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ORIGINAL ARTICLE

Molecular Phylogenetics of *Trichostrongylus* Species (Nematoda: Trichostrongylidae) from Humans of Mazandaran Province, Iran

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Abstract: The present study was performed to analyze molecularly the phylogenetic positions of human-infecting *Trichostrongylus* species in Mazandaran Province, Iran, which is an endemic area for trichostrongyliasis. DNA from 7 *Trichostrongylus* infected stool samples were extracted by using in-house (IH) method. PCR amplification of ITS2-rDNA region was performed, and products were sequenced. Phylogenetic analysis of the nucleotide sequence data was performed using MEGA 5.0 software. Six out of 7 isolates had high similarity with *Trichostrongylus colubriformis*, while the other one showed high homology with *Trichostrongylus axei* registered in GenBank reference sequences. Intra-specific variations within isolates of *T. colubriformis* and *T. axei* amounted to 0-1.8% and 0-0.6%, respectively. *Trichostrongylus* species obtained in the present study were in a cluster with the relevant reference sequences from previous studies. BLAST analysis indicated that there was 100% homology among all 6 ITS2 sequences of *T. colubriformis* in the present study and most previously registered sequences of *T. colubriformis* from human, sheep, and goat isolates from Iran and also human isolates from Laos, Thailand, and France. The ITS2 sequence of *T. axei* exhibited 99.4% homology with the human isolate of *T. axei* from Thailand, sheep isolates from New Zealand and Iran, and cattle isolate from USA.

Key words: *Trichostrongylus colubriformis*, *Trichostrongylus axei*, human, PCR, ITS2-rDNA region, phylogenetic analysis, Mazandaran, Iran

INTRODUCTION

Trichostrongylus species are parasitic nematodes of the small intestine of ruminants, rodents, pigs, horses, birds, and humans with a worldwide distribution [1,2]. There are more than 30 species of *Trichostrongylus* most of which are parasites of herbivores. At least 10 species have been reported from humans where people and herbivorous animals are in close contact [3,4]. Human infections occur mainly via ingestion of filariform larvae from contaminated vegetables or water or rarely by penetrating through the skin [3]. It is common locally in many countries, including Iran, Iraq, Egypt, Ethiopia, Laos, Thailand, South Korea, China, Japan, and United States [5]. Most human infections cause no clinical symptoms and symptomatic individuals may present with abdominal pain, diarrhea, and eosinophilia [6,7].

Classification of *Trichostrongylus* spp. by conventional morphological methods is relatively reliable on *Trichostrongylus* males. However, these methods are laborious and cannot be relevant to recognize female worms [8]. Finding the characteristic eggs of *Trichostrongylus* in stool samples is a routine diagnostic method, but is not helpful to differentiate the species [8].

In recent years, PCR-based techniques are applied for species identification and phylogenetic analysis of *Trichostrongylus* nematodes in ruminants worldwide [9-13]. There are also a few studies on identification and genetic characterization of *Trichostrongylus* samples from humans, such as third-stage larvae [3,7,14] and eggs in stool samples [15], by the detection of DNA. Investigations have shown ribosomal DNA sequencing (particularly ITS2 region) as a useful tool for differentiation of *Trichostrongylus* species and analysis of genetic variations and

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phylogenetic relationships [3,9,10,13,15]. In Iran, using morphological methods, human infections with 8 species of *Trichostrongylus* were reported, including *T. capricola* [16], *T. lerouxi* [17], *T. orientalis, T. vitrinus, T. axei, T. colubriformis, T. probolurus,* and *T. skrjabini* [4]. Among those, *T. orientalis* and *T. colubriformis* were detected more frequently in regions where the prevalence of infection was high [4]. Above mentioned species were also reported in ruminants in Iran, by morphological [4,18,19] and molecular methods [9], indicating zoonotic potential of those species. However, phylogenetic studies on human isolates of trichostrongyliasis in Iran are lacking. Therefore, we tried the molecular phylogenetic analysis based on ITS2 region of ribosomal DNA with fecal egg samples of *Trichostrongylus*, collected from residents in Mazandaran Province, northern Iran.

MATERIALS AND METHODS

Study area and sample collection

Mazandaran Province is situated on the southern coast of the Caspian Sea in the north of Iran (53°6' E, 36°23'N) (Fig. 1). It has a humid weather with annual average rainfall of 977 mm. This province is geographically divided into 2 parts; the coastal plains and the mountainous regions [20]. Most rural residents are farmers, and domestic animal husbandry can expose them to zoonotic parasites, such as *Trichostrongylus*. This province has previously been known prevalent for *Trichostrongylus* species, both in domestic animals [4] and humans [4,15]. In this study, 7 human trichostrongyliasis cases were detected by formalin-ether concentration technique during a study on evaluation of molecular and parasitological methods for the diagnosis of strongyloidiasis in fecal samples [21]. They were 5 males and 2 females with ages ranging between 23 and 57 years old, residing in Mazandaran Province, northern Iran. For extraction of genomic DNA, fecal samples of the patients were kept in 70% ethanol at room temperature.

Molecular and phylogenetic analysis

DNA of the samples was extracted by using in-house (IH) method as described by Repetto et al. [22] and modified by Sharifdini et al. [21]. Briefly, 1 g of stool samples was diluted in 10 ml of PBS and was subjected to 5 cycles of freezing and thawing. Next, 500 µl of the PBS diluted stools were incubated overnight with 500 µl GTES buffer at 37˚C, followed by 3 times freezing–thawing. Then, 200 mg of glass beads were added and shaken rigorously for 5 min. The suspension was incubated for 12 hr in nematode lysis buffer at 37˚C. Then, the samples were extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and DNA was precipitated with an equal volume of isopropanol and 1 ml of 100% ethanol, respectively. The pellet was washed with 300 µl of 70% ethanol, dried and eluted in 100 µl of TE buffer and stored at −20˚C until PCR amplification.

The ribosomal DNA internal transcribed spacer 2 (ITS2) region was amplified by forward (NC1: 5-ACGTCTGGTTCAGGGTTGTT-3) and reverse (NC2: 5-TTAGTTTCTTTTCCTCC-

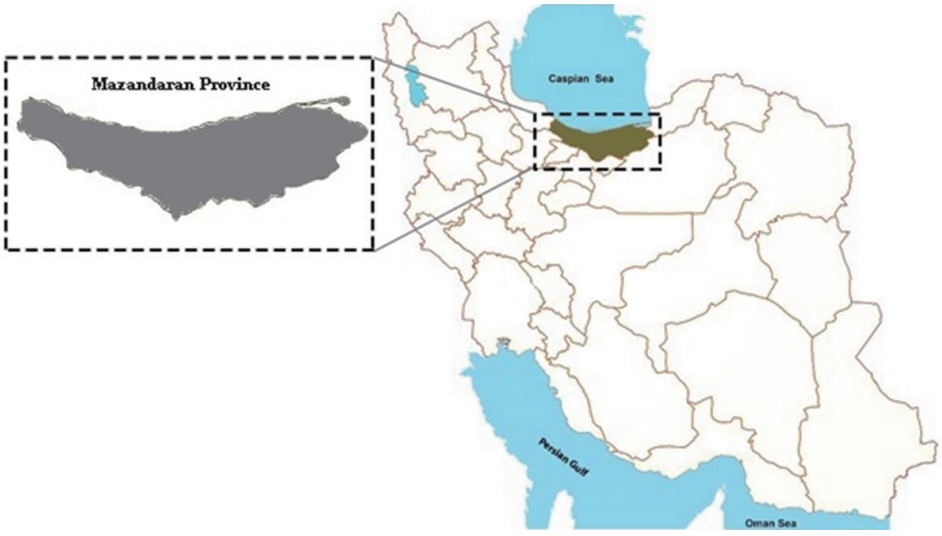

Fig. 1. Map of Iran showing geographical location of Mazandaran Province.

GCT-3) primers [23]. The PCR reactions were performed in a final reaction volume of 30 µl containing 15 µl of PCR mix which included 1.25 U Taq DNA polymerase, 200 µM of dNTPs, and 1.5 mM $MgCl_2$ (2x Master Mix RED Ampliqon, Copenhagen, Denmark), 10 pmol of each primer, and 4 µl of DNA sample. The PCR program was an initial denaturation step at 95˚C for 6 min followed by 35 cycles of 94˚C for 45 sec (denaturation), 60˚C for 90 sec (annealing), and 72˚C for 60 sec (extension), followed by a final extension at 72˚C for 5 min.

The PCR products were run on a 1.5% agarose gel. DNA sequencing was performed using an ABI 3130xl platform (Applied Biosystems, Foster City, California, USA). The sequence results were edited and analyzed by the Geneious software (www.geneious.com) and compared with sequences deposited in GenBank by BLAST program (http://www.ncbi.nlm.nih.gov/). A phylogenetic tree was constructed using the maximum likelihood method based on the Tamura 3-parameter model, and pairwise comparisons were determined of the level of sequence differences within and among species using MEGA 5.0 software. Bootstrap analysis was done based on 1,000 replications.

RESULTS

Microscopical examination of all 7 stool sedimentations revealed the presence of *Trichostrongylus* eggs with their morphological characteristics, including elongated oval shape, and slight tapering at one end. However, alignments of ITS2 sequences of these isolates separated them as *T. colubriformis* (Fig. 2A) and *T. axei* (Fig. 2B).

All samples were successfully presented amplification of about 328 bp for the ITS2 gene. Comparisons of the sequences from these isolates with other available reference sequences in GenBank, using BLAST system, revealed that 6 isolates had high similarity (more than 95%) with *T. colubriformis*, and the other one had high homology with *T. axei*. These sequences were deposited in GenBank database (accession nos.: KP663663, KP663664, KF989494, KF989495, KF989496, and KF989497 for *T. colubriformis* and KF840722 for *T. axei*).

The multiple alignments of ITS2 sequences of *Trichostrongylus* spp. obtained in this study are available in GenBank. Intraspecific variation within isolates of *T. colubriformis* and *T. axei* amounted to 0-1.8% and 0-0.6%, respectively; while interspecific sequence differences among *Trichostrongylus* nematodes were significantly higher, being 1.8-7.0%. Based on these variations, 21 isolates of *T. colubriformis* and 5 isolates of *T. axei* were classified into 5 and 2 haplotypes for ITS-2, respectively (Fig. 3). *Trichostrongylus* species of the present study were in a cluster with the relevant reference sequences from previous studies (Fig. 4).

DISCUSSION

Infection with species of *Trichostrongylus* is common among herbivores in most parts of Iran. *T. colubriformis* [4,18,19,24,25], *T. vitrinus* [4,18,19,24,25], *T. axei* [4], *T. capricola* [4,19], *T. probolurus* [4,18,19,24,25], *T. longispicularis* [19], *T. orientalis* [4], *T. lerouxi* [26], *T. skrjabini* [4], and *T. hamatus* [25] were reported in different animals, such as sheep [4,18,19], goat [4,19],

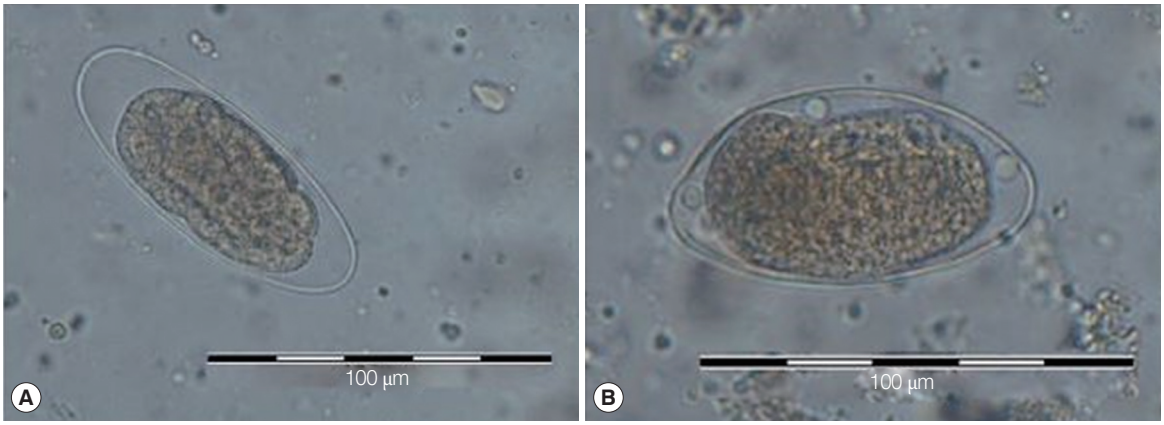

Fig. 2. Light microscope view of *Trichostrongylus colubriformis* (A) and *Trichostrongylus axei* (B) eggs in stool samples of infected people showing morphological characteristics, including elongated oval shape and slight tapering at one end.

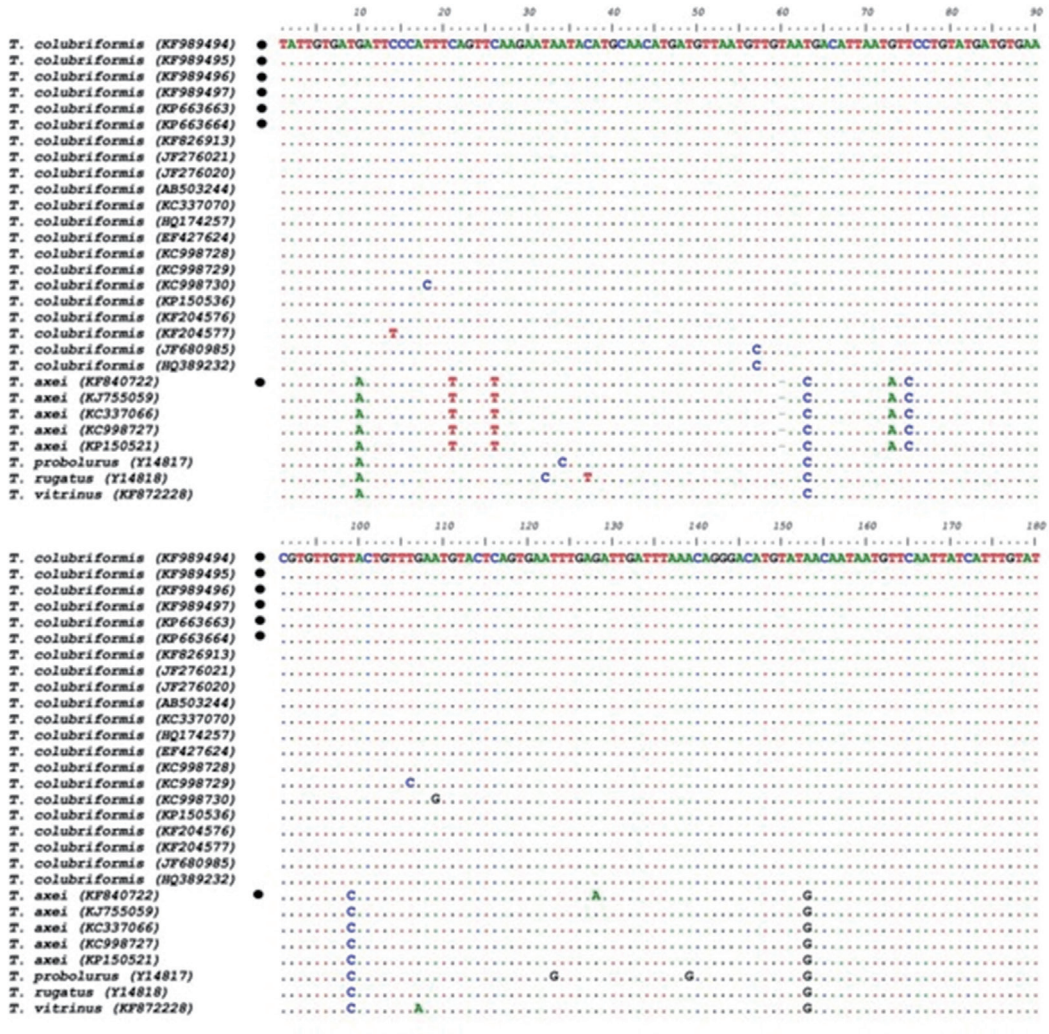

Fig. 3. Alignments of the ITS2 sequences for all *Trichostrongylus* isolates in the present study and reference sequences retrieved from GenBank. Sequences with circle mark were inferred from this study.

cattle [4,19], camel [24,25], and buffalo [4,19] using morphology. Predominant species among different herbivores in most parts of the country like Mazandaran Province are *T. colubriformis, T. vitrinus*, and *T. axei* [4]. Considering human infections, high prevalence of infection [4], as well as variety of *Trichostrongylus* species including *T. orientalis* [4], *T. colubriformis* [4], *T. vitrinus* [4], *T. axei* [4], *T. capricola* [16], *T. probolurus* [4], *T. skrjabini* [4], and *T. lerouxi* [17], has been reported from Iran, back to 1970's, indicating *T. colubriformis* and *T. orientalis* as predominant species [4]. Recently, among human geohelminths, the prevalence of some species especially *Ascaris lumbricoides* and hookworms are sharply declined [27]; however, *Trichostrongylus* spp. [15,28,29] and *Strongyloides stercoralis* [21,29,30] are more frequently reported due to zoonosis of the former and ability of auto infection of the latter parasite, respectively.

In this study, in spite of the availability of few human *Trichostrongylus* samples (n=7), using ITS2 sequence analysis, 6 of them were determined as *T. colubriformis*, and the other one as *T. axei*. This result is compatible with the result of a recent molecular study on human trichostrongyliasis in Mazandaran Province, in which *T. colubriformis* was accounted as the most probable common species of *Trichostrongylus* in humans [15]. Human trichostrongyliasis is caused by using animal feces as a fertilizer for agriculture and gardening [31]. In the north of Iran, many domestic animals, such as sheep, goats, and cows graze almost freely around, and contamination risk of vegetables in the fields with animal feces is high. Additionally, in

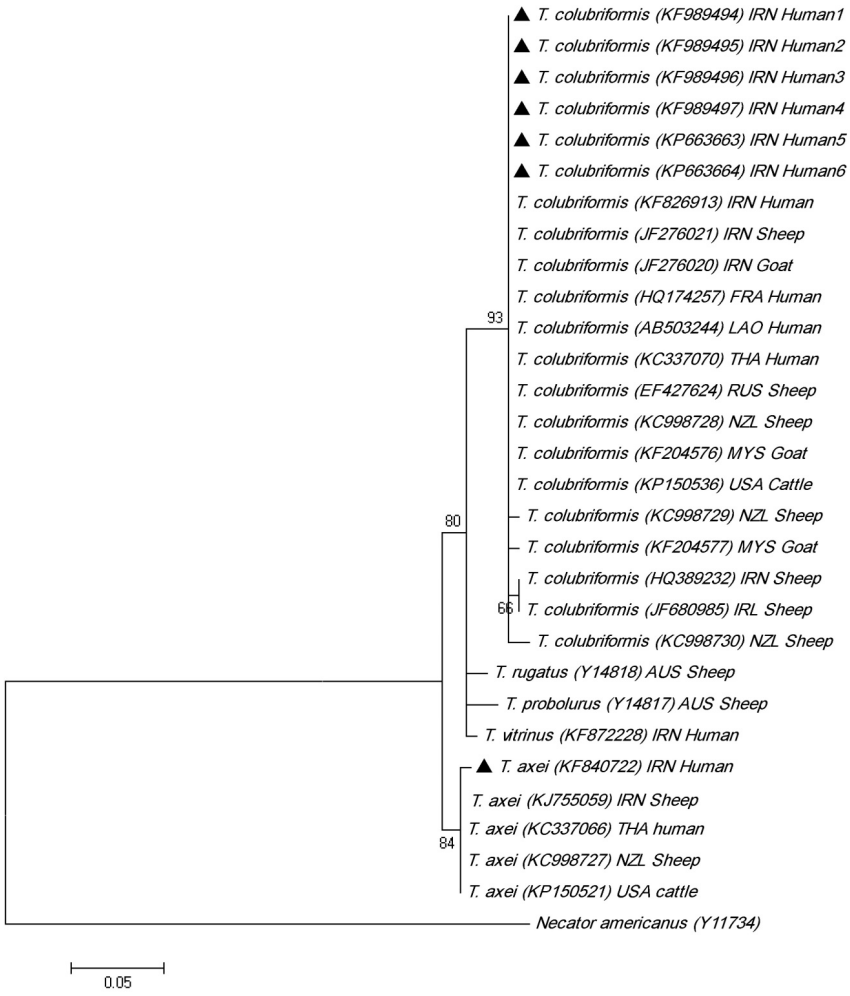

Fig. 4. Phylogenetic tree of isolates of *Trichostrongylus* spp. obtained in this study (▲) and reference sequences retrieved from GenBank based on ITS2 nucleotide sequences and constructed using the Tamura 3-parameter model in MEGA software version 5. GenBank sequences of *Trichostrongylus* spp. included with *Necator americanus* as an out group. Iran (IRN), France (FRA), Thailand (THA), Laos (LAO), Russia (RUS), Malaysia (MYS), United States (USA), Ireland (IRL), Australia (AUS), and New Zealand (NZL) are represented with country codes (ISO 3166-1 a-3 codes).

gardening practices, use of fresh sheep and cattle residues as fertilizer is common. Therefore, *T. colubriformis* is among more frequent geohelminths of humans in the study area due to its high zoonotic capability and its high prevalence in domestic animals [4]. While, the prevalence of *T. orientalis*, similar to human hookworms and also *A. lumbricoides*, has decreased due to not using human nigh soil as fertilizer in the study area, as in most other parts of the country.

Phylogenetic sequence analysis is a useful tool to gain information on an organism's evolutionary relationships. The existence of genetic variation among *Trichostrongylus* nematodes has been confirmed previously [9,10,32]. However, only a few studies have analyzed molecular-phylogenetic characterization of human trichostrongyliasis [3,14,33]. This study is the first phylogenetic analysis of *Trichostrongylus* species from humans in Iran. BLAST analysis of the isolates indicated that sequences of all 6 *T. colubriformis* had 100% homology with each other and with the previously registered sequences from the human (KF826913), sheep (JF276021), and goat (JF276020) in Iran. Among GenBank sequences of *T. colubriformis*, 1 sequence from sheep in Iran (HQ389232) had 1 nucleotide difference in ITS2 with that of *T. colubriformis* in this study. The latter sequence, in phylogenetic tree, was placed in 1 group along with a sheep isolate of *T. colubriformis* sequence from Ireland (JF680985). Sequences of *T. colubriformis* isolates in present study also presented 100% homology with *T. colubriformis* in

humans from France (HQ174257), Thailand (KC337070), and Laos (AB503244). These sequences also showed 100% similarity with *T. colubriformis* from sheep (EF427624 and KC998728), goat (KF204576), and cattle (KP150536) in other countries. For these sequences, 99.4% similarities were obtained with *T. colubriformis* isolates from sheep in Ireland (JF680985), sheep in New Zealand (KC998729) and goat in Malaysia (KF204577). *T. colubriformis* sequences of this study presented 98.8% homology with the sequences of *T. colubriformis* from sheep in New Zealand (KC998730).

Human infections with *T. axei* were reported among the literatures in Iran [4] and Italy [34]. Recently, *T. axei* was also found in humans in Thailand [3] and Iran [15] by molecular techniques. The ITS2 sequence of *T. axei* in this study exhibited 99.4% homology with the human isolate of *T. axei* from Thailand (KC337066), sheep isolate from New Zealand (KC998727), sheep isolate from Iran (KJ755059), and cattle isolate from USA (KP150521). *T. axei* of the current study (KF840722) has 1 nucleotide difference with the reference sequences of *T. axei* from Iran and other countries.

To sum up, *T. colubriformis* was found to be the most probable dominant human species in the study area, but further investigation with higher sample size is recommended. This species is also the most possible cause of human trichostrongyliasis infection in Laos [14], Thailand [3], and France [7]. It might also be the main zoonotic species of *Trichostrongylus* in some other parts of the world which have not been investigated yet.

Current phylogenetic analysis clarified the relation of human *Trichostrongylus* species from an endemic area of trichostrongyliasis in Iran and those of human and animal species of *Trichostrongylus* registered in GenBank. Based on pairwise comparisons, there was 100% homology among all 6 ITS2 sequences of *T. colubriformis* in the present study and most previously registered sequences of *T. colubriformis* from human and herbivores animals. High zoonotic capacity of this species is probably one of the main reasons of current higher occurrence of human trichostrongyliasis in the study area than that of human hookworms which are not zoonotic. Comparative characterization of *Trichostrongylus* species based on molecular approach from human and different livestock in every endemic area, using several gene targets, will be interesting and can be beneficial to understand the rate of zoonosis of each species.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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