolates collected from sheep revealed 100% and 99.9% sequence identity, respectively. Also, three isolates from camel had 99.64–100% and 99.7–100% homology. Two isolates from sheep had 100% identity in their 18SrRNA gene and were categorized together, but showed 99.9% similarity in the Cox1 gene, not clustering together. Phylogenetic analysis of the Cox1 gene classified nearly all the isolates into *L. arctica* clade. It can be concluded that 18 S rRNA and Cox1 genes sequencing can be a proper method for the analysis of phylogenetic relationships of *L. serrata* among different hosts in different parts of Iran, possibly helpful for infection control and prevention.

Keywords *Linguatula serrata* · 18S rRNA · Cox1 · Phylogeny · Iran

Introduction

Linguatulosis is a rare zoonotic parasitic disease caused by the larvae and nymphal stages of *Linguatula serrata*, an aberrant arthropod that belongs to the subclass Pentastomida, the adult form of the parasite is worm-like and inhabits the upper respiratory tract of terrestrial, carnivorous vertebrates with worldwide distribution. The range of intermediate hosts is wide and including cattle, goat, sheep, camels, rodents and other herbivorous mammals (Razavi et al. 2004; Villedieu et al. 2017). Larval development occurs in an intermediate host, which ingests eggs in the sputum or feces of definitive host. The larvae hatch within the intestine molt several times and encyst in different tissues. Finally, the definitive host become infected after the intermediate hosts' nutrition viscera (i.e., liver, lungs). Humans can also act as accidental aberrant hosts (Gjerde 2013). Nasopharyngeal linguatulosis, known as Halzoun or Marrara syndrome in human, often occurs after the feeding of viscera infective (i.e., liver, lung, and lymph nodes) with the nymph of infected animals. The syndrome has been reported in many countries of South-East Asia, the Middle East, and Africa. (Beaver et al. 1984; Drabick 1987). Although there are few reports on basic morphological descriptions, there are several studies unraveling the prevalence rate in domestic dogs, sheep and goats, cows and buffalo, camels and wild animals (Young 1975; Oluwasina et al. 2014). However, few reports have been published on the molecular characters of the tongue worm; hence the phylogenetic relationship of pentastomids has remained elusive. It has been confirmed that nuclear 18 S subunit ribosomal RNA (18s rRNA) is a possible marker to genetic analysis of eukaryotes (Alborzi et al. 2013; Fonseca et al. 2014). Because of extremely

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conserved nature and slow rate of developmental mutation, 18 S rRNA sequencing is a promising tool for intra species distinction at higher taxonomic levels. Recently, the mitochondrial cytochrome C oxidase subunit 1 (Cox1) gene has been used for phylogenetic and population genetics analysis in various insect species according to its high mutational rate compared to 18 S rRNA (Mohanta and Itagaki 2017). Previous data based on scanning electron microscopy and biological characters have classified pentastomids to Arthropoda, but recently there have been suggested as a separate phylum (Møller 2009; Christoffersen and De Assis 2013). A close relationship between the Pentastomida and the crustacean subclass Branchiura have been demonstrated with molecular tests. A molecular identification and phylogenetic analysis of 18 S rRNA and Cox1 gene between *L. serrata* and *L. arctica* from reindeer exhibited 99.9% and 90.2% sequence similarity, respectively (Gjerde 2013). In addition, 18 S rRNA and Cox1 sequencing showed a small variation among four *L. arctica* isolates. As for *L. arctica*, there is little data regarding the development of preadults and adults in the respiratory passages and maxillary sinuses of ruminants. The aim of this study was the molecular characterization and phylogenetic analysis of *L. serrata* in camels, sheep and goats from Iran using DNA sequence data of Cox1 and 18 S rRNA.

Materials and methods

Sample collection and preparation

During this study, the mesenteric lymph nodes (MLNs) were obtained from various ruminants including sheep, goats, and camels at Isfahan and Shiraz slaughterhouses, and transferred to our laboratory. The samples were cut into small pieces and immersed in a glass petri dish containing digested 100 ml of preheated digestion fluid and then incubated at 37 °C for 24 h, and finally, digested contents were examined with a stereomicroscope. The isolated nymphs were identified using morphological characteristics, stored in 70% ethanol, and kept at − 20 °C for the next steps.

DNA extraction and polymerase chain reaction (PCR)

Total genomic DNA was extracted from nymphs using phenol-chloroform method. This protocol was performed based on a modified method recommended by Sambrook and Russel (Sambrook and Russell 2006). DNA extract quantity (concentration and extraction efficiency) were evaluated and stored at − 20°C for future use.

Two such selected primer pairs were targeting the 18S rRNA gene and Cox1 gene (Ghorashi et al. 2016). The first set partial 18S rDNA amplicons were amplified in 1 round with the use of the forward primer (18S-F 5-CCA TGGTTGTCACGGGTGACG − 3' and 18 S-R 5-CTTGCG ACGATCCAAGAATTT − 3') and was based on 18 S rDNA sequences of *L. serrata* available in Gene bank (Accession no. JX088397). The second one (*Cox1*-F 5'-CAATAT ACGCCCAGCAAAAT − 3' and *Cox1*- R 5'-TGGTAA ATAGGAAGATGAAA- 3') was based on available *Cox1* gene sequences in Gene bank (Accession no. KF029447). All PCR reactions were performed in a 25 µl reaction mixture, containing 20 µl of Taq DNA Polymerase Master Mix (Thermo Fisher Scientific, USA), 4 µl template DNA and 1 µM each primer. PCR cycling included an initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 10 s, and a final extension at 72 °C for 1 min. The PCR products were electrophoresed on 1.5% agarose gel (CinnaClon Bioscience Co., Iran) in tris–acetate–EDTA (TAE) buffer, stained with Safe stain (CinnaClon Bioscience Co., Iran) and were visualized under ultraviolet light.

DNA sequencing and phylogenetic analysis

The PCR fragments were sequenced using specific primers and a capillary DNA analyzer by BioNeer Co. (Seoul, South Korea). The newly amplified sequences were compared with previous ones using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and Clustal W software. Phylogenetic trees were constructed by means of the neighbor-joining (NJ) method and maximum parsimony (MP) methods using MEGA6 software (http://www.megasoftware.net/) (Tamura et al. 2013). Support values for internal nodes were estimated using a bootstrap resampling procedure with 1000 replicates (Felsenstein 1992). Three *L. serrata* from camel was deposited in the GenBank database under accession number MG913255.1, MG913254.1 and MG913253.1. Four *L. serrata* from goat and sheep were identified and submitted to the GenBank with accession No.: MG913252.1, MG913251.1, MG913250.1, MG913249.1.

Results

In the present study, the sequences of 18 S rRNA and Cox1 genes were detected in *L. serrata* samples and successfully amplified. In the PCR assay, DNA template produced specific products with 595 bp and 542 bp for 18 S rRNA and Cox1 gene regions, respectively. The obtained sequences were compared with previous ones using BLAST (https://blast.ncbi.nlm.nih.gov/BLAST.cgi). GenBank (http://www.ncbi.nlm.nih.gov) accession numbers were provided for nucleotide sequences of the partial Cox1 and 18 S rRNA gene of *L. serrata* isolates. The comparison between the

sequences from 18s rDNA and *Cox1* gene regions with previous published data revealed that our isolates were *L. serrata*. The sequence alignment and the percentage of sequence identity showed 99.6–100% nucleotide sequence similarity level among our isolates. Based on 18 S rRNA and *Cox1* sequences, two isolates collected from sheep revealed 100% and 99.9% sequence identity. Also, three isolates from camel had 99.64–100% and 99.7–100% homology. The analysis of the 18 S rRNA and *Cox1* sequences of *L. serrata* isolates revealed homology to isolates of *L. serrata* obtained from GenBank. Based on the analyses of *L. serrata* 18 S rRNA gene and *Cox1* gene, our isolates had similarities with previous reports of *L. serrata* in different hosts. Nucleotide sequence in the 18 S rRNA gene from the *L. serrata* isolated from human (FJ528908) showed (97%) nucleotide similarity to our isolates. The sequence identity in the *Cox1* gene between *L. serrata* isolates and *L. arctica* (KF029443, Rangifer) isolate was 93–94.5% In the phylogenetic analysis, two isolates from sheep had 100% identity in their 18 S rRNA gene and were categorized together. However, these isolates showed sequence similarity (99.9%) in their *Cox1* gene and did not cluster together Phylogenetic analysis of isolates based on the *Cox1* gene set nearly all *L. serrata* isolates into the clade *L. arctica* (Fig. 1).

Discussion

Linguatulosis should be considered a public health subject for both the human and animals population in Iran. Linguatulosis should be considered a public health subject for both the human and animals population in Iran. The prevalence of infection of the *L.serrata* nymph in ruminants in numerous studies has been reported in different regions which the infection was higher in goats than in other ruminants of Iran (Hajipour and Tavassoli 2019).

The presence of *L. serrata* in sheep, goat and camel is a public health concern, since linguatulosis is a zoonotic disease and these animals can be hazardous for public health. *L. serrata* causes economic loss in ruminants without specific clinical symptoms which necessitates its proper diagnosis (Oryan et al. 2008; Alborzi et al. 2013; Mohammadi et al. 2020; Raele et al. 2022). *L. serrata* isolates have been identified from various animals mostly in dogs (20–76.5%) (Oryan et al. 2008; Christoffersen et al. 2013). A previous study showed a high prevalence of *L. serrata* in goats and sheep which was associated significantly with higher age, colder seasons and more exposure to dogs. In the present study, the two regions including 18 S rRNA and *Cox1* genes were amplified and the sequence analysis confirmed the existence of *L. serrata*. We observed that 18 S rRNA had more conserved regions, being more advantageous for phylogenetic analysis purposes. The amplified specific sequences were analyzed to evaluate phylogenetic relation of *L. serrata* from various livestock sources. Based on 18 S rRNA and *Cox1* sequences, two isolates collected from sheep revealed 100% and 99.9% sequence identity, respectively. Also, three isolates from camel had 99.64–100% and 99.7–100% homology. The analysis of the 18 S rRNA and *Cox1* sequences of *L. serrata* isolates showed homology to isolates of *L. serrata* obtained from GenBank. Based on the analyses of *L. serrata* 18 S rRNA gene and *Cox1* gene, our isolates had some similarities with previous reports of *L. serrata* in different hosts. Moreover, we observed that two isolates from sheep had 100% identity in their 18 S rRNA gene and were categorized together. However, these isolates showed sequence similarity (99.9%) in their Cox1 gene and did not cluster together. Similar to our results, sequence analysis of the Cox1 gene of *L. serrata* exhibited > 99% similarity to others in GenBank in the lung tissue of a vicuña (*Vicugna vicugna*) from Cuzco Peru. Another study in Australia showed the presence of *L. serrata* in human eye using 18 S rRNA sequencing as a proper apparoach. The 18 S rRNA sequencing had been used to classify other pentastomids for possible phylogenetic typing. However, a study by Mohanta using 18 S rRNA and Cox1 gene sequencing suggested that more additional loci are needed for phylogenetic analysis of *L. serrate* (Mohanta and Itagaki 2017). Similarly, their results demonstrated that 18 S rRNA had no intraspecific variation, but Cox1 gene sequences showed 99.7–99.9% homology. The sequencing of the Cox1 gene revealed that pentastomids are more related to Nematoda than to Arthropoda. Thus, morphology-based classification is insufficient and molecular analyses are essential to clear the phylogenetic relationships of the *Pentastomida*. In addition, next generation sequencing of approximately 100 bp of the V9 region of the 18 S rRNA gene by Lie exhibited a proper informative phylogenetic analysis compared to full-length sequencing of 18 S rRNA (Koehsler et al. 2011; Lie et al. 2014; Mohanta and Itagaki 2017). In another study, similar to our results, it has been revealed higher sequence diversity in the Cox1 gene than 18 S rRNA of *L. serrata* isolated from various livestock ruminants and dogs and there was no relationship between nucleotide variations, host species and geographical locations (Hajipour et al. 2016; Naude et al. 2018). In addition, their findings highlighted a close association between *L.serrata* and *L.arctica*. Revealing the genetic variety of *L.serrata* isolates gives data for understanding the evolution of these isolates in various regions worldwide. It seems from existing data that Cox1 gene has a higher mutation rate than the 18 S rRNA. These genes are suitable candidates for phylogenetic analysis of *L. serrata*, but more studies are needed in this attention (Shekarforoush 2004; Ghorashi et al. 2016; Hajipour et al. 2016; Mohammadi et al.2020). It can be resulted that these regions (18 S rRNA gene and Cox1 gene) are needed more investigations for future studies

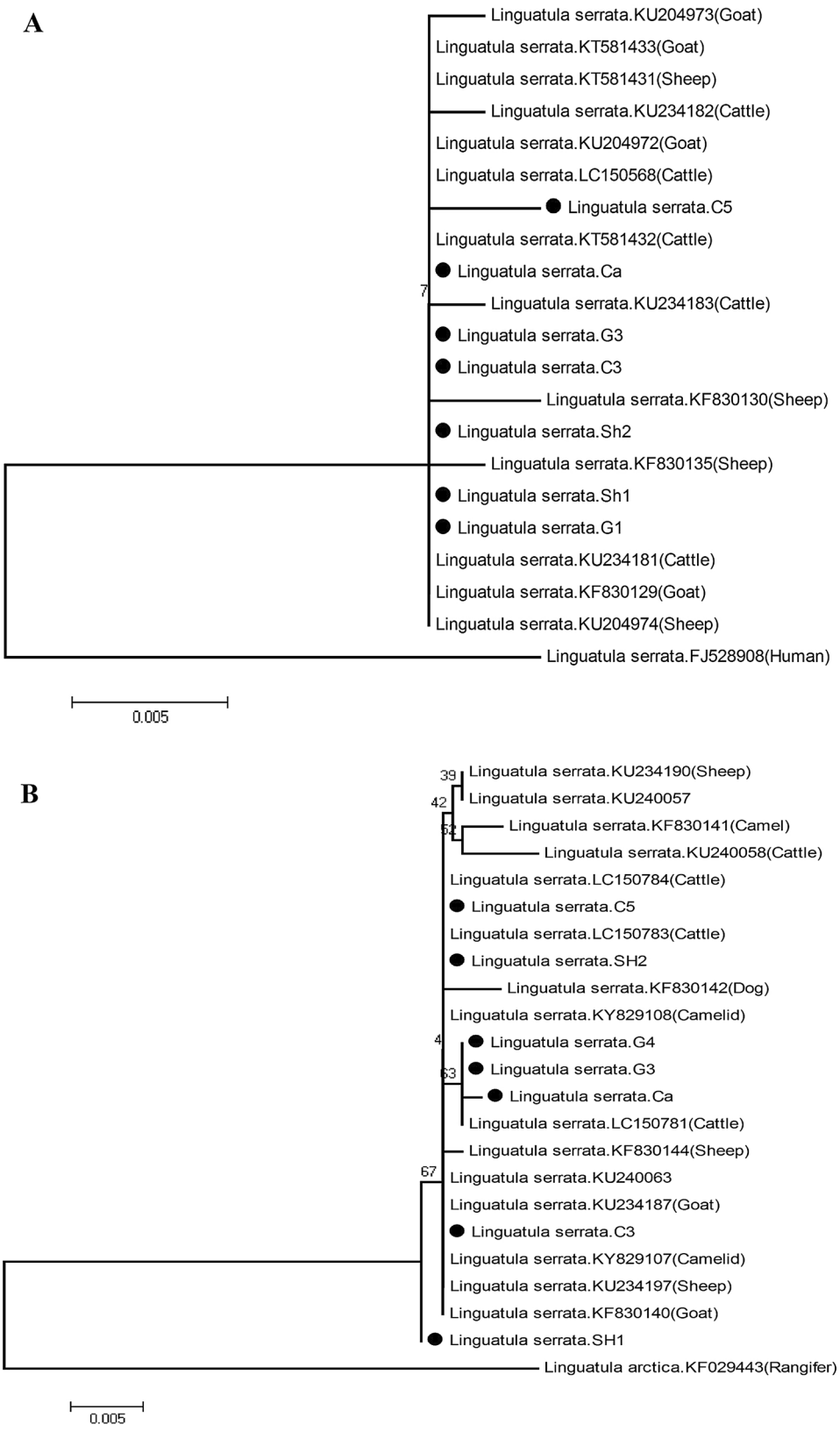

Fig. 1 Phylogenetic tree based on 18 S rRNA (**A**) and Cox1 DNA sequences data (**B**) constructed according to the NJ method algorithms, which black spots represent the position of Iranian isolates of L. serrata. Numerals above the branches indicate bootstrap values (%) from 1000 replicates. The scale bar indicates the proportion of sites changing along each branch

investigating the phylogenetic relationships of *L. serrata*. However, from our results and previous data, 18 S rRNA sequencing seems to be more advantageous.

Conclusion

Our data showed that sequencing of 18 S rRNA and Cox1 genes is possibly helpful for the phylogenetic typing and understanding of evolutionary process of *L. serrata* isolates in livestock. The data highlighted that 18 S rRNA sequencing has more advantageous results than Cox1 gene sequencing because of more conserved regions. However, more investigations using other conserved genes are needed for this purpose. The lack of sufficient samples and geographical diversity of isolates were limitations of the present study. Studying on genetic diversity among *L. serrata* isolates provides information for the parasite evolution in various regions of the world and different sources, possibly helpful for infection control and prevention.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical considerations Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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