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Original Article

Molecular Detection of *Dipylidium caninum* in Isolated Fleas of Dogs in Meshkinshahr County, Northwest of Iran

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Abstract

Background: Fleas (Insecta: Siphonaptera) are considered as highly specialized bloodsucking on mammals such as humans, livestock, dog, cat, rabbit, squirrels, rats, and mice. The desire for blood feeding from warm-blooded animals has led to becoming an intermediate host for some tapeworms like *Dipylidium*. The aim of this study was to detect *D. caninum* larval infection in fleas of dogs living in Meshkinshahr County, northwest of Iran.

Methods: Fleas were collected from 42 dogs using brushing the hair in Meshkinshahr for one year (2014-2015). After the morphological study, fleas were preserved in 90% ethanol for molecular identification. After DNA extraction, the 28S ribosomal RNA gene (~670 bp) of *D. caninum* was amplified using specific primers. Finally, the PCR products were sequenced.

Results: Over, 974 fleas were collected from the dogs. In the morphological study, three species *Ctenocephalides canis*, *Ct. felis*, and *Pulex irritans* were identified. PCR and sequence analysis results showed that 4 isolates *Ct. Canis* were infected with *D. caninum*. Also, no positive specimens were isolated from the other two species.

Conclusion: *Ct. canis* is reported as the most important species of fleas in transferring *D. caninum* in that region.



Introduction

Different species of arthropods such as fleas, ticks, mites, and lice actually live on the body of dogs as ectoparasites. Fleas are a group of ectoparasites that their body is laterally compressed. They are directly involved in the transmission of some diseases such as bubonic plague, murine typhus, tularemia and listeriosis (1). They can serve as a host for *Dipylidium caninum* and *Hymenolepis diminuta* (2). One of the most important families of that species involved in the transmission of the disease to humans is polycidae family. The *Ctenocephalides canis*, *Ct. felis*, *Pulex irritans*, *Xenopsylla cheopis* and *X. austria* are species in this family (3). Several studies have shown that three flea's species *Ct. canis*, *Ct. felis* and *P. irritans* have already been reported from dogs (4). *Ct. canis* and *Ct. felis* are known as intermediate host of many pathogens like *D. caninum* in dogs that some of them have a zoonotic role (5).

D. caninum is a cosmopolite tapeworm parasite in the small intestines of dogs and cats is considered as a major public health problem in many parts of the world (6). Gravid segments are passed complete in the feces or appear in the perianal region of the host. After eating of an egg by the intermediate host (flea *Ctenocephalides* spp.), an oncosphere is released into the flea's intestine. The oncosphere enters the intestinal wall, grows in the flea's hemocoel, and change into a cysticercoïd larva. The dogs becomes infected by ingesting the adult flea infected by the cysticercoïd (7). Human become accidental hosts after ingestion of infected fleas. The infection is more prevalent in infants and young children due to their playing behaviors and their nearness with the dogs and cats (8).

Meshginshahr is an endemic area for canine visceral leishmaniasis, *Dirofilaria* and other pathogens parasites which some of them are pathogenic and zoonotic (9). The aim of this

study was to detect *D. caninum* larval infection in fleas of dogs living in Meshkinshahr County, northwest of Iran.

Materials and Methods

Study area

Ardabil Province is located in the North West of Iran and Meshginshahr County is located in Ardabil Province (Fig. 1).

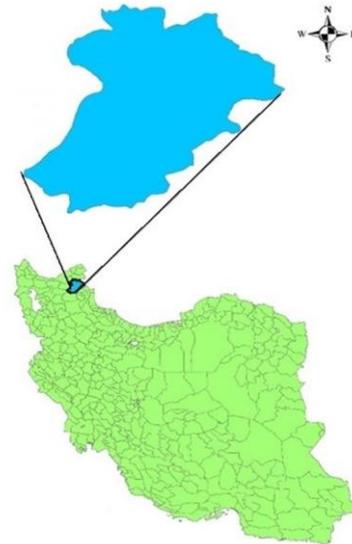


Fig. 1: Meshginshahr County (area of study) in Ardabil province, North West of Iran.

Sample population

In this area, 42 dogs were examined for *D. caninum* infection and flea infestation. The dogs' age ranges were between 1 and 5 years old and were examined from Meshkinshahr and seven different villages, named Parikhhan, Sarikhanloo, Urkandi, Ahmad Bigloo, Agh-balagh, Koojangh.

Ethics approval

This study was authorized by the Ethics Committee of Tarbiat Modares University (No. 6572- 1393.10.14), Tehran, Iran. We

conducted this study in accordance with the guidelines proposed by Helsinki Declaration.

Collecting fleas

The fleas were collected from the body of dogs using brushing hair. In some cases, the fleas were collected by the use of forceps. Samples collected from each dog were finally stored in 96% ethanol for further study.

Morphological study

Fleas were cleaned with water and immersed in lactophenol for 1 hour. Then each of them was fixed in between microscope slides and cover glass using lactophenol. After mounting, all fleas were counted at $\times 40$ and identified at $\times 400$ microscopically (10).

Molecular analysis

DNA is extracted from fleas using the method of Ish-horowicz (11). In PCR, a 670 bp fragment was amplified at the 28S rDNA locus. The specific primers were used to identify the contamination of fleas and blood. The primers were (5'-CAC ATT CAA CGC CCG ACT CCT GTA G-3') for forward and (5'-GCA TGC AAG TCA AAG GGT CCT ACG-3') for reverse (12). The PCR was performed for each flea species specimen separately. The amplification conditions were: 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for the 30s; annealing at 52 °C for 30s, and extension at 72 °C for 40 s; with a final extension step at 72 °C for 10 min. The amplified product was visualized follow-

ing DNA staining (FluoroDye DNA Fluorescent) of agarose (1.5%) gels. The PCR products were sequenced by the ABI3730XL sequence analyzer (Macrogen, Korea). The sequences were edited and aligned using ClustalW

(<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) and compared with reference sequences from GenBank.

For 28s ribosomal DNA sequences of various orders of Eucestoda, the phylogenetic tree was built with the maximum likelihood (ML) algorithm using molecular evolutionary genetics analysis (MEGA) software (version 7.0), including the sequence of representative *D. caninum* isolate from our study.

Results

Morphological study

Forty-two dogs, consisting of 20 females (47.62%) and 22 males (52.38%) were examined. A total of 974 fleas belonging to the genus *Ctenocephalides* and *Pulex* were collected from each dog separately in different fixed localities in Meshginshahr County. The geographical features of the studied area and dispersion of the captured hosts are shown in Table 1.

The dog flea, *Ct. canis* was the most common flea infesting 100% of dogs, and *P. irritans* was identified on 8/42 (19.47%), and *Ct. felis* was identified on 7/42 (16.66%) respectively.

Table 1: Geographical features of the studied Area and dispersion of the captured hosts

Area	Geographical Features		No. dogs (%)	No. collected fleas (%)
Meshkinshahr	N382246	E474054	7 (20)	101 (9.6)
Parikhan	N382452	E473852	5 (14.4)	152 (14.4)
Sarikhanloo	N383105	E473110	3 (8.5)	28 (2.7)
Urkandi	N382115	E473804	6 (17.1)	351 (33.3)
Ahmad Bigloo	N382441	E473336	3 (8.5)	187 (17.7)
Aghbalagh	N382102	E474023	62 (5.7)	6 (0.6)
Koojanganh	N382918	E473053	8 (22.8)	204 (19.4)
Ahmad Abad	N382200	E473534	1 (2.9)	24 (2.3)

Molecular identification of *D. caninum* in fleas

Molecular analysis was performed on fleas (192 isolates of *Ct. canis*, 12 isolates of *Ct. felis*, and 18 isolates of *P. irritans*) taken from dogs in order to identify *D. caninum*. Only 4 isolates

of *Ct. Canis* were positive for the DNA of *D. caninum* (Fig. 2). None of the *Ct. felis* and *P. irritans* were infected with *D. caninum* (Table 2). Also, PCR was carried out on the fleas of healthy dogs and none of them were infective.

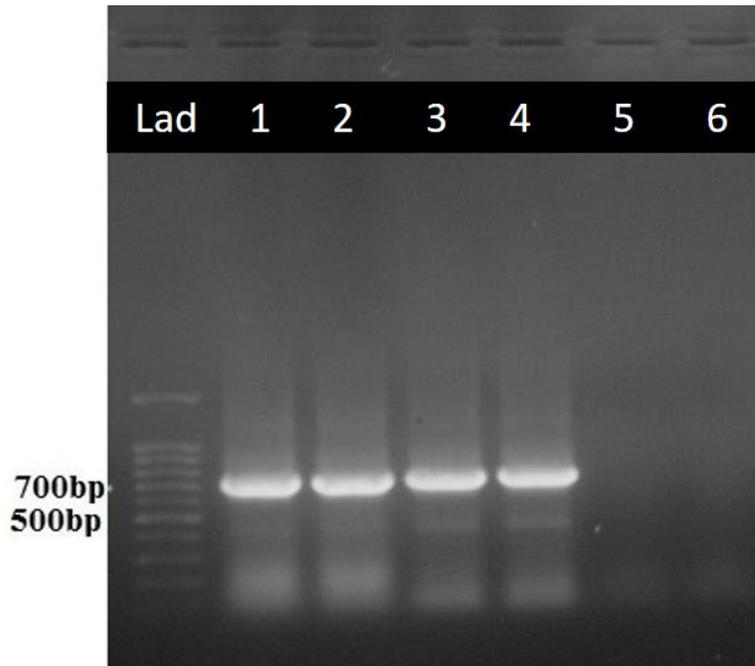


Fig. 2: The results of PCR based on rDNA extracted *D. caninum* from fleas collected from dogs in Meshginsahr County. The amplified ~670 bp product from the positive sample *D. caninum* was subjected to electrophoresis in 1.5% agarose gel. A 100-bp ladder (Lad), Positive samples (1-4), Negative samples (5, 6)

Table 2: The results of fleas morphological study, PCR based *D. caninum* from fleas, collected from dogs in Meshginsahr County

<i>Species of flea</i>	<i>No.</i>	<i>No fleas were examined by PCR</i>	<i>PCR positive</i>
<i>Ct. canis</i>	930	192	4
<i>Ct. felis</i>	12	12	0
<i>P. irritans</i>	32	18	0

Multiple alignment and phylogenetic analysis

To assess genetic diversity among isolates, multiple alignments of the various isolates with the sequences produced in the present study was generated.

The phylogenetic analysis was constructed using Maximum Likelihood (ML) for 28s ribosomal DNA (Fig. 3) and revealed the sequence of our isolated clustered to dipylidiidae of cyclophyllidean cestodes. The sequence is accessible under GenBank Accession No. MG774549.

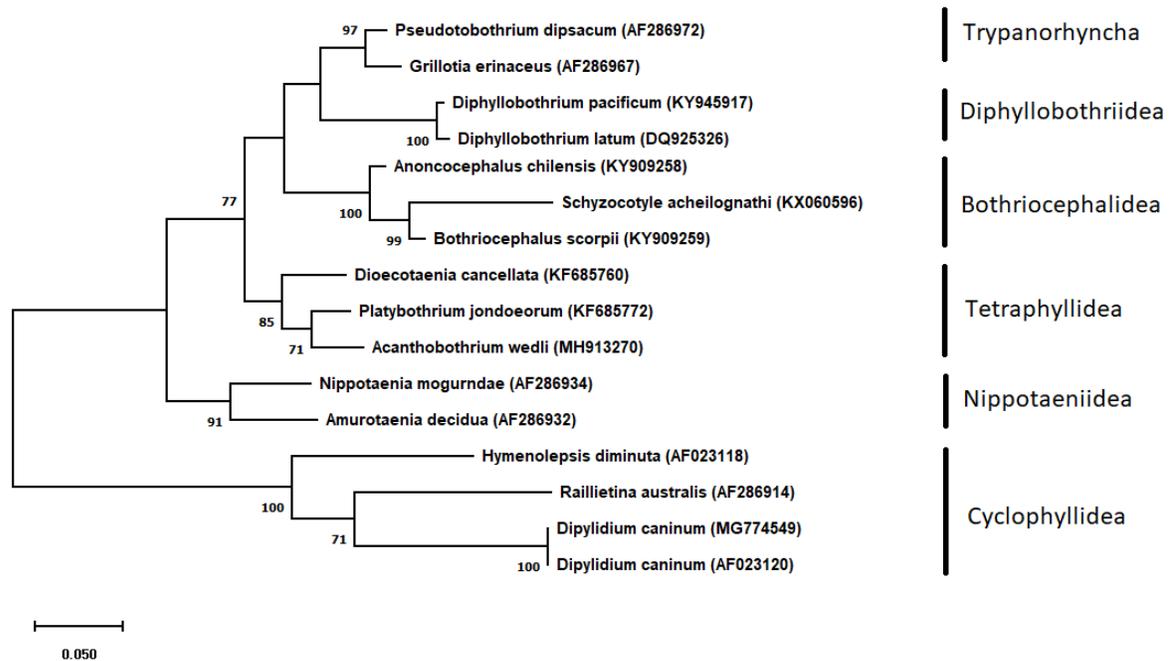


Fig. 3: Phylogenetic relationships based on 28s ribosomal DNA sequences of various orders of Eucestoda inferred by the maximum likelihood (ML) algorithm. Maximum likelihood algorithms employing bootstrapping of more than 500 replicates. The number above branches are bootstrap values greater than 75%

Discussion

There are many reasons that fleas might represent efficient agents for the natural transfer of infective pathogens. Some of them include the duration of blood feeding, the mode of obtaining blood, digestion, and contact with the host, and frequency of host exchange. In this study, 42 Sheepdogs (20 females and 22 males) from Meshkinshahr district in Ardabil province were examined for *D. caninum*. In a systematic analysis conducted in Iran, the rate of infection of *D. caninum* was estimated at 20.45% of dogs in Iran (13). The prevalence of this infection among dogs have been reported in Mexico (2.3-2.8%) (14), Italy (0.7%) (15), Nigeria (0.2-75%) (16–19), Brazil (36.8-45.7%) (20–23), India (23.33%) (24) and Poland (4.07%) (25).

A total of 947 fleas were identified in three genera including *Ct. canis* (95.48%), *Ct. felis* (1.24%), and *P. irritans* (3.28%). In a study conducted in 2010 in Iran, contrary to this study, *Ct. felis* and *P. irritans* had the highest frequency (42% and 26.5%, respectively) compared to *Ct. canis* (16.5%) (26). The prevalence of *Ct. felis* and *P. irritans* isolated from dogs in different parts of Europe was 76.4%, 7.5%, and 15.9%, respectively (12). In contrast to our study and that of Europe, in the United States, *Ct. felis* has the highest frequency (61%) compared to the two *Ct. canis* (21.2%) and *P. irritans* (12.7%) (27).

Based on dissection and microscopic examination of the fleas for searching cysticeroid larvae of *Dipylidium*, Hinaidy (1991) examined fleas of cats and dogs in Austria. He reported flea infestation with cysticeroid of *D. caninum* at rates of 2.3% in *Ct. felis* of cats, 1.2% of

dogs, and 3.1% in *Ct. canis* of dogs (28). In another study, *Ct. felis* from dogs, were dissected and examined for the presence of cysticercoid in Brazil. The percentage of flea infestation was 0.4 (29).

This is the first molecular study on fleas of dogs for the detection of cysticercoid larvae of *D. caninum* in Iran. PCR assay, detected *D. caninum* in 4/192 (2.08%) cases of *Ct. canis*. No positive samples were observed in *Ct. felis* and *P. irritans* isolates. Furthermore, none of the fleas isolated from healthy dogs were infected with cysticercoid larvae of *D. caninum*. Based on PCR detection of *D. caninum* DNA in *Ct. felis* fleas collected from cats, the infection rates of the parasite in fleas have been calculated in Europe 2.23%, the Czech Republic 3.13%, France 1.05%, Germany 1.44%, Hungary 5.79%, Portugal 0.68%, Romania 16.67% and Slovenia 1.26%. In dogs, the infection rates of the parasite in fleas have been calculated in Europe 5.2%, France 3.63%, Sicily (Italy) 14.39%, and in Albania, Bulgaria, Germany, Hungary, Romania 0% (12).

There is a low risk for dipylidiasis in humans and most of the reported cases are related to infants and children (30). It is either due to the more resistance of adults to *D. caninum* or the higher probability of accidental ingestion of a contaminated flea by children. The parasite's load on humans is directly related to the number of cysticercoid larvae in the flea and the number of swallowed fleas because the parasite does not multiply in the human body (31,32). So far, there have been numerous reports of infection in infants and children from around the world, including the United States (33), Poland (34), Chile (32), China (35), and Greece (36). However, there are reports of human dipylidiasis in adults (37,38). Risk factors for infection include contact with animals, contaminated soil, and public playgrounds (38). Ground contamination has been directly linked to stray and free-ranging animals, which are less health-friendly. Collecting stray animals, such as dogs and cats, from urban areas and providing veterinary care for them will

increase public health and will reduce the risk of transmitting zoonotic infections.

Conclusion

The parasite found in *Ctenocephalides* fleas highlights the need to establish a regular control program for flea and *Dipylidium* of dogs in Meshkinshahr.

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Conflict of interest

The authors declare no conflict of interest.

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