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FIRST DETECTION OF *LEISHMANIA* DNA IN TICKS COLLECTED FROM DOMESTIC AND WILD ANIMALS IN ALGERIA

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Abstract. Herewith, we report the detection of *Leishmania major* and *Leishmania infantum* DNA in ticks collected from diverse animal species in two sites in northern Algeria (Algiers and Tizi-Ouzou). Between December 2019 and April 2020, 422 ticks were collected from dogs, cows, turtles and boars during a period when sand flies, the main vectors, are not very active where the main vectors, sand flies, are not very active. A total of 237 ticks (56.2%) were identified as *Rhipicephalus sanguineus,* followed by 131 *Hyalomma marginatum* (31%), 20 *Ixodes ricinus* (4.7%), 16 *Dermacentor marginatus* (3.8%), 10 *Rhipicephalus turanicus* (2.4%), 7 *Hyalomma aegyptium* (1.7%) and 1 *Hyalomma detritum* (0.2%). The DNA was extracted and the polymerase chain reaction was performed to detect the potential presence of *Leishmania* spp. DNA. The results were positive in four of the collected species*: Rhipicephalus sanguineus, Rhipicephalus turanicus, Dermacentor marginatus* and *Hyalomma aegyptium*. Restriction Fragment Length Polymorphism (RFLP) PCR performed to identify the *Leishmania* species gave profiles of *Leishmania major* and *Leishmania infantum*. The presence of *Leishmania* spp. DNA in ticks from Algeria is reported here for the first time. To the best of our knowledge, *Leishmania major* DNA is detected for the first time in ticks. These findings do not confirm the role of ticks as an active vector of *Leishmania* parasites. However, it is suggested to not overlook the current knowledge during the establishment of Leishmaniasis control strategies until further information.

INTRODUCTION

Leishmaniasis is a disease of public importance. More than 20 species of *Leishmania* cause the disease in humans. The main clinical manifestations that occur depend on the responsible parasite species and subspecies as well. Three main forms of leishmaniasis are observed: visceral, cutaneous and mucocutaneous (WHO 2023).

In Algeria, leishmaniasis causes serious public health problems. Visceral leishmaniasis is due to *Leishmania infantum*. This species is also associated to northern cutaneous leishmaniasis (Belazzoug et al. 1985). In 1990, Izri et al. isolated promastigotes from *Phlebotomus* (*Larroussius*) *perniciosus* in Tizi-Ouzou (Izri et al. 1990).

Zoonotic cutaneous leishmaniasis caused by *Leishmania major* is widely distributed. It is found in areas bellow 300 m in altitude but may be found at even at 1000 m (Dedet et al. 1984). In 1992, Izri et al. (1992) isolated *Leishmania major* from *Phlebotomus papatasi* and confirmed the role of this sand fly as a vector of the parasite. This parasite species extended from Sub-Saharan regions to arid and semi-arid ones and its distribution area is worryingly extending to the north. *Leishmania major* and *Leishmania infantum* can be found in the same areas. *Leishmania tropica* was also identified in the centre-north and north-west of Algeria (Bachi et al. 2019).

Sand flies are the only proven biological vectors of *Leishmania* spp. (Ready 2013). The occurrence of other possible arthropod vectors as fleas and ticks has been discussed but has not yet been demonstrated (Coutinho et al. 2005; Coutinho and Linardi 2007; Dantas-Torres et al. 2010). Multiple studies suggest that ticks could participate as a secondary vector in the transmission of the pathogen, it is particularly the case of *Leishmania infantum* (Dantas-Torres et al. 2010; Solano-Gallego et al. 2012). Ticks (Acari: Ixodida) are the second important vectors, after mosquitoes, of a wide range of viruses, parasites and bacteria causing human diseases. They are the most important vectors of animal pathogens (Yu et al. 2015).

As leishmaniasis occurs in Algeria in very significant

numbers and ticks have been suspected to be vectors of the disease, the aim of this study was to investigate the potential presence of *Leishmania* spp. DNA in ticks removed from numerous hosts in different regions of Algeria. The study was done in the regions where and during a period when sand flies are not very active and sand fly roosts are rare.

MATERIALS AND METHODS

Study area

This study was carried out in two provinces in northern Algeria. The first one is Tizi-Ouzou province, an approximately 3000 km² area with the temperate Mediterranean climate and a population of 1,238,070 inhabitants. Ticks were collected from mountainous and rural localities. The second one is Algiers, the capital. It is approximately a 1200 km² area with the Mediterranean climate and a population of 2,988,145 inhabitants. Here, ticks were collected from domestic pets (Figure 1).

Tick collection and identification

Ticks were collected from December 2019 to April 2020 in the two provinces. In the first one, Tizi-Ouzou, the dogs *Canis lupus familiaris* (Linnaeus, 1758) and cows *Bos taurus* (Linnaeus, 1758) were examined with the verbal consent and in the presence of the owners. Ticks from boars *Sus scrofa* (Linnaeus, 1758) were obtained during hunting trips allowed by local authorities (Figure 2). In the second province, ticks were removed from dogs *Canis lupus familiaris* (Linnaeus, 1758) and turtles

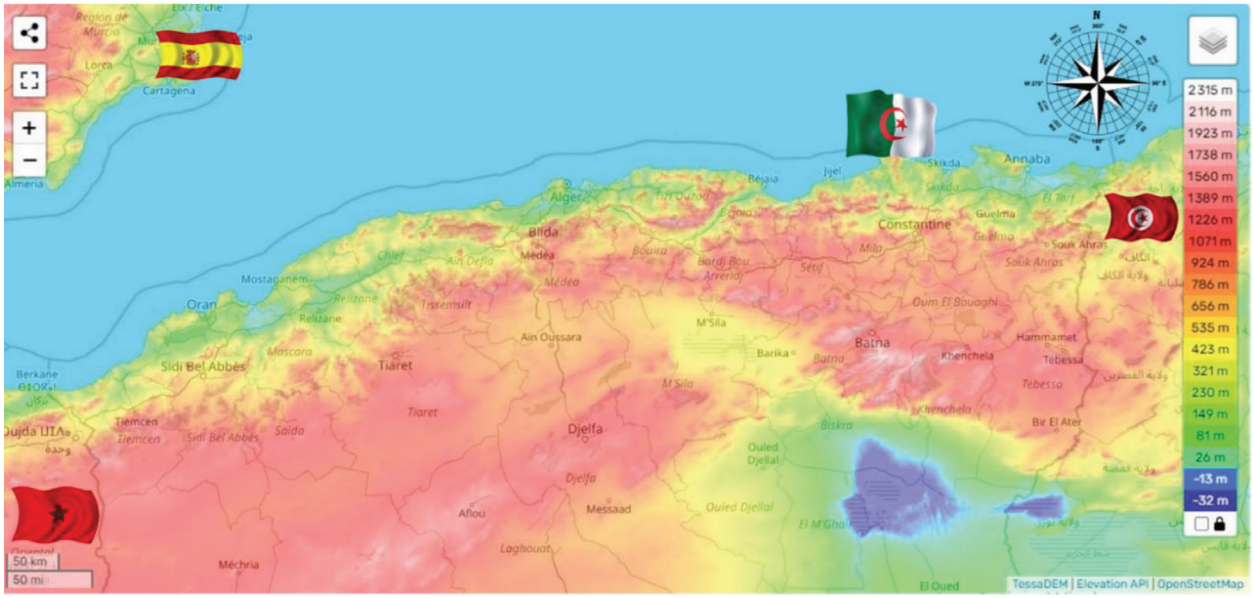

Figure 1. Map showing the altitudes of northern Algeria.

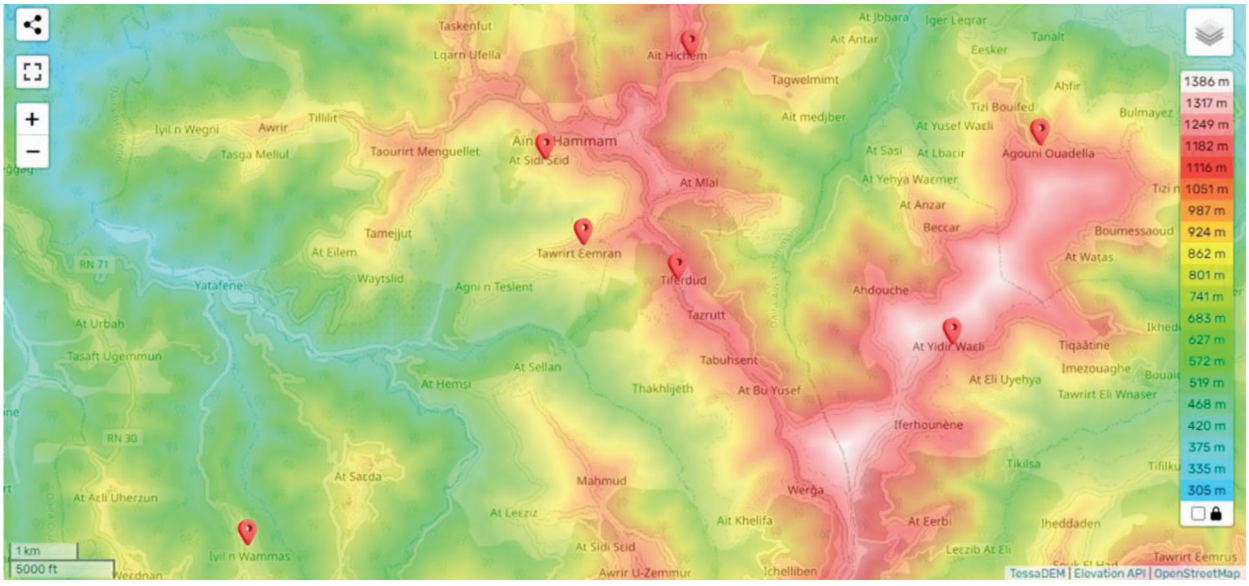

Figure 2. Sampling localities in Tizi-Ouzou province.

Figure 3. Sampling localities in Algiers province.

Testudo greaeca (Linnaeus, 1758) that were examined with the verbal consent of the owners (Figure 3).

After removal, all ticks were immediately placed into vials containing 70% ethanol, and sores of animals were cleaned with hydrogen peroxide when it was possible. Morphological identification to the species level was performed using taxonomic keys (Walker et al. 2003).

DNA extraction and PCR testing

Ticks were rinsed in three consecutive baths of distilled water during 10 min per bath, then dried on paper towels. A longitudinal and symmetrical dissection was performed using a new sterile blade for each tick in order to prevent potential cross contamination. The first half-tick part was stored at -80°C, while the second one was used for DNA extraction and polymerase chain reaction (PCR).

DNA extraction was performed using the Invitrogen™ PureLink™ Genomic DNA Mini Kit according to the manufacturer directions. DNA extracts were screened at first by a conventional PCR using the primers Lei70L (5'-CGCAACCTCGGTTCGGTGTG-3') and Lei70R (5'-CGCGGTGCTGGACACAGGGTA-3') (Spanakos et al. 2002). The reaction was carried out in a 50 µl final volume. The tube contained 10 µl of extracted DNA, 11.6 µl of RNase- and DNase-free water, 25 µl of Master mix 2X, and 1.7 µl of each primer concentrated at 20 µM. PCR was performed in a Biorad Thermal cycler (Biorad MyCycler, France) following a protocol of the first denaturation at 95°C for 15 min, followed by 40 cycles of 95°C for 1 min, 65°C for 1 min and 72°C for 1 min 30 s. The final elongation step was added for 10 min at 72°C. The obtained PCR products were loaded on 2% agarose gel stained with BET and observed under UV for the visualization of 345 base pair bands specific to the *Leishmania* genus.

ITS amplification

The ITS1 region was PCR amplified to identify *Leishmania* species using the primers L5.8S (5'-TGATACCACTTATCGCATT-3') and LITSR (5'- CTTGGATCATTTTCCGATG-3') (El Tai et al. 2001). PCR was performed in a total volume of 50 µl containing 5 µl of 10X buffer, 5 µl of 50 Mm $MgCl_2$, 1 µl of 2.5 Mm deoxynucleotide triphosphates, 0.4 µl of 2.5 U *Taq* DNA polymerase, 25.6 µl nuclease-free water, 1.5 µl of each primer, and 10 µl of Lei70 positive DNA template.

PCR was performed in a Biorad Thermal cycler (Biorad MyCycler, France) following a protocol of the first denaturation at 94°C for 4 min, followed by 40 cycles of 94°C for 40 s, 53°C for 30 s, and 72°C for 1 min. The final elongation step was added for 10 min at 72°C. The obtained PCR products were loaded on 2% agarose gel stained with BET and observed under UV for the visualization of 350 base pair bands corresponding to *Leishmania* spp.

Restriction Fragment Length Polymorphism (RFLP) analysis

Without prior purification, 8 µl of the ITS PCR products were digested during three hours with 1.5 µl of *HaeIII* restriction enzyme 2.500U (Promega), 3 µl of recommended buffer, and 12.5 µl of nuclease-free water at 37°C. The resulting fragments were loaded on 3% agarose gel stained with BET. Electrophoresis was performed at first at 75 V for 10 min then at 80 V for 2 hours. Under UV, results were inspected for species-specific profiles.

RESULTS

A total of 422 adult ticks were collected from various animals in the two provinces in northern Algeria. Seven species belonging to four genera were identified: *Rhipicephalus sanguineus*, *Rhipicephalus turanicus, Dermacentor marginatus*, *Hyalomma marginatum*, *Hyalomma detritum, Hyalomma aegyptium*, and *Ixodes ricinus* (Table 1).

Rhipicephalus sanguineus was the most frequent species (39.8% males, 16.4% females), followed by 131 *Hyalomma marginatum* (23.2% males, 7.8% females), 20 *Ixodes ricinus* (1.4% males, 3.3% females), 16 *Dermacentor marginatus* (1.9% both males and females), 10 *Rhipicephalus turanicus* (2.4% males), 7 *Hyalomma aegyptium* (1.4% males, 0.2 % females) and 1 *Hyalomma detritum* (0.2% males).

PCR results of detection of the genus *Leishmania* with Lei70 were positive for 125 ticks (29.62%). The four tick species that gave the positive result were: 99 *Rhipicephalus sanguineus* (79.2 %), followed by 16 *Dermacentor marginatus* (12.8 %), 6 *Rhipicephalus turanicus* (4.8%) and 4 *Hyalomma aegyptium* (3.2 %). No amplification was observed for *Hyalomma marginatum*, *Hyalomma detritum* and *Ixodes ricinus* (Figures 4, 5).

The PCR RFLP performed on ITS PCR products showed bands of 200 bp and 150 bp corresponding to *Leishmania major* and bands of 200 bp, 80 bp and 60 bp corresponding to *Leishmania infantum*. Figure 6 illustrates the obtained profiles.

The *Leishmania infantum* profiles were obtained from *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* ticks collected on dogs, while *Leishmania major* profiles were obtained from ticks collected from boars and turtles (Figure 6).

Our results showed that *Rhipicephalus sanguineus* was the most frequently collected tick species in dogs in Tizi-Ouzou and Algiers, while *Rhipicephalus turanicus* was collected only from the dogs in Algiers. The two species showed the presence of *Leishmania infuntum* DNA. For wild boars in our study, we were able to find *Leishmania major* DNA only in *Dermacentor marginatus* ticks in Tizi-Ouzou. For turtles, a few ticks of the species *Hyalomma aegyptium* showed the pres-

Table 1. Collected tick species from two northern localities in Algeria.

Host	Identified ticks	Male	%	Female	%	Total	Species %
Dogs (TO)	*Rhipicephalus sanguineus*	144	34.1	61	14.5	**205**	**56.2**
Dogs (A)	*Rhipicephalus sanguineus*	24	5.7	8	1.9	**32**	
	Rhipicephalus turanicus	10	2.4	0	0	**10**	**2.4**
Cows (TO)	*Hyalomma marginatum*	98	23.2	33	7.8	**131**	**31**
	Hyalomma detritum	1	0.2	0	0	**1**	**0.2**
Boars (TO)	*Ixodes ricinus*	6	1.4	14	3.3	**20**	**4.7**
	Dermacentor marginatus	8	1.9	8	1.9	**16**	**3.8**
Turtles (A)	*Hyalomma aegyptium*	6	1.4	1	0.2	**7**	**1.7**
Total		**297**	**70.4**	**125**	**29.6**	**422**	**100**

*TO: Tizi-Ouzou, **A: Algiers.

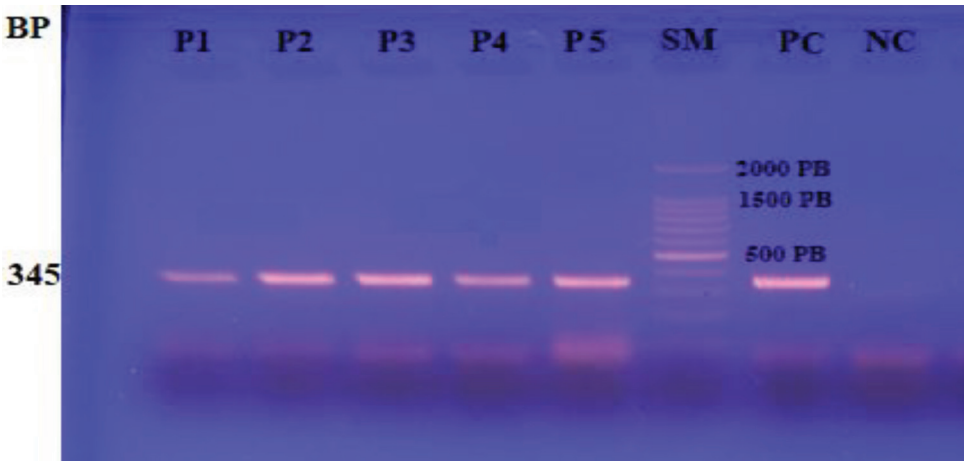

Figure 4. Lei70R / Lei70S PCR profile (Spanakos et al. 2002) targeting the 18S rDNA gene for collected ticks. **SM:** 100 BP DNA ladder, **P1:** *Rhipicephalus sanguineus* (Algiers), **P2:** *Rhipicephalus sanguineus* (Tizi-Ouzou), **P3:** *Rhipicephalus turanicus* (Algiers), **P4:** *Hyalomma aegyptium* (Algiers), **P5:** *Dermacentor marginatus* (Tizi-Ouzou). The results show a band of 345 bp corresponding to *Leishmania* spp. **PC:** Positive control, **NC:** Negative control.

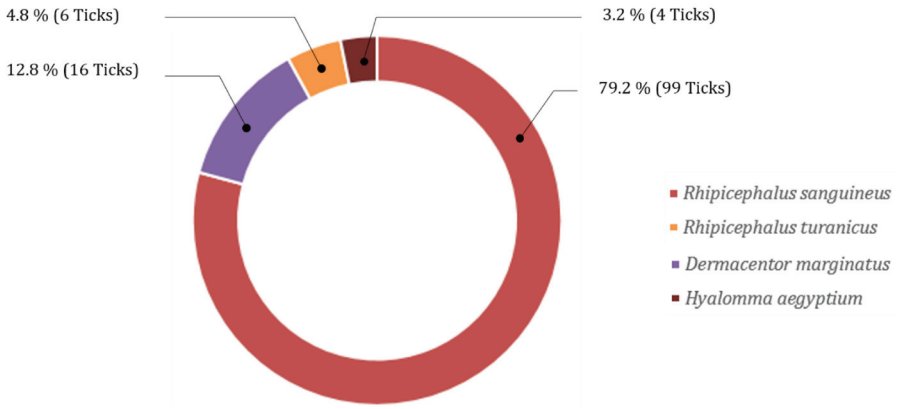

Figure 5. Prevalence of *Leishmania* positive samples according to tick species.

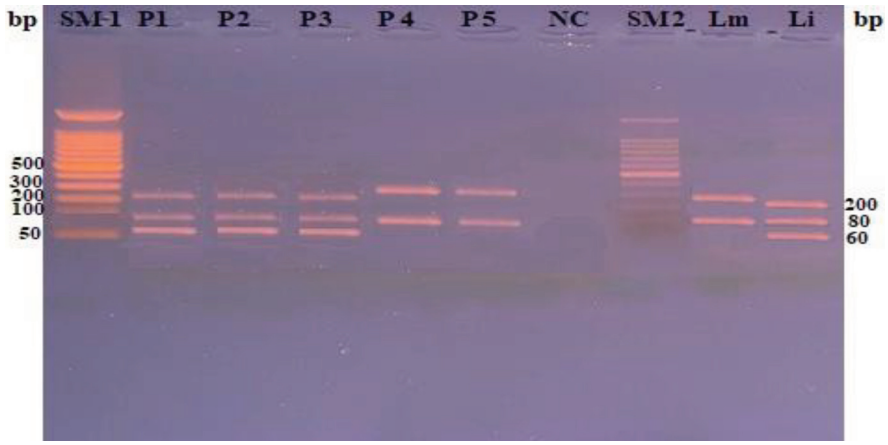

Figure 6. Electrophoresis results of PCR-RFLP on ITS1 of *Leishmania* spp. detected in collected ticks. **SM1:** 100 bp DNA ladder, **SM2:** 50 bp DNA ladder, **Lm:** Positive control *Leishmania major*, **Li:** Positive control *Leishmania infantum*. **NC:** Negative control, lanes **P1–P5:** PCR RFLP products. **P1–P2–P3:** The results show three bands of 200 bp, 80 bp and 60 bp, respectively, corresponding to *Leishmania infantum* ITS1 digested with *HaeIII* restriction enzyme: **P1:** *Rhipicephalus sanguineus* (Algiers)**, P2:** *Rhipicephalus sanguineus* (Tizi-Ouzou), and **P3:** *Rhipicephalus turanicus* (Algiers). **P4–P5:** The results show two bands of 200 bp and 150 bp, respectively, corresponding to ITS1 *Leishmania major* digested with *HaeIII* restriction enzyme: **P4:** *Hyalomma aegyptium* (Algiers), **P5:** *Dermacentor marginatus* (Tizi-Ouzou).

ence of *Leishmania major* DNA in Algiers (Figure 7). The number of ticks positive to *Leishmania infantum* is more important than the number of ticks positive to *Leishmania major* (Figure 7).

DISCUSSION

In our study, seven tick species belonging to the genera *Hyalomma*, *Rhipicephalus*, *Dermacentor* and *Ixodes* were identified. Ticks can cause several afflictions. During the blood meal, the neurotoxins contained in saliva may cause host paralysis that can lead to respiratory failure and death if not treated properly and early (Nutall and Labuda 2008). Moreover, the molecules contained in tick saliva participate in tick-borne pathogens transmission (Nutall and Labuda 2008). The identified tick species are vectors of several bacterial, viral and parasitic pathogens that affect human and animal health (Trotta et al. 2012). Many tick-borne microorganisms such as *Babesia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Borrelia* spp., tick-borne encephalitis virus TBEV, Crimean Congo Hemorrhagic Fever Virus CCHFV, Powassan virus POWV (Madisson-Antenucci et al. 2020) and Bourbon Virus (Kosoy et al. 2015) lead to several diseases and can affect many tissues and organs as the bone and bone marrow (Farooq and Moriarty 2021). Previous studies suggested the role of ticks in the transmission of *Leishmania* spp. but it remains uncertain (Dantas-Torres et al. 2010; Colombo et al. 2011; Solano-Gallego et al. 2012; Trotta et al. 2012).

In Algeria, it is important to know the distribution of both the parasite and the vectors. Hence, we collected ticks from domestic and wild animals: dogs, cows, turtles and boars in order to investigate the potential presence of the *Leishmania* spp. in these arthropods. Some sampling localities are >1000 m of altitude. It is particularly the case for the first province, Tizi-Ouzou.

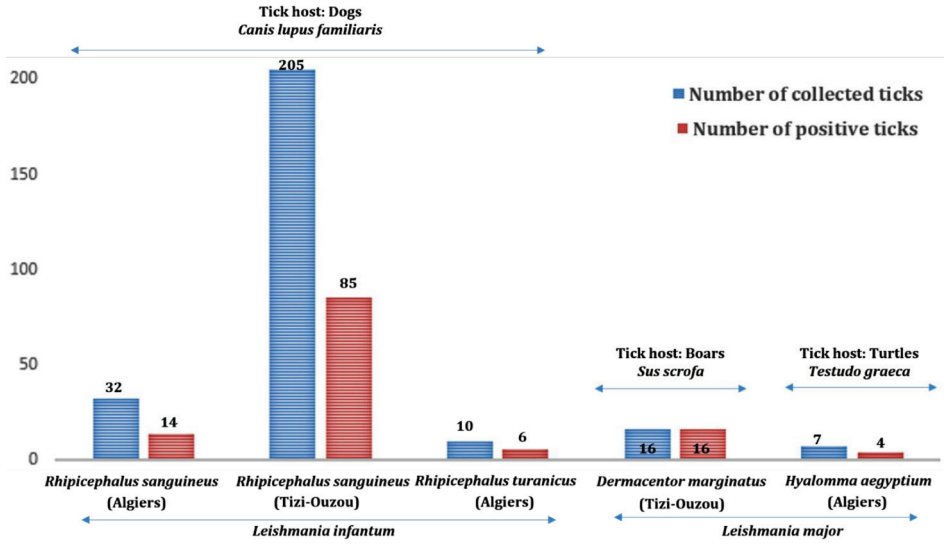

Figure 7. Distribution of *Leishmania infantum* and *Leishmania major* according to tick species, host and provinces.

The sampling period was from December 2019 to early April 2020. Vectors are apparently not very active at this period of the year.

In this study, we used Lei70L and Lei70R primers in the conventional PCR performed to detect the genus *Leishmania* in the collected ticks. These primers target the conserved region of the su-rRNA gene. Other methods and primers targeting different regions may be used as well. This is the case in other studies that used real-time PCR or other primers as MC1/MC2 (Dantas-Torres et al. 2010), RV1/RV2 (Millán et al. 2016) and ITS1-F/ITS1-R (Viol et al. 2016) for conventional PCR that targets different regions and obtained results as well.

Here, the genus *Leishmania* was detected in different species. These results concord with previous works that suggested the role of wild mammals in the epidemiology of leishmaniasis (Millán et al. 2016).

Millán et al. (2016) detected the genus *Leishmania* in *Amblyomma tigrinum* ticks and *Pseudalopex* foxes in Argentina, and Rojas-Jaimes et al. (2022) detected *Leishmania* spp. by using quantitative PCR in *Rhipicephalus microplus* and *Amblyomma sabanerae* ticks in Peru. In a study done by Viol et al. (2016), *Leishmania* spp. promastigotes were identified in salivary glands, intestine and ovaries of dissected engorged females collected from dogs in Brazil (Viol et al. 2016; Rojas-Jaimes et al. 2022).

In order to identify *Leishmania* spp. species in the collected tick samples, an ITS1-RFLP PCR was performed. This technique is cost-effective and has high sensitivity for the identification of different *Leishmania* spp. species. In this study, results of RFLP PCR showed the profiles of three bands of 200, 80 and 60 bp and two bands of 200 and 150 bp corresponding to *Leishmania infantum* and *Leishmania major*, respectively.

Leishmania infantum was detected in *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* ticks collected from dogs. This was expected since dogs are the main reservoir of visceral leishmaniasis. This result agrees with previous studies where *Leishmania infantum* DNA was detected in ticks collected from various hosts and regions from the globe as Brazil and Italy. Campos and Costa (2014) also suggested that *Rhipicephalus sanguineus* ticks may have epidemiological importance. Magri et al. (2022) detected *Leishmania infantum* RNA in *Ixodes ricinus,* while in our study this tick species was negative for the genus *Leishmania* (Dantas-Torres et al. 2010; Solano-Gallego et al. 2012; Campos and Costa 2014; Magri et al. 2022).

Leishmania major was detected in *Hyalomma aegyptium* ticks collected from turtles in Algiers and in *Dermacentor marginatus* ticks collected from boars in Tizi-Ouzou. In a previous study under laboratory conditions, the PCR performed to detect *Leishmania major* in unfed *Rhipicephalus sanguineus* nymphs molted from larvae fed on infected Gerbils *Meriones unguiculatus* was negative (Bilgic et al. 2017). To the best of our knowledge, *Leishmania major* is detected here in naturally infected ticks for the first time.

In the recent years, the geographical limit between north cutaneous leishmaniasis due to *Leishmania infantum* and the zoonotic cutaneous leishmaniasis due to *Leishmania major* in Algeria has been crossed. Many authors explain this by the climatic changes and environmental modification, while others suggest the extension of the vector territory because of changes in their ecosystem (Bachi et al. 2019). The host rodents may also be affected by those variations. For example, *Meriones shawii*, one of the reservoirs of cutaneous leishmaniasis, is now present in the humid and subhumid zone (Meunier et al. 2020). Population displace-

ment and the travelling of the owners with their animals to regions where cutaneous leishmaniasis is endemic may also be a reason for the infestation. Other studies suggest the possible participation of other mammals and other arthropods in the transmission of the parasite in different regions of the old and new world (Millán et al. 2016; Dantas-Torres et al. 2010; Coutinho and Linardi 2007).

Sand flies, the vectors, live in many roosts like the hollows of trees, rodent burrows, stables, cellars and houses. They are active at dusk and during night. Their flight is silent and jerky, hence, they are found near their hosts and roosts. Their distribution is also related to environmental factors, for example, rain and wind can stop their displacement (Bachi et al. 2019).

In our study, the period and the localities of sampling did not correspond to the activity period of the sand fly, and the location of some sampling points are not favourable for the vector development. However, the present PCR results are not sufficient to prove that ticks are the biological vectors of the parasite. This may be due to the tick life cycle or to their blood meal. Animals infected during a previous season favourable for sand flies could remain chronically infected in a season non-favourable for the sand flies, and the remnants of blood directly ingested from the blood meal upon these hosts could be the main reason for the PCR-positive result.

The involvement of ticks in the transmission and epidemiology of leishmaniasis requires more investigations. A complete cycle of *Leishmania* spp. must be established *in vitro*; then, its potential occurrence should be checked *in vivo*. The area and the period of sampling and host ranges should also be broader for a better understanding of the transmission process. Interestingly, a study performed on ticks collected from naturally infected dogs suggested that the parasite may have viability inside the ticks. The same study suggested that adult ticks molted from infected larvae may conserve a viable genetic material of *Leishmania infantum* (Colombo et al. 2011).

CONCLUSION

To the best of our knowledge, this paper reports for the first time the presence of *Leishmania* DNA in ticks collected from wild and domestic animals in Algeria and northern Africa. It is also the first report of the presence of *Leishmania major* DNA in ticks. However, these results are not a proof that ticks are vectors of *Leishmania* or *Leishmania major*. Further investigation is desirable to improve the knowledge about the role of ticks in the transmission of this protozoan parasite.

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