


Prevalence and phylogeography of *Taenia hydatigena* metacestodes from goats of India

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Research Article

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Abstract

The study determined the prevalence and genetic population structure relationships of *Cysticercus tenuicollis* (*Taenia hydatigena* metacestode) retrieved from the goats slaughtered in north India. An overall prevalence of 9.62% (59/613) was recorded. Genetic population structure relationships were assessed by targeting partial cytochrome c oxidase subunit 1 mitochondrial gene sequence. Phylogenetic tree analysis revealed that all the present study representative isolates ($n = 7$) formed a major clade and grouped with *T. hydatigena* isolates retrieved from sheep, goats, pigs and dogs, originating from China, Iran, Nigeria, Ghana and Poland. However, a single isolate from Himachal Pradesh (isolate 3) formed a subgroup within the clade. The neutrality and diversity indices revealed high values of haplotype diversity [$H_d = 0.99695$ (0.95238–1.0000)] and low nucleotide diversity ($\pi = 0.49276$), which was indicative of demographic expansion and low gene flow, suggesting that Indian *T. hydatigena* isolates were not genetically differentiated. Tajima's D (–1.26988) and Fu and Li's D statistics values (–0.74556) were negative, demonstrating deviations from neutrality and both pro-pounded recent population expansion or purifying selection. Results highlighted a low genetic diversity of *T. hydatigena* metacestodes across the geographical range of north India.

Introduction

Cysticercosis caused by *Cysticercus tenuicollis*, larval stage (metacestode) of a canine tapeworm *Taenia hydatigena*, is endemic in many parts of the world (Boufana *et al.*, 2015; Ohiolei *et al.*, 2019). A 2 distinct hosts relationship in the life cycle involves dogs and other wild canids as definitive hosts and small ruminants (sheep and goats) and pigs as intermediate hosts, resulting in domestic and sylvatic cycles of disease transmission (Abbas *et al.*, 2021). Rarely, other animals such as cattle, deer, wild boars and other wild ungulates also act as intermediate hosts to the parasite (Cengiz *et al.*, 2019; Filip *et al.*, 2019; Sgroi *et al.*, 2019). The intermediate hosts acquire the infection by ingesting the taeniid eggs from contaminated pastures, which further results in development of metacestodes (*C. tenuicollis*) (Cengiz *et al.*, 2019). However, the definitive hosts get infected either by consuming the offal (mesenteries, omentum and less commonly the liver) of slaughtered intermediate hosts containing the cysticerci or by predation and hence, completing the domestic and sylvatic cycles, respectively (Sgroi *et al.*, 2019). In infected animals, mortality occurs due to hepatitis cysticercosa, peritonitis and pneumonia (Scala *et al.*, 2016). The infection in the intermediate hosts also holds huge economic importance as it leads to mortality of heavily infected lambs and kids and also condemnation of heavily infected organs (especially liver) and carcasses (Ohiolei *et al.*, 2019).

In India, small ruminant (goat and sheep) farming/rearing plays a very important role in the livelihood of landless (pastoralists), small and marginal farmers (Chakraborty and Gupta, 2017). It has been estimated that small ruminant farming contributes around 14, 4 and 15% of meat, milk and skin/hide production, respectively, and hence, adds approximately \$314 014 671 per annum to the rural economy (Chakraborty and Gupta, 2017). Despite being a potent benefactor to the underprivileged and poorest section of the society, the goat farming sector experiences many constraints including poor management practices leading to low productivity, less number of productive breeds, a very limited veterinary care especially to the animals reared under pastoralism and also issues related to marketing (Chakraborty and Gupta, 2017).

In the past decade, only 2 abattoir-based studies demonstrated the occurrence of *C. tenuicollis* in small ruminants with 4.22% (Singh *et al.*, 2015) and 18.87% (Nimbalkar *et al.*, 2011) prevalence in north India and Maharashtra state, respectively. However, no such study has been carried out in definitive hosts. The transmission of various taeniid parasites becomes extremely successful in India as most ideal conditions aid in establishment, propagation and dissemination of these parasites (Moudgil *et al.*, 2019). The most common practices which result in easy transmission of taeniid parasites involve improper disposal of offal at unregulated abattoirs, easy access of dogs to the offal, absence of proper meat inspection and no or very limited public awareness about the various disease conditions (Singh *et al.*, 2012).

Globally, a few reports targeting mitochondrial gene sequences (partial *cox1* and *nad1*) had reported considerable levels of genetic diversity among *T. hydatigena* populations from different hosts and geographical regions (Kedra *et al.*, 2001; Boufana *et al.*, 2015; Braae *et al.*, 2015; Rostami *et al.*, 2015; Omar *et al.*, 2016; Adwan *et al.*, 2018; Ohiolei *et al.*, 2019; Sarvi *et al.*, 2020; Ohiolei *et al.*, 2021).

In India, majority studies pertaining to incidence, pathology, biochemical and enzyme profiles/activities of *C. tenuicollis* and *T. hydatigena* started in 1980s (Pathak *et al.*, 1982, 1984; Pathak and Gaur, 1982). However, the disease condition in definitive and intermediate hosts is poorly documented from India. The existing few reports exhibit an infection rate ranging from 2.23 to 37.03% in small ruminants (Pathak and Gaur, 1982; Nath *et al.*, 2010; Singh *et al.*, 2015). Since the data pertaining to basic research related to *T. hydatigena* are scant, a very little research regarding detailed studies related to molecular confirmation, phylogeny, molecular phylogeography and population-genetic structure of the parasite has been carried out in India. Hence, the present preliminary study was envisaged to perform phylogenetic analysis and to assess neutrality indices and haplotype/genetic diversity of *C. tenuicollis* isolates retrieved from slaughtered goats of north India by targeting partial cytochrome c oxidase subunit 1 (*cox1*) mitochondrial gene sequence.

Materials and methods

Study area

The present study targeted 3 geographically different locations of north India, Hisar, Palampur and Chandigarh, belonging to 2 different states (Haryana and Himachal Pradesh) and a union territory (Chandigarh), respectively. The study targeted the arid zone of Haryana state involving the district of Hisar (29.09°N, 75.42°E) having an elevation of 215 m above mean sea level and an average annual rainfall of 450 mm. Palampur (32.11°N, 76.54°E), located in the foothills of north-west Himalayas at an elevation of 1472–2350 m above mean sea level, receives an average annual rainfall of 1578 mm. Chandigarh (30.73°N, 76.78°E) possesses an elevation of 321 m above mean sea level and an average annual rainfall of 792 mm.

Study design, sample size estimation and collection of parasitic stages

Total goat population in Haryana, Himachal Pradesh and Chandigarh is 336 338, 1 108 413 and 998 units, respectively. The sample size was estimated to be 215 (Haryana = 73, Himachal Pradesh = 73 and Chandigarh = 69) by assuming the prevalence 5% [based on the study carried out in neighbouring state Punjab (Singh *et al.*, 2015)], confidence level 95% and desired absolute precision or margin of error 5% (Thrusfield, 2007). In the present cross-sectional study, a total of 613 goats (both male and female adult animals of all breeds) slaughtered at Hisar ($n = 207$), Palampur ($n = 201$) and Chandigarh ($n = 205$) were randomly selected and screened visually or palpated for the presence of *C. tenuicollis* infection during February to August 2021. *Taenia hydatigena* metacestodes (*C. tenuicollis*) were carefully collected/dissected out and morphologically identified as per Loos-Frank (2000). The cysticerci exhibiting calcification and caseation were avoided and only viable cysticerci were collected for the phylogenetic study. The cysticerci were cleaned with normal saline and transported to laboratory at 4°C (under cold chain) and further kept in 70% ethanol at –20°C until genomic DNA extraction. Before proceeding for the genomic DNA extraction, the excised portions (preferably scolices) were washed

repeatedly (minimum 5 times) with phosphate-buffered saline (pH = 7.4) for the removal of ethanol (Kilinc *et al.*, 2019).

Genomic DNA extraction, PCR amplification and sequencing

Out of 59, 7 [Himachal Pradesh ($n = 3$; isolates 1–3), Haryana ($n = 2$; isolates 4–5) and Chandigarh ($n = 2$; isolates 6–7)] randomly selected representative isolates of *C. tenuicollis* were subjected to molecular analysis. Genomic DNA was extracted from the excised portions of the cysticerci; preferably scolex, using a DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The retrieved DNA was stored at –20°C until further use. Mitochondrial *cox1* gene was targeted for partial amplification of DNA sequences through PCR using primers; forward: 5'-TGCATTTAGCTGGTGCCTCAAGTA-3' and reverse: 5'-ACAAACACGCCGGGTAACC-3' (Filip *et al.*, 2019). The 25 µL PCR reaction mixture contained 12.5 µL of master mix (GoTaq Green Mater Mix, Promega, Madison, WI, USA), 0.75 µL of each primer (forward and reverse) (10 pmol), 1 µL of genomic DNA template and 10 µL of nuclease-free water. Amplification reactions were carried out in thermal cycler (Bio-Rad T100™ Thermal cycler, USA) using the conditions: initial denaturation (95°C for 1 min), denaturation (35 cycles of 95°C for 20 s), annealing (35 cycles of 56°C for 20 s), extension (35 cycles of 72°C for 40 s) and final extension (72°C for 5 min) (Filip *et al.*, 2019). The amplified PCR products were electrophoresed on 1.25% agarose gel along with 100 bp marker (DNAMark™ 100 bp, G-Biosciences, Saint Louis, Missouri, USA) and were visualized under gel documentation system. The genomic DNA of *T. hydatigena* adult parasites and *Echinococcus granulosus* retrieved from definitive hosts (dogs) were used as positive and negative controls, respectively, for amplification reactions. The amplicons of approximately 830 bp obtained in the present study were subjected to custom sequencing (GeneBio Solutions, Dehradun, India). Sanger sequencing (by carrying out chain termination PCR) was performed on the amplicons in both the directions (forward and reverse) separately by using the aforementioned primers. Each isolate was sequenced in triplicate to eliminate any error in sequencing.

Data analysis

DNA sequences retrieved were analysed for misread sequences using BioEdit software and final alignment of resulting gene sequences was carried out (Hall, 1999). The NCBI BLAST algorithm (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was employed for the identification and assessment of homology of the sequenced products by comparing them with reference sequences of different hosts archived in GenBank [goat ($n = 2$) – JN831297, KR337823; sheep ($n = 1$) – MT784879; small ruminants ($n = 8$) – MK945752, MK945753, MN175590–MN175595; swine ($n = 2$) – MF630923, MF630926; dog ($n = 3$) – MT784873, MT784890, MT784891]. The phylogenetic analysis was then performed using MEGA X (Molecular Evolutionary Genetic Analysis) software by constructing phylogenetic tree for representative isolates of *T. hydatigena* and other *Taenia* sp. [*Taenia ovis* (AB731675.1), *Taenia serialis* (AB731674.1) and *Taenia multiceps* (JX507220.1)] using maximum-likelihood method (Kumar *et al.*, 2018). Bootstrap analyses were conducted using 1000 replicates and the distance scale was estimated at 0.05. The sequence of *Moniezia expansa* (AB099693.1) was included in the phylogenetic analysis as out-group species to root the tree. Population neutrality indices [Tajima's D (Tajima, 1989) and Fu and Li's D statistics values (Fu and Li, 1993)], nucleotide diversity (π) and haplotype diversity (Hd) were estimated using DnaSP v.5 software. Median-joining network (Bandelt *et al.*, 1999) based haplotype

diversity was inferred based on the sequences of mitochondrial *cox1* using PopART (<http://popart.otago.ac.nz>). Fisher's exact test was used to determine the association between the prevalence of *C. tenuicollis* among different geographical locations (Haryana, Himachal Pradesh and Chandigarh). Significance was set at a 2-tailed *P* value <0.05.

Results

Prevalence studies

Out of 613 slaughtered goats screened, 59 (9.62%) showed the presence of *C. tenuicollis* (Fig. 1) attached to greater omentum, mesentery and even liver. Infection rates were highest in the slaughtered goats examined from Himachal Pradesh (12.43, 25/201, *P*=0.11) followed by Haryana (8.69%, 18/207, *P*=0.66) and Chandigarh (5.36%, 11/205, *P*=0.013).

Molecular analysis

The partial amplification of *cox1* gene and a PCR product size of approximately 830 bp confirmed the presence of *C. tenuicollis* (Fig. 2). An amplicon of 830 bp was also obtained for *T. hydatigena* adult parasites (positive control) whereas no amplification was observed for the DNA extracted from *E. granulosus* (negative control), validating the specificity of the primers.

GenBank archived DNA sequences of *T. hydatigena*, retrieved from different intermediate (goats, sheep and pigs) and definitive hosts (dog) showed high similarity (>99%) with the present study isolates (Fig. 3). Phylogenetic tree analysis revealed that all the 7 isolates formed a major clade and grouped in a different cluster with *T. hydatigena* isolates retrieved from sheep, goats, pigs and dogs, originating from China, Iran, Nigeria, Ghana and Poland (Fig. 3). However, a single isolate (isolate 3) from Himachal Pradesh formed a subgroup within the clade.

A total of 10 *cox1* nucleotide GenBank archived sequences of *T. hydatigena* from China, Iran, Nigeria, Ghana and Poland were included with the present study isolates. The haplotype and nucleotide diversities (*H_d* and π) of the present study isolates were 0.99695 (0.95238–1.0000) and 0.49276, respectively, whereas the neutrality indices were negative with Tajima's *D* and Fu and Li's *D* statistics values of -1.26988 and -0.74556 , respectively.

The haplotype network of *T. hydatigena* had all 17 (7 from present study and 10 retrieved from GenBank) haplotypes arranged within a star-like configuration (Fig. 4) with a main central haplotype (MT784873.1, retrieved from dog), separated from other haplotypes by 1–22 mutational steps. A total of 55 polymorphic regions containing 34.54% (19/55) parsimony informative sites were detected in 17 isolates analysed in this study. In Indian isolates, 27 polymorphic regions with 6 parsimony informative sites (68, 89, 235, 254, 365 and 487) and 21 singleton variable sites were recorded. The haplotype network containing Indian isolates of the present study also exhibited star-like configuration (Fig. 5) and the haplotypes were separated by 2–14 mutational steps. The 7 present study isolates subjected to haplotype network analysis represented 7 different haplotypes (Fig. 5).

Discussion

Taenia hydatigena is ubiquitous in distribution. Scanty information is available on prevalence and morphological characterization of the parasite in small ruminants in north India (Singh *et al.*, 2015). To the best of the authors' knowledge, this is the first study from India assessing the genetic population structure relationships of *C. tenuicollis* from goats. The overall prevalence (9.62%) of cysticercosis recorded in this study was higher than



Fig. 1. *Cysticercus tenuicollis* (*Taenia hydatigena* metacestode) retrieved from slaughtered goats.

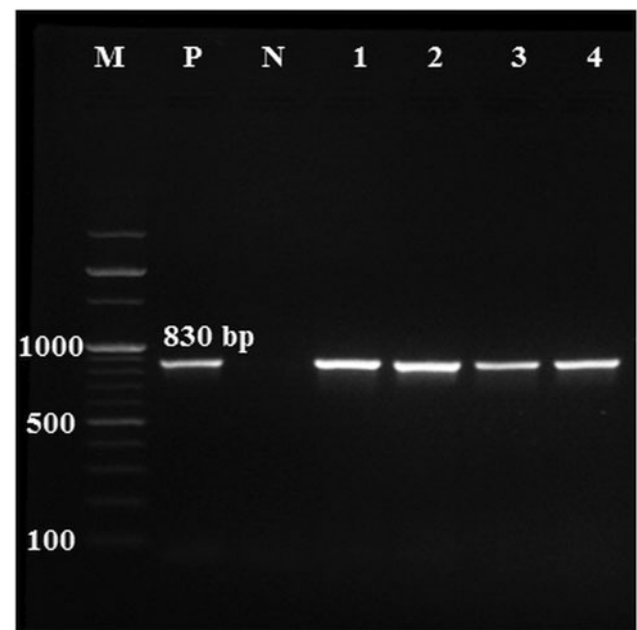


Fig. 2. PCR amplification targeting the mitochondrial *cox1* gene. M: 100 bp plus marker; P: positive (*Taenia hydatigena*) control; N: negative template (*Echinococcus granulosus*) control; 1–4: PCR products.

reported in a previous study (4.83%, Singh *et al.*, 2015). These rising trends of the parasitic infections can be attributed to the improper disposal of offal from open space non-gazetted abattoirs, making offal easily accessible to the scavenger dogs (Singh *et al.*, 2012). This leads to easy completion of life cycle of the parasite in dogs feeding on contaminated offal and its subsequent transmission to other healthy susceptible animals. High prevalence of *C. tenuicollis* infection in goats slaughtered in Himachal Pradesh can be attributed to this offal-based transmission since large migratory goat herds are usually guarded by dogs. These guard dogs are being fed with the offal from diseased goats (often infected with cysticercosis) by the goat herder. These infected dogs contaminate the pastures (feces containing eggs), thereby setting up the transmission cycle (Moudgil *et al.*, 2019).

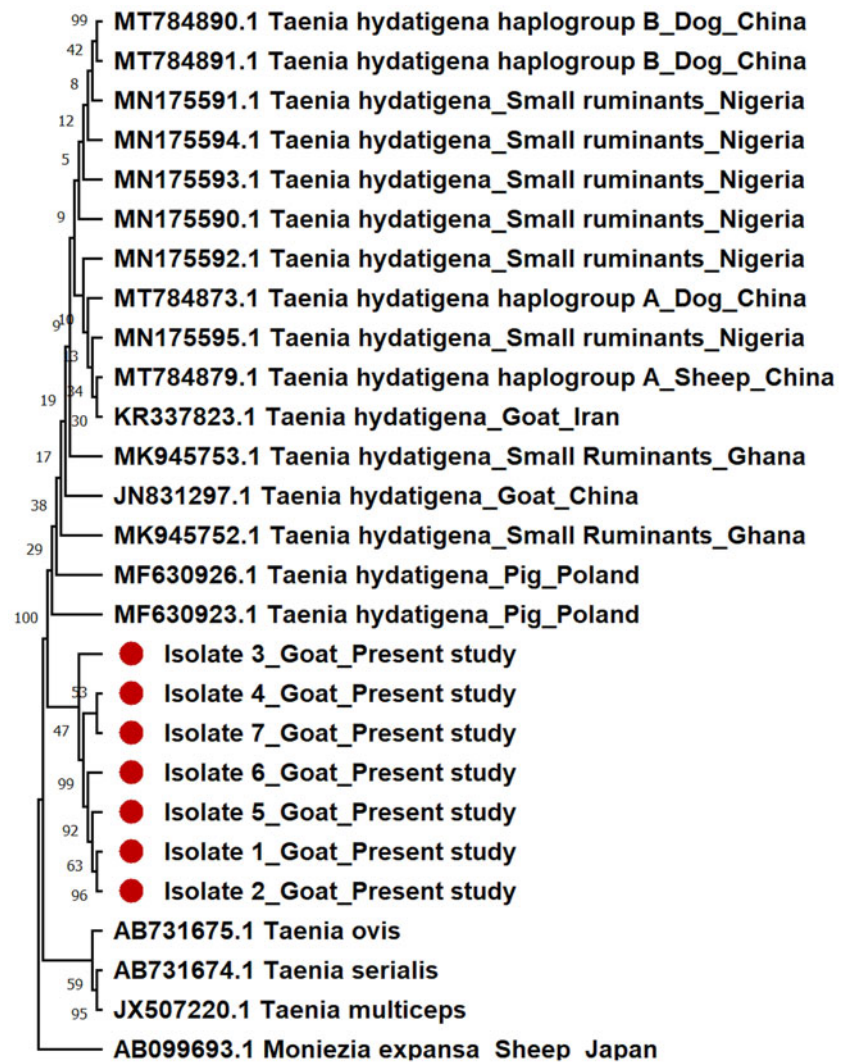


Fig. 3. Phylogenetic tree of *Cysticercus tenuicollis* (*Taenia hydatigena* metacestode) isolates retrieved from goats in relation to different GenBank archived isolates of intermediate and definitive hosts based on mitochondrial *cox1* gene. The phylogenetic tree was constructed by the maximum-likelihood method using MEGA X software. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. *Moniezia expansa* (AB099693.1) was used as out-group species to root the tree.

Mitochondrial DNA genes are usually considered as most potent targets for genealogical studies (Celik *et al.*, 2021). Also, for phylogenetics and phylogeography of taeniid cestodes, mitochondrial *cox1* gene is the most promising candidate to assess and establish intra- and interspecies variants (Bowles *et al.*, 1992; Adwan *et al.*, 2018). This is due to relatively higher mutation and evolutionary rates, conserved structure, maternal inheritance, absence of recombination and high genetic divergence of mitochondrial DNA (Ohiolei *et al.*, 2019; Moudgil *et al.*, 2021). Mitochondrial DNA also holds high diagnostic values as it is present in more than 1 copy per cell (Celik *et al.*, 2021).

Phylogenetic analysis of *cox1* DNA sequences revealed that all the isolates of the present study were accurately identified as *T. hydatigena* as they clustered with other GenBank archived isolates retrieved from different regions of the world. The present study *T. hydatigena* isolates also exhibited relative distance from other *Taenia* species (*T. ovis*, *T. serialis* and *T. multiceps*), in concordance to previous study (Ohiolei *et al.*, 2019). The neutrality tests (Tajima's *D*, Fu and Li's *D* statistics) based on selective neutrality of nucleotide variability exhibited negative values, indicating deviations from neutrality and both propounded excess of polymorphic sites and recent population expansion or purifying selection (Adwan *et al.*, 2018; Cengiz *et al.*, 2019). The neutrality and diversity indices revealed high values of *H_d* and low π , which was indicative of demographic expansion and low gene flow, suggesting that Indian *T. hydatigena* isolates were not genetically differentiated (Adwan *et al.*, 2018). We observed 7 different

haplotypes corresponding to 7 different isolates of north India, which is an indication of cross-fertilization driven gene flow in mature *T. hydatigena* parasites (Kilinc *et al.*, 2019). The prevailing conditions will definitely affect *T. hydatigena* host adaptation and life cycle in future as new strain and genotype emergence would take place due to intra-species variation due to incessant nucleotide changes as a result of cross-fertilization (Kilinc *et al.*, 2019).

The consumption of meat infected with *C. tenuicollis* does not cause any harm to human beings, however it remains a threat to livestock production (Ohiolei *et al.*, 2021). The genetic diversity of *T. hydatigena*/*C. tenuicollis* within populations is poorly understood due to limited studies (Ohiolei *et al.*, 2021). A proper understanding of the genetic diversity is required for epidemiological investigation, anthelmintic treatment, vaccine/drug development and control (Ohiolei *et al.*, 2021). Recent studies had reported a high variation between *T. hydatigena* populations, establishing its 2 haplogroups (Ohiolei *et al.*, 2019, 2021). However, in the present study, we had observed a low genetic diversity of *T. hydatigena* across north India.

While analysing the haplotype diversity through median-joining network, we observed a central haplotype in a star-like population structure considering the different isolates from different regions of the world. Our finding was in agreement with Boufana *et al.* (2015). However, while considering the 7 isolates retrieved in the present study, we did not find any central haplotype. Similar findings were also reported by Ohiolei *et al.* (2021), which indicated uncertainty about the existence of a central or

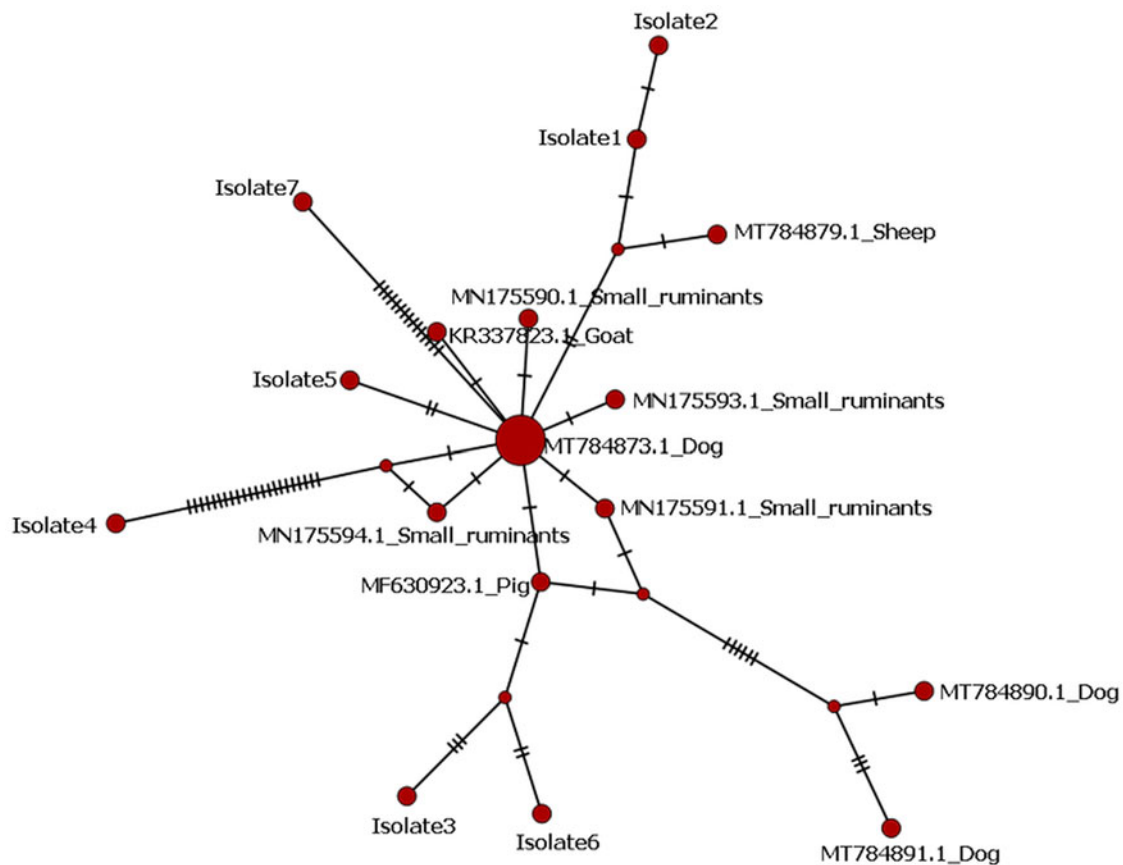


Fig. 4. Median-joining network of *Cysticercus tenuicollis* isolates from different hosts and geographical regions using partial mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene. Circle size is relative to haplotype frequency. Bars/hatch marks indicate the number of haplotype substitutions/mutations.

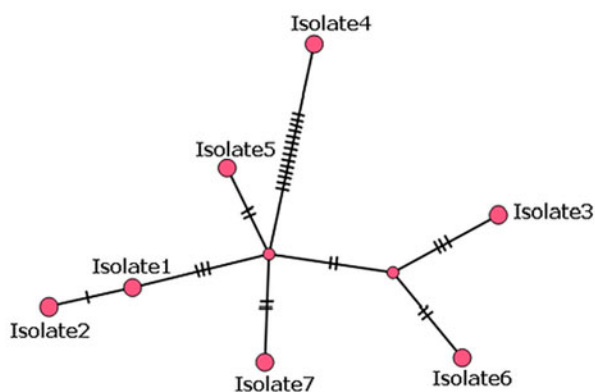


Fig. 5. Median-joining network of north Indian *Cysticercus tenuicollis* isolates retrieved from goats using partial mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene. Circle size is relative to haplotype frequency. Bars/hatch marks indicate the number of haplotype substitutions/mutations.

widespread haplotype of *T. hydatigena*/*C. tenuicollis* (Kilinc *et al.*, 2019; Ohiolei *et al.*, 2019; Ohiolei *et al.*, 2021). Extensive DNA sequence analysis data targeting different mitochondrial genes from a wide range of hosts and different geographical locations are requisite to resolve the issues of existence of central or widespread haplotype of *T. hydatigena* (Ohiolei *et al.*, 2021).

Conclusion

The results of the present pilot study on phylogeographical analysis of *C. tenuicollis* from goats highlighted a low genetic diversity of *T. hydatigena* metacestodes across north India. Considering the

present study as first step, future detailed studies involving different mitochondrial genes, vast host range and different geographical locations of India are warranted for understanding the molecular ecology and population genetics of *T. hydatigena* prevailing in India. This will also help in designing and implementation of effective control strategies.

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Author contributions. A. D. M. was involved in conceptualization, sample collection, investigation, methodology and writing. A. K. N. was involved in sample collection, investigation and laboratory work. S. V. was involved in supervision and editing. S. D. T. drafted, reviewed and edited the manuscript. D. S. was involved in sample collection and investigation. All the authors contributed to the review.

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Conflict of interest. None.

Ethical standards. No studies involving laboratory animals or invasive techniques were conducted. The samples were collected from slaughtered animals at abattoirs.

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