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Molecular detection of *Leishmania infantum* in road-killed carnivores from North of Iran, Golestan Province

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ABSTRACT

Visceral leishmaniasis (VL) is an emerging zoonosis disease in countries of the Mediterranean basin caused by *Leishmania infantum*. Although domestic dogs are the main vertebrate hosts, many wild carnivores have been considered playing a role in the spreading of VL. Sporadic numbers of dog and human VL have been reported in Golestan Province in North Iran. The present study was performed to detect the *L. infantum* DNA in wild carnivores. Forty road-killed carnivores including jackals (*Canisaureus*= 20), red fox (*Vulpesvulpes*= 3), wild cat (*Felissilvestris*= 4), jungle cat (*Felischaus*=7), stone marten (*Martesfonia*=2) and least weasel (*Mustelanivalis*= 4) were necropsied and tested for *L. infantum* using PCR. PCR testing was performed on the spleen tissue samples, targeting the minicircle of kinetoplast DNA (kDNA). None of the animals showed typical VL lesion at postmortem examination. Among forty carnivores, just 2 male jackals were detected positive by PCR. The kDNA positive jakals were found in the dry area with lower rainfall comparing those were source of negative samples. Briefly, this study for the first time reports *L. infantum* infection occurrence in jackals in Golestan Province where VL is occurring as an emerging disease. The results support the possible active role of wild canids such as jackals in *L. infantum* life cycle. More studies on wild mammals' population seem necessary for better understanding of their role in *L.infantum* disseminating.

1. Introduction

Visceral leishmaniasis (VL), a systemic lethal disease caused by protozoan parasite *Leishmania infantum* with a broad geographic distribution, is an important zoonotic disease transmitted to the human and mammals by sand flies particularly in tropical and Mediterranean regions (Baneth et al., 2008)

The disease frequency has been increasing in recent decades globally. Domestic dogs have been introduced as the main vertebrate reservoirs for VL (Kuhls et al., 2011). Nevertheless, the participation of other infected mammals, rather than dogs, in the transmission cycle of *L. infantum* in urban areas, was already proposed for cats and opossum (Millán et al., 2011; Schallig et al., 2007).

Some wild canids' species such as Crab-eating fox, puma and skunk have been found to be infected with *L. infantum*. Albeit their role in *L. infamnum* transmission is not clear (Dahroug

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et al., 2010; Souza et al., 2010; Beck et al., 2008).

So far, at least 7 endemic foci of this disease from some areas of Fars province in the South, Ardabil and East Azarbayejan provinces in the Northwest, Khorramabad county in the West, Khuzestan and North Khorasan provinces in the Southwest and Northeast, Qom and Saveh counties in the central of Iran, have been investigated and approved. Every year sporadic cases of VL are reported from other parts of Iran (Mohebaliet al., 2005).

In recent years, VL infection of dogs and humans has been reported in arid and semi-arid parts of Golestan Province. Fakhar et al. have introduced Golestan Province as a new emerging region for VL infection (Fakhar et al., 2014).

Also, Namroodi and her colleagues have reported 15.3%, 8%, 8.3% *L.infantum* seropostivity in dogs, wild rodents and golden jackals' population respectively, in Golestan Province (Namroodi et al., 2013; 2014; 2015).

Golestan is one of the northern provinces of Iran, located in the geographic range of 36° 30' to 38° 8' N latitude and 53° 51' to 56° 22' E altitude, is bounded on the north by Turkmenistan, west by Mazandaran province and Caspian Sea, south by Semnan, and east by North Khorasan province. The climate diversity of the Golestan province is notable, as the southern areas have mountainous climate, central and southern west areas have moderate semi Mediterranean climate, and the northern parts have arid and semi-arid climate (Sharbati, 2012) (Figure 1).

Some wild Carnivora species that are face to danger of extinction, such as Persian leopard and *Vulpes corsac*, live in Golestan Province.

Contrary to the numerous reports of *L. infantum* contamination of dogs, much remains to study in terms of the putative roles of wild carnivores as *Leishmania* spp reservoirs. Study of *L. infantum* contamination in wild carnivores, especially in the areas where sporadic VL occurring, is of fundamental importance to improve understanding of *Leishmania* spp ecology.

There are many available diagnostic approaches, including serological, parasitological and molecular tests. Among the mentioned methods, molecular tests have more sensitivity and susceptibility in detection of *L.infantum* in tissue samples (Bodelão et al., 2014).

So in this study *L.infantum* infection of road-killed carnivores was surveyed in Golestan Province by PCR to clear the possible role of these animals in *L. infantum* life cycle.

Fig 1. Location of Golestan Province in Iran

2. Materials and Methods

2.1. Animals and sampling

Road-killed wild carnivores from Golestan Province consisting of Canidae, Felidae, and Mustelidae family were surveyed in this study during 2015-6 (Table 1). Sample collection was opportunistic since the animals were obtained through vehicle collision. Animals were surveyed for macroscopical features of VL before and during necropsy. Spleen samples were collected during necropsies and preserved frozen at -20 C^o until analyzed. Information about sex, species, and climate of locations where the animals were found was documented in separate sheets (Data not shown).

2.2. DNA extraction and PCR

Total genomic DNA from spleen samples was extracted using a commercial kit (Bioneer Co, South Korea) following the manufacturer's protocol.

A previously reported PCR method with primers (LINR4 and LIN17) targeting the parasite's minicircle of kinetoplast DNA (kDNA) from *leishmania* spp was used in this study (Fakhar et al., 2012). These primers are of the kDNA minicircle families that are used to

identify the *Leishmania* genus. The amplification reaction was carried out in a total of 25 µl containing 250 µM of each deoxynucleoside triphosphate (dNTPs), 2 Mm MgCl2, 1U Taq polymerase (Cinagene, Tehran), 1 µM LINR4 primer (5′-GGG GTT GGT GTA AAA TAG GG-3′), 1 µM LIN17 primer (5′-TTT GAA CGG GAT TTC TG-3′) and 5 µl of extracted DNA, in 1X PCR buffer (Vivantis, Malaysia) and overlaid with mineral oil.

The mixture was incubated at 94ºC for 5 min and PCR program was run for 30 cycles that consisted of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 60 seconds. After the last cycle, the extension was continued for a further 5 min. Reference strains of *L. infantum* and deionized water were used as positive and negative control respectively. PCR products were visualized under UV light after electrophoresis in a 2% agarose gel stained with ethidium bromide. Samples that yielded a 720 bp product were considered positive.

2.3. Statistical analysis

Basic comparison of prevalence values between sexes, species and geographic regions was performed by means of chi-squared tests. Statistical uncertainty was assessed by calculating the 95% confidence interval (CI) for each of the proportions.

3. Results

None of the sampled animals showed typical VL lesion at postmortem examination. *L.infantum* DNA was not detected among all the sampled carnivores except for 2 of the 20 jackals (Table 1). Statistically significant differences in prevalence were observed between species. Detection areas of PCR-positives samples were drier, with less grassland and lower rainfall compared to negative samples.

Table 1. Data on sampled road-killed animals, including species and PCR results on spleen samples

Family	Species	Number of PCR positive samples
Canidae	*Canisaureus*= 20	2
	Vulpesvulpes= 3	0
Felidae	*Felissilvestris*= 4	0
	Felischaus= 7	0
Mustelidae	*Martesfonia*= 2	0
	Mustelanivalis=4	0

4. Discussion

Diseases at the wildlife-livestock-human interface are an increasing concern for public health, animal health and animal conservation authorities worldwide. Also, wildlife-associated infectious diseases are at top of human emerging diseases. Basic epidemiologic data would constitute the foundation for targeted prevention and control measure of wildlife-associated disease (Jones et al., 2008).

Visceral Leishmaniasis was described for the first time in 1908 by Nicolle and Comet (Nicolle and Comet, 1908). From then on, different reports from different parts of the world were published about that.

The Carnivora order includes many wild species that are susceptible to VL. After first report of *L. infantum* infection in crab-eating fox, serological and molecular surveys of *L.infantum* in wild carnivore species have been performed globaly (Courtenay et al., 1996).

Reports of human and dog VL in Golestan Province can be considered as a potentially emerging threat for this province.

The present work aimed to describe possible hosts for *L.infantum* in wild carnivores in this new emerging area, Golestan province. Testing of the presence of *L.infantum* DNA in spleen samples of road-killed carnivores instead of detecting antibody presence was selected because the aim was to measure the occurrence of effective infection rather than detecting exposure.

Among 40 wild carnivores, *L. infantum* was just detected in 2 road-killed jackals. Jackals are found in wild - domestic environment and have wide home range. So they are able to colonize different habitats in different ecotypes. Their high abundance and adaptability to peri-domestic environment reinforce the importance of this species in disseminating of *L. infamtum* in new areas such as Golestan Province (Ziaie, 2006).

A previous report by Namroodi and her colleagues indicates *L. infantum* seropositivity in 8.3% of jackals population in Golestan Province. The importance of this species as reservoirs depend on their ability to transmit the infection to sandflies rather than on their infection rate; it is also a function of their ability to introduce the pathogen into dog population. Also it is said that a reservoir host is a species on which the

parasite depends for its survival and thus serves as a source of infection for other susceptible hosts, including man (Dressen, 1990; Faust et al., 1975).

These findings together with detection of 8.3% *L.infantum* seropositivity in jackal's population by Namroodi and her colleagues in Golestan Province, shows the reservoir potential of golden jackals for *L. infantum.*

Survey on *L.infantum* infection of wild canids is very limited in Iran. Nadim and his colleagues performed the first survey on *L.infantum* infection in wild canids in Iran (Nadim et al., 1970). They detected sign of *L. infantum* infection in one out of 30 sampled canids (foxes and jackals). Also Hamidi and his colleagues surveyed *L. infantum* infection in 161 jackals in Mazandaran Province. Their results showed that 0.04 % of sampled jackals were infected by *L. infantum* (Hamidi et al., 1978). Compared to the mentioned similar studies in Iran, *L. infantum* infection of jackals in this study seems relatively high.

Considering all reports of *Leishmania* spp infection in wildlife in the world, the red fox is apparently the species with the highest frequency of reported cases, although, clinical disease has not been reported in this species. Foxes have been introduced as the wild canine reservoir of VL in southern Europe where foxes are the most abundant wild canid and in some places the prevalence of infection among this population was detected similar to that of dogs in the same areas (Libert et al., 2012; Dahroug et al., 2010; Dipineto et al., 2007; Portús et al., 2002; Criado-Fornelio et al., 2000).

Courtenay and his colleagues, however, reported opposite scenario. They monitored immunologically and clinically a total of 37 Crab-eating foxes for *L.infantum* and reported that Crab-eating fox populations do not maintain an independent transmission cycle of domestic dogs. The implication was that they were unlikely to introduce the parasite into Leishmania-free dog populations (Courtenay et al., 2000). *L. infantum* DNA was not detected in sampled foxes in this study. It seems that low number of sampled foxes was the reason to this results. Nevertheless, the relatively low populations of foxes in Golestan Province and the short dispersion area of sandfly raises doubts to the role of foxes as primary reservoirs of VL in Golestan Province.

None of the sampled wild felids were contaminated with *L. infantum* in this study. Commonly wild felids are occasionally diagnosed with VL and their *L.infantum* contamination has been reported in France (Barbary lion) and Brazil (puma and jaguar). So far, all the infected felids were asymptomatic and their role in life cycle of *L.infantum* is not clear.

There are several reports of *Leishmania spp* infection in wild canids in Europe. Gray wolves (*Canis lupus*), red foxes (*Vulpes vulpes*), Egyptian mongooses (*Herpestesich-neumon*), genets (*Genetta genetta*), Iberian lynxes (*Lynx pardinus*), pine martens (*Martes martes*), and beech martens(*Marte sfoina*) have been diagnosed as asymptomatic carriers in Spain and Portugal (Sastre et al., 2008; Sobrinoet al., 2008; Millán et al., 2011; Mu˜noz-Madrid et al., 2013;Criado-Fornelio et al., 2000).

In a similar study on the road-killed mammals in Brazil, *Leishmania* spp was detected in tissues of Brazilian guinea pig, crab-eating foxes, white-eared opossum, crab-eating raccoon, porcupine and lesser anteater, highlighting the important role of wild mammals in *Leishmania* spp life cycle (Bodelão et al., 2014).

Del Rio and his colleague reported widespread of *L. infantum* infection among wild carnivores including Eurasian badger, foxes, stone martens, wild cats, pole cats and weasels, in peri-endemic areas in Spain (Del Ríoa et al., 2014). In this study *L. infantum* DNA was not detected in weasels.

However, the low number of sampled weasels prevented determining if they were exposed to *L. infantum*.

The source of *L. infantum* infection of jackals is not known in this study. None of the epidemiological studies of *L. infantum* infection in wildlife rule out dog involvement as a source of infection for wildlife (Quinell and Courtenay, 2009). More molecular studies seems necessary for definite diagnosis of *L. infantum* source in this study.

There was not meaningful relation between sex and *L. infantum* infection of sampled carnivores.

The relatively small number of examined carnivores and low infection challenge in the sampled carnivores could partly account for the

lack of an association between PCR results and sex of sampled animals in this study.

Tow *L. infantum* contaminated jackals were detected in arid areas with warm weather. It could be due to suitable conditions for sandfly vector growth in these regions (Mohebali et al., 2005).

Clinical signs of VL have not been recognized in golden jackals. However, since they roam in rural areas and as VL is an important fatal disease of humans and animal health, conservation authority in Iran should be aware of the indirect consequence on conservation and wildlife management caused by their potential role as *L. infantum* reservoirs.

This study highlights that wild canids such as jackals may maintain and serve as a source of VL infection to sand fly vector, providing a constant source of *L.infantum* infection to a peri-domestic transmission system in Golestan Province. Generally, once an infectious disease such as VL is recognized in a new area, the disease becomes endemic and its limitation is confirmed to be very challenging (Diniz et al., 2008; Peterson et al., 2009) This shows the importance of performing of more studies on the role of wild canids in *L. infantum* cycle in Golestan Province.

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