Original Article

Molecular Identification of *Leishmania infantum* kDNA in Naturally Infected Dogs and Their Fleas in an Endemic Focus of Canine Visceral Leishmaniasis in Iran

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Abstract

Background: Fleas (Insecta: Siphonaptera) are considered as highly specialized bloodsucking on mammals such as dogs. The existence of three factors, namely a vast distribution area, different hosts, and digestive system with a specific mechanism for digesting blood has led to species of fleas who nourish from mammals be introduced as the potential vectors of diseases. The aim of this study was to assess *Leishmania infantum* natural infection of dog fleas in northwest Iran in 2018.

Methods: A total of 20 infested domestic dogs (*Canis lupus familiaris*) were randomly selected from 5 villages. Fleas were collected using brushing against dog hairs and fine forceps. Then, they were morphologically identified and preserved in ethanol for molecular assay. The kinetoplast DNA of the parasite was used for detection of *Leishmania infantum* using a semi-nested polymerase chain reaction (PCR) assay.

Results: The human flea, *Pulex irritans*, and the cat flea, *Ctenocephalides felis* were identified on 40% and 35% of dogs, respectively. The results of PCR indicated that *L. infantum* was found in the *Ctenocephalides canis* (75%) and *C. felis* (66.7%) collected from infected dogs. No leishmanial infection was observed in *P. irritans*.

Conclusion: It is concluded that fleas could be infected by *Leishmania infantum*, but maintenance of the parasite and their vectorial competence needs to be determined.

Keywords: *Ctenocephalides canis*; *Ctenocephalides felis*; *Leishmania infantum*; *Pulex irritans*

Introduction

Fleas (Insecta: Siphonaptera) are considered as highly specialized bloodsucking on mammals such as humans, livestock, dog, cat, rabbit, squirrels, rats, and mice (1). They transmit agents of some diseases such as bubonic plague, murine typhus, tularemia, and listeriosis (2). One of the most important families of fleas that transmit dangerous diseases to humans is Pulicidae. *Ctenocephalides canis*, *C. felis*, *Pulex irritans*, *Xenopsylla cheopis*, and *X. austria*, are species of this family (3). Several studies have shown that already various species of fleas have been reported from dogs and in most of these studies, three species *C. canis*, *C. felis*, and *P. irritans* have been observed (4). *Ctenocephalides canis* and *C. felis* are the known vector of many pathogens of dogs that some of them have a zoonotic role (5). Due to the biological flexibility of *C. canis*, *C. felis*, and the global distribution of dogs and cats, these species are

the most common flea in the world. In addition, these insects can transfer a great variety of bacterial, viral, and fungal diseases from dogs to humans and other hosts (5).

Leishmaniasis is endemic in many parts of the world and considered as a major public health problem. There are two forms of this disease in Iran: cutaneous and visceral leishmaniasis. Visceral leishmaniasis is a life-threatening disease caused by *Leishmania infantum* and is one of the most important diseases transmitted by vectors. *Leishmania* parasites are transmitted by sand flies infected with the parasite. In the Old World, *Phlebotomus* (Diptera: Psychodidae) is the only known biological vector of *Leishmania* spp. parasites (6). In the Mediterranean area, Canidae has a definite role in the transmission of visceral leishmaniasis (6). Previous studies have shown that even if there are no *Phlebotomus*, healthy dogs may receive *Leishmania* parasites from infected dogs. It shows that other species of arthropods such as fleas, ticks, mites, and lice may be involved (7). Fleas can receive many types of microorganisms during blood-feeding. This study was focused on the fleas gathered from healthy dogs and those infected with *Leishmania* parasites and assessing natural infection of dogs to *Leishmania* spp. and their fleas in Meshkin-Shahr County in Ardabil Province.

Materials and Methods

Study area

Meshkin-Shahr County locates in the Ardabil Province in the northwest of Iran (Fig. 1). Meshkin-Shahr County with geographical coordinates 38° 44’ N and 47° 40’ E and an altitude of 4811 meters above sea level (8).

Sample population

In this study, a population of 20 dogs and their fleas were examined to detect *Leishmania* parasites. The dogs were collected from five different villages, named Ahmad Abad, Kojanagh, Parikhan, Ur Kandi, and Sarikhanlou in Meshkin-Shahr County, Ardabil Province in 2018. The dogs’ age range was between 1 and 5 years old, which was estimated by the teeth.

Blood collection and DAT examination

Blood samples from dogs were prepared. A blood sample (8ml) was collected from the saphenous vein of each dog. Then, each blood sample was divided into two parts. One part was used for Direct Agglutination Test (DAT) and another part was transferred to the laboratory for molecular tests. Blood samples were stored at -20 ℃ until tested. The collected blood samples were centrifuged at 800g for 5–10 min and the sera were separated and stored at -20 ℃ until examined by DAT. Afterward, samples were tested according to the protocol described by Harith et al. (9).

Collection and morphological identification of fleas

All fleas were collected from the body of dogs using brushing against their hair. In some cases, fleas were collected by fine forceps. Then, the collected specimens from each dog were finally conserved in 70% ethanol and identified by the standard keys. At the laboratory, fleas were cleaned by water and immersed in lactophenol for 1 hour. Finally, temporary mounts were prepared with lactophenol to identify the collected fleas. After mounting, the slides of fleas were evaluated using a light microscope (×400) and identified by the standard keys (10).

Molecular study

In this study, the method of Ish-Horowitz was used to extract the DNA of *L. infantum* from fleas (11). Also, DNA of parasites was extracted from blood samples using the Genomic DNA Extraction Kit (Bioneer) and stored at -20 ºC for later use. For the detection and identification of species of *Leishmania* parasites, the sequence of kinetoplast DNA (kDNA) was used. A Semi-nested PCR assay was carried out to detect the *Leishmania* infection of

blood samples and collected fleas from healthy and infected dogs. The primers introduced by Aransay et al. (2000) were applied for the detection of leishmanial infection of blood samples and fleas. The sequences of the primers were LINR4 (5'- GGG GTT GGT GTA AAA TAG GG -3') for forward and LIN17 (5'- TTT GAA CGG GATTTCTG -3') and LIN19 (5'- CAGAACGCCCCTACCCG -3') for reverse used in a semi-nested PCR technique (12). In the semi-nested PCR assay, the kDNA was amplified using LIN primers (length 720bp). The PCR test was performed individually for each species of fleas. In the first round of the PCR, 8µl of the master mix, 1µl of each primer (LINR4, LIN17), 2µl of sterile distilled water, and 3µl of DNA were used. The amplification conditions were 94 ºC for 5min, followed by 30 cycles of denaturation at 94 ºC for 30sec; annealing at 52 ºC for 3sec and extension at 72 ºC for 40sec with a final extension step at 72 ºC for 5min. The second round of the PCR was performed with 8µl of the master mix, 1µl of each primer (LINR4, LIN19), 3µl of sterile distilled water, 2µl of the product of the first round of the PCR, in 33 cycles (94 ºC for 30sec, 58 ºC for 30sec and 72 ºC for 1min) and a final extension at 72 ºC for 10min. PCR products were visualized by UV rays in a 1.5 % agarose gel after electrophoresis with a safe stain solution.

Results

Twenty dogs, including seven females (35%) and 13 males (65%) were examined. Using DAT (8), a high level (titer) of antibody (above 1/320) was observed in 12 out of 20 dogs and eight dogs did not have a high level (titers) of antibody. A total of 974 fleas belonging to the genus *Ctenocephalides* and *Pulex* were collected from dogs in different localities. Three species *C. canis*, *C. felis*, and *P. irritans* were identified which *C. canis* was the most abundant (95.48%) followed by *P. irritans* (3.28 %) and *C. felis* (1.23%). The dog flea, *C. canis* was the most common flea on all dogs (100%), and *P. irritans* and *C. felis* were found on 8 (40%) and 7(35%) of dogs respectively (Fig. 2, Table 1). Out of 12 types of a blood sample which were positive by DAT, 10 samples were positive using semi-nested PCR method (Fig. 3). The molecular test was done on 192 *C. canis*, 12 *C. felis*, and 18 *P. irritans* that had been collected from infected dogs to identify *Leishmania* parasite. 160 (83.3%) of *C. canis* (Fig. 4) and 9(75%) of *C. felis* (Fig. 5) were infected with *L. infantum* and no infection was observed in *P. irritans*. By semi-nested PCR, it was found that the collected fleas from healthy dogs are not infected with *L. infantum* (Table 1).

Table 1. The number of collected fleas from healthy and infected dogs and the results of *Leishmania infantum* molecular tests in fleas collected from dogs using the PCR method, Meshkin-Shahr County, Ardabil Province in 2018

Species	**The number of collected fleas**		**The number of tested fleas**		**Fleas infection with *Leishmania infantum***			
					The infected fleas with healthy host		**The infected fleas with infected host**	
Canis lupus familiaris	Healthy dog	Infected dog	Healthy dog	Infected dog	Number	%	Number	%
Ctenocephalides canis	72	858	72	192	0	0	160	83.4
Ctenocephalides felis	0	12	0	12	0	0	9	75
Pulex irritans	10	22	10	18	0	0	0	0

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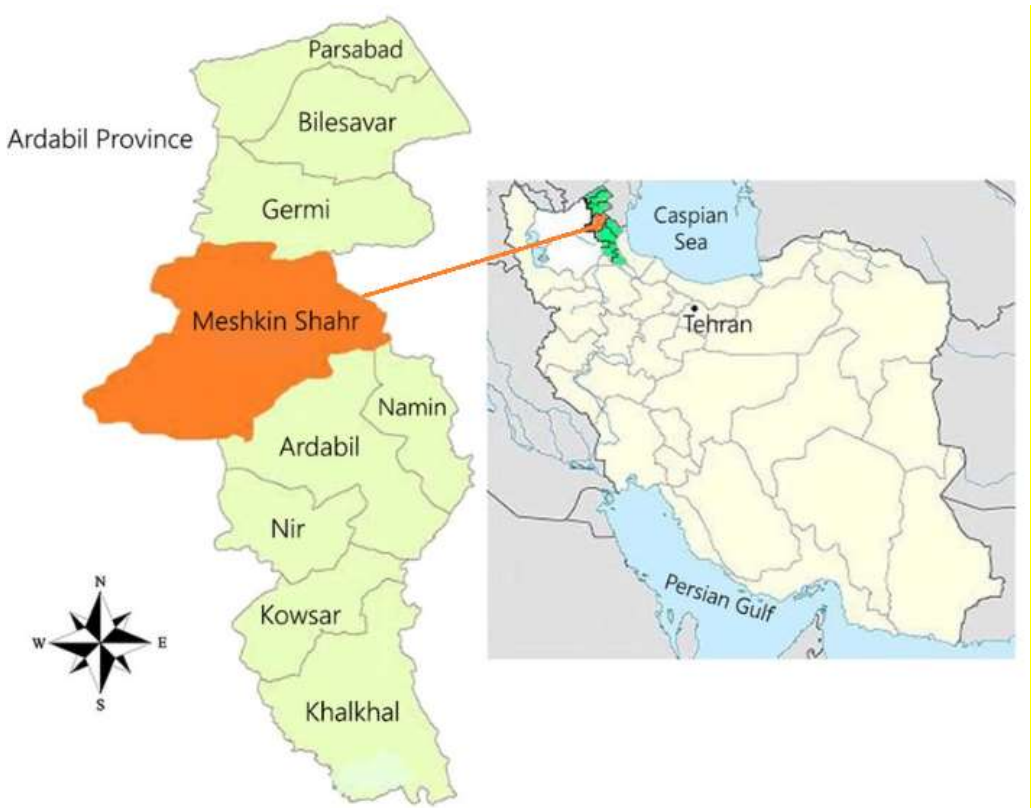

Fig. 1. Location of Meshkin-Shahr County in Ardabil Province, Iran, 2016

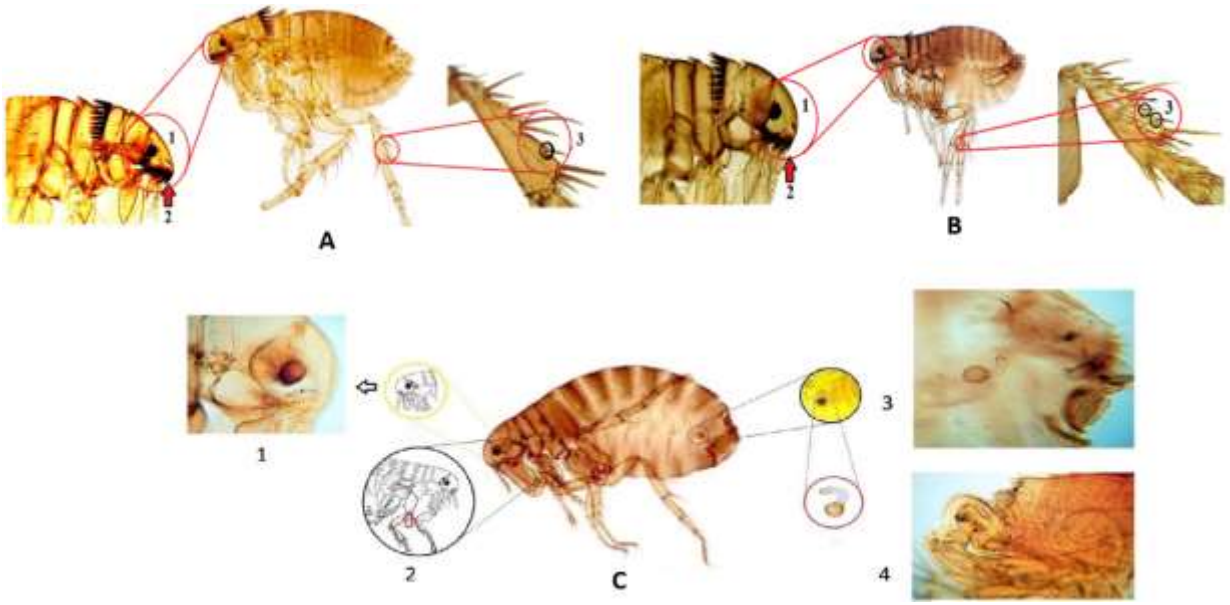

Fig. 2. A: Adult of *Ctenocephalides felis*; 1 shape of the head, 2 length of the first spine of the genal comb, and 3 One short, stout bristle in the interval between the postmedian and long apical bristles of the dorsal margin of the hind tibia, **B: Adult of *Ctenocephalides canis*;** 1 shape of the head, 2 length of the first spine of the genal comb, 3 Two short, stout bristles in the interval between the postmedian and long apical bristles of the dorsal margin of the hind tibia, **C: Adult of *Pulex irritans*;** 1 Eye hair in this type is under the eye, 2 the borderline of moral rood does not exist in this type, 3 the first part of spermatheca not distinguishing, 4 the picture of the ending part in the male gender

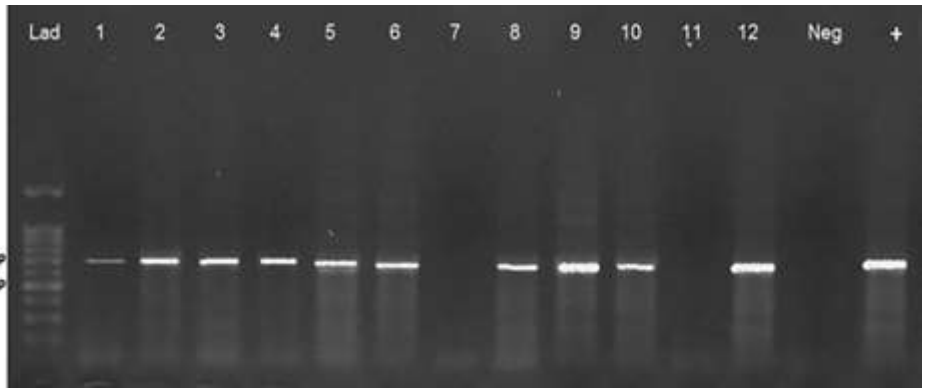

Fig. 3. Agarose gel (1.5%) electrophoresis of semi-nested PCR products (720bp) of DNA extracted from blood samples of *Leishmania*-free and infected dogs with *L.eishmania infantum*. Lad: A 100bp ladder (SinClon, Iran); lanes 1–6, 8–10, and 12: PCR products of infected blood; lanes 7 and 11: PCR products of *Leishmania*-free blood samples; Neg: a negative control free of DNA template and lane +: positive control for *L. infantum*

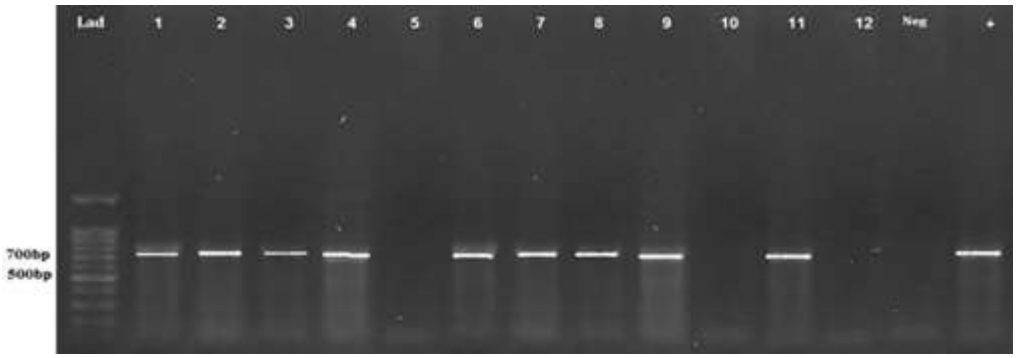

Fig. 4. Agarose gel (1.5%) electrophoresis of semi-nested PCR products (720bp) of DNA extracted from *Ctenocephalides canis* collected from *Leishmania*-free and infected dogs with *Leishmania infantum*. Lad: A 100bp ladder (SinClon, Iran); lanes 1–4, 6–9 and 11: PCR products of infected fleas; lanes 5, 10, and 12: PCR products of *Leishmania*-free fleas; Neg: a negative control free of DNA template and lane +: positive control for *L. infantum*

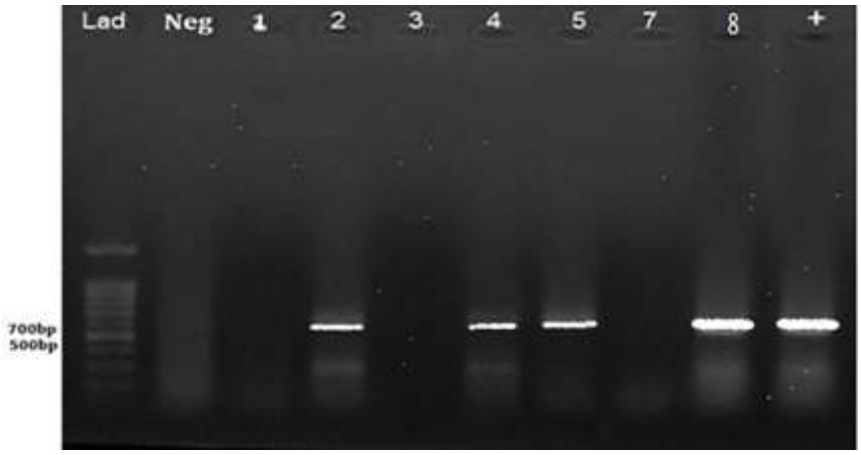

Fig. 5. Agarose gel (1.5%) electrophoresis of semi-nested PCR products (720bp) of DNA extracted from *Ctenocephalides felis* collected from infected dogs with *Leishmania infantum*. Lad: A 100bp ladder (SinClon, Iran); Neg: a negative control free of DNA template; lanes 2, 4, 5, and 8: PCR products of infected fleas; lanes 1, 3, and 7: PCR products of *Leishmania*-free fleas and lane +: positive control for *L. infantum*

Discussion

Fleas are one of the most important arthropods in transmitting many diseases. There are many reasons that fleas may have a role in the transmission of infectious pathogens. Some of them include a long time of blood-feeding, the mode of obtaining blood, digestion, and contact with the host, and the frequency of host exchange. In the present study, 12 out of 20 (60%) dogs were infected with *L. infantum* using DAT. But PCR assay confirmed leishmanial infection in 10 out of them (Fig. 2). Three species of fleas collected including *C. canis*, *C. felis*, and *P. irritans*. All the caught dogs (100%) were infested with 974 fleas. The fleas are the most common ectoparasites in domestic dogs of the Meshkin-Shahr area. In recent studies, the probable role of fleas in the transmission of *Leishmania* parasites has been discussed. In our study, *L. infantum* was observed in 9/12 (75%) of *C. canis* (Fig. 3) and 4/6 (66.67%) of *C. felis* (Fig. 4) using semi-nested PCR and *P. irritans* fleas did not infect with the parasite. Also, no infection was observed in fleas collected from *Leishmania*-free dogs by PCR assay. Lainson and Shaw (1985) isolated agent of visceral leishmaniasis from 8 *Lutzomyia longipalpis* and 3 *Lutzomyia antunesi* on the island of Maraio (13). There are sporadic cases of visceral leishmaniasis on this island (13). In another study conducted, kDNA of *L. infantum* was isolated from salivary glands of collected ticks (*Rhipicephalus sanguineus*) from infected dogs in southern Italy. In this study Dantas-Torres et al. performed by PCR technique, kDNA of *L. infantum* in the larvae of *R. sanguinus* tick was isolated after 4 months of experimental infection, which represents the possibility of transovarial transmission of *Leishmania* parasites in the ticks (14). Dabaghmanesh et al. (2016) collected a total of 180 *Leishmania*-free ticks collected from fields and bred on lab rodents, were divided into eight groups, and allowed to feed on a dog for fixed periods of time. The infection rate was significantly higher in female than male ticks. The rates were higher among nymphs than adult ticks. The kDNA of *L. infantum* was not detected in ticks 24h post-feeding. It was, however, positive among the second to fourth groups of nymphs and adult ticks. Eggs and unfed larvae recovered from the third and fourth adult groups were 100% PCR-positive. The data revealed the passage of *L. infantum* kDNA in nymphs and adults of brown dog tick following fixed time intervals post blood feeding on an infected dog (15). Coutinho and Linardi in 2007 conducted a study on the possibility of transmission of *Leishmania chagasi* caused by devouring collected fleas (*C. felis*) from infected dogs in the Golden Hamsters (*Mesocricetus auratus*. In this study, of the fleas collected, 4/207 (1.9%) showed the presence of promastigotes in smears stained by Giemsa, whilst 43/144 (29.9%) exhibited positive PCR assay for DNA of *Leishmania* parasites. Fourteen of the hamsters tested PCR amplification and 4 of them by indirect fluorescent antibody test (IFAT) were positive with *Leishmania* parasites. In addition, out of 16 infected hamsters, 11 had been infected peritoneally and 5 orally (16).

In 1932, Wenyon reviewed most existing theories about the transmission of *Leishmania* infection and believed that ticks have no role in the transmission of visceral leishmaniasis in the Mediterranean region (17). Prior to this study, similar cases on the likelihood of transmission of the disease had not been made on dogs. The results are very important because it has been shown in practice that *R. sanguinus* ticks have failed to transfer *L. infantum* from infected dogs to healthy dogs.

Conclusions

Visceral leishmaniasis has been increasing worldwide, principally due to a substantial rise in human and domestic animal traffic contributing to spreading leishmanial infection in low or non-endemic areas (18). Female sand flies of

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some species of the genus *Phlebotomus* are the main and proven vectors of *L. infantum* transmission in humans and dog. There has long been speculation about the role of fleas as biologically or mechanically vectors of *L. infantum* and recent studies have reinforced this hypothesis (15, 18). The human flea, *P. irritans*, and the cat flea, *C. felis* were identified on 40% and 35 % of dogs, respectively. The results of PCR indicated that *L. infantum* was in the collected *C. canis* (75%) and *C. felis* (66.7 %) from infected dogs. The result of the present study confirms that fleas can be infected by *L. infantum*. But it is not yet clear whether these insects as vectors are able to transmit *Leishmania* parasites from an infected host to healthy hosts and the vectorial competence of fleas needs to be determined. Given this, it is recommended that the Xenodiagnoses test be carried out to determine the probable role of *Leishmania* spp. transmission.

Acknowledgements

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Ethical considerations

The protocols conducted in this study followed the guidelines of the institutional ethical committee (Tarbiat Modares University). The protocols were approved by TMU ethical committee under registry TMU-3674.

Conflict of interest statement

Authors declare that there is no conflict of interest.

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