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Short communication

# First detection of *Leishmania infantum* kinetoplast DNA in hair of wild mammals: Application of qPCR method to determine potential parasite reservoirs

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ABSTRACT

The data presented in this paper describe the application of a method for a reliable and non-invasive diagnosis of leishmaniosis in wild reservoirs, based on the detection of *Leishmania infantum* kinetoplast DNA (kDNA) in hair samples by Real Time PCR (qPCR). The study has been performed on 68 ear/leg hair samples from 5 different wild species (*Vulpes vulpes*, *Canis lupus*, *Martes foina*, *Rattus norvegicus* and *Erinaceus europaeus*) from several geographic areas of West and North Spain. The presence of *Leishmania* kDNA was detected in 14 of the 68 analyzed samples, being the highest quantity of DNA observed in foxes. This is the first report of the presence of *Leishmania* in a hedgehog. The kDNA remained stable under the exposure of hair to different environmental conditions (freezing or high temperature, ultraviolet rays or treatment with tanning salts). This detection method could constitute a suitable alternative for the search of the parasite in wild hosts, due to the numerous advantages that hair samples present for collection, transport and storage processes.



## 1. Introduction

Leishmaniosis are worldwide distributed zooantroponoses, caused by *Leishmania* protozoan and transmitted by sandfly vectors bite. The World Health Organization recommends the surveillance of wild and domestic hosts for epidemiological and prevalence analysis, as an important component of control strategies for zoonotic leishmaniosis (WHO technical report series, no. 949). Up to date, *Leishmania* parasites have been isolated from more than 70 different wild and domestic animal species belonging to several families: *Canidae, Muridae, Cricetidae, Cuniculidae, Leporidae, Procyonidae, Mustelidae, Felidae, Equidae* and *Bovidae*, among others. It is currently known that a large number of animals may be hosts of dermal, mucosal and visceral *Leishmania* species (Quinnell and Courtenay, 2009) that are responsible of human infections in the New World. In the Old World a relevant number of reservoirs have also been described (Reyes and Arrivillaga, 2009); several species of rodents behave as responsible of cutaneous forms of the disease, whereas the domestic dog is considered to be the target host and main reservoir of visceral leishmaniosis caused by *L. infantum*, playing a key role in the transmission to humans (Ashford, 1996). In Spain, the first *Leishmania* isolation in a wild mammal was reported by Morillas Márquez et al. (1985) in black rat (*Rattus rattus*). Since then, several studies in wild animals have been performed (Portús et al., 2002; Sobrino et al., 2008; Millán et al., 2011; Molina et al., 2012). The searching of new wild reservoirs presents many difficulties. Most of times, anesthesic darts or traps are employed for the capture of alive specimens (Fowler, 2008). Sample collection causes additional stress to animals, mainly when lymph node or bone marrow samples are required for the detection of viscerotropic *Leishmania* species. Other additional problems come from the storage of samples in field trials and the need of specific facilities, which sometimes require a great economical support. For these reasons, most of the studies have been performed in corpses. In these cases fresh samples are difficult to obtain with the inconvenient of the tissues degradation that could interfere with the final results.

Several direct or indirect methods have been usually performed for the diagnosis of leishmaniosis. The employment of the highly sensitive Real Time PCR (qPCR) method for *Leishmania* DNA detection in skin biopsies, spleen or blood samples of wild animals

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has been already reported (Talmi-Frank et al., 2010a,b; Gil de Mendonça et al., 2011).

Recently, it has been proposed the hair as a suitable sample to consider in the diagnosis of *Leishmania* infection by qPCR in the canine model (Belinchón-Lorenzo et al., 2013). The development of this method opens a new stage in the searching of animal hosts of *Leishmania* species. Thus, the objective of the present study has been to determine whether it is possible to apply this technique to detect the presence of *Leishmania* kDNA in wild animals that could be reservoirs of the disease. The stability of the parasite DNA has been also tested when hair is exposed to different conditions as freezing, desiccation, putrefaction, ultraviolet rays or tanning salts treatment.

## 2. Materials and methods

After extraction process, the quantity of parasite DNA present in each sample was estimated using a standard curve experiment, following the protocol described by Belinchón-Lorenzo et al. (2013). The purity, quality and concentration of exogenous DNA obtained from the samples after our extraction method was not assessed, but it was enough and suitable to allow correct amplifications by qPCR.

Samples were tested in triplicate and considered positive when threshold cycle (Ct) was lower than 35 (Applied Biosystems Application note, 2006) and when the amplification was detected in all the replicates.

### 2.1. Leishmania kinetoplast DNA (kDNA) detection by qPCR

A total of 68 samples (15–20 hairs from each animal) were carefully extracted with tweezers from clean zones of corpses (without presence of blood, wounds, *etc.*), to ensure the absence of contamination with other type of cells. The tweezers were always properly disinfected between samples. Hairs that proceeded from foxes (*Vulpes vulpes*), rats (*Rattus norvegicus*), beech martens (*Martes foina*), wolves (*Canis lupus*) and a hedgehog (*Erinaceus europaeus*) were analyzed (Table 1). All the corpses were collected in several areas of the West (Extremadura) and North (Asturias and Galicia) of Spain.

### 2.2. Stability study of Leishmania kDNA in hair

Hair samples from a *Leishmania* positive fox were exposed to 4 types of different environmental conditions (Table 2), in order to determine if they could affect to the qPCR result:

- Freezing: 5 hairs were analyzed before and after storage for 10 months at −80 °C.
- High temperatures (desiccation): 10 hairs were introduced in a stove at 56 °C for 4 months.
- Faecal contamination (putrefaction conditions): three samples of 5 hairs were incubated at 37 °C with a stool sample:

  - Two of them were placed in a homogenized filtered solution of 1 g of stool sample in 10 ml of distilled water and incubated for 24 and 72 h, respectively.
  - The other one was placed on 1 g of stool sample and incubated for 24 h.

- Ultraviolet rays exposure: 5 hairs were introduced in a sterile microtube and exposed to the short wave ultraviolet C rays (λ: 200–283 nm) of a laminar flux cabin for 72 h.
- Tanning salts treatment: a total of 5 hairs were deposited for 3 days in a solution that it is normally employed for fur tanning: 0.2 g of potassium alum salt [$AlK(SO_4)_2 \cdot 12H_2O$] plus 0.2 g of sodium chloride salt (NaCl) in 5 ml of warm water.

After the performance of the respective processes, samples were stored at −80 °C until DNA extraction and analysis, that were carried out together with a control sample (the same number of hairs from the same animal, but without any treatment), in each case.

## 3. Results

### 3.1. Detection and quantification of Leishmania kDNA by qPCR

As shown in Table 1, qPCR method was able to detect and quantify the presence of *Leishmania infantum* kDNA in the hair of 14 out of 68 analyzed samples (including 6 of 9 foxes, 3 of 3 beech martens, 2 of 6 rats, 2 of 49 wolves and the hedgehog) of the two body sections tested. The estimated number of parasites in all positive samples were quite variable, ranging from 0.41 to more than 11,000. Wolves were the species in which the lowest number of positive samples was found. The highest quantity of parasite DNA was observed in foxes, being the estimated number of parasites in hair of two individuals (F1 and F2) up to 1000 times greater than the observed in other species.

### 3.2. Stability study of Leishmania kDNA in hair

The different assays performed to analyze the stability of *L. infantum* kDNA in hairs showed a high rate of preservation to physical and chemical degradation agents (Table 2).

The storage of hair samples at freezing temperatures (−80 °C) did not affect the qPCR results. It was also found that *Leishmania* kDNA was able to resist in hair 4 months under desiccation conditions (56 °C). Moreover, after 72 h of exposure, neither the short length wave (UVC rays) nor the treatment with alum salts solution (used in fur tanning) degraded the totality of the parasitic genetic material contained in hairs, since the qPCR results remained positive after the influence of these external factors.

In contrast, the putrefaction conditions for 1 or 3 days at 37 °C due to the exposure of hair to faecal contamination did not allow to amplify the kDNA in none of the samples analyzed.

## 4. Discussion

The current knowledge of the ecology of many parasitic diseases such as leishmaniosis has required a century of hard work to identify the potential hosts involved in the epidemiology of this disease in the different geographic areas. These studies in mammal reservoirs, especially wild animals, have needed in most cases many costly techniques to allow the detection of *Leishmania* parasites. Most of works are based on *post mortem* analysis, or *in vivo* sampling of animals trapped by laborious procedures and their subsequent release into natural habitats. In captive animals from zoos the problem is not minor because the sample collection entails a complex manipulation and requires deep sedation to avoid the stress of the individuals (Fowler, 2008).

The presence of foreign parasitic DNA in hair and epidermal keratinocytes of *Leishmania* infected animals (Belinchón-Lorenzo et al., 2013) has opened a suitable new scientific field to analyze the physiology and function of these epithelial cells during leishmaniosis, and possibly in other diseases. The qPCR-hair method allowed us to continue the search of other parasite reservoirs. This technique could be applied to epidemiological trials with significant methodological advantages. Hair samples are obtained by non-invasive ways without the need of anaesthesia. Thus, they could be collected in alive animals by rapid catch and release with lower

**Table 1**
Summary of species, geographic origin, sampling body area, Real Time PCR results and assessment of the 68 hair samples analyzed.

| Region | Species | Animal ref. | Body area of hair sample | qPCR results* | |
|---|---|---|---|---|---|
| | | | | Ct (assessment) | Estimated no. of parasites |
| Extremadura (West Spain) | Fox (*Vulpes vulpes*) | F-1 | Leg | 18.63 (+) | 11,111.13 |
| | | F-2 | Leg | 18.67 (+) | 10,651.27 |
| | | F-3 | Ear | 37.07 (−) | – |
| | | F-4 | Ear | 36.79 (−) | – |
| | | F-5 | Ear | 36.11 (−) | – |
| | | F-6 | Ear | 28.14 (+) | 33.88 |
| | | F-7 | Ear | 32.32 (+) | 1.85 |
| | | F-8 | Ear | 29.78 (+) | 10.69 |
| | | F-9 | Ear | 27.60 (+) | 49.71 |
| | Beech marten (*Martes foina*) | M-1 | Leg | 33.72 (+) | 0.56 |
| | | M-2 | Ear | 29.88 (+) | 10.00 |
| | | M-3 | Ear | 30.87 (+) | 5.08 |
| | Rat (*Rattus norvegicus*) | R-1 | Leg | 34.22 (+) | 0.50 |
| | | R-2 | Leg | 31.83 (+) | 1.87 |
| | | R-3 | Leg | 36.03 (−) | – |
| | | R-4 | Leg | 35.78 (−) | – |
| | | R-5 | Leg | 35.58 (−) | – |
| | | R-6 | Leg | N.D** (−) | – |
| Asturias & Galicia (North Spain) | Hedgehog (*Erinaceous europaeus*) | H-1 | Ear | 33.51 (+) | 0.81 |
| | Wolf (*Canis lupus*) | W-1 | Ear | 33.49 (+) | 0.41 |
| | | W-2 | Ear | 31.37 (+) | 2.99 |
| | | W-3 to W-49 | Ear | > 35 (−) | – |

Cut-off established: positive result (+): Ct values < 35.
Negative result (−): Ct values ≥ 35.
* Mean values of the three replicates of each sample.
** kDNA not detected at Ct = 40

stress than other conventional sampling methods. Moreover, the stability of the DNA in the samples is not affected by the temperature or environmental conditions in which they are stored and transported, since hairs are very resistant due to the hydrophobic nature of the cuticle and to the presence of keratin (Gilbert et al., 2006). This stability was demonstrated for endogenous DNA in all keratinized tissues (Bengston et al., 2012) and in mouse DNA samples after freezing and storing for 44 months (Garzel et al., 2010). But in our study, data suggested that hair protects also he foreign kDNA against external degradation. As expected, the storage of hair samples at freezing temperatures (−80 °C) and under desiccation did not affect the qPCR results, since both freezing and desiccation are processes that significantly reduce the free water required for the activity of enzymes and microorganisms and therefore provide desirable conditions for DNA conservation. For the same reason, the parasite DNA was neither degraded in the presence of tanning salts, since they are also employed to avoid the growth of microorganisms.

However, parasite DNA also could be extracted from hair and amplified by qPCR after maintenance at room temperatures and under the UVC rays, described by Guerrero-Beltrán and Barbosa-Cánovas (2004) as the most potent, damaging and denaturalizing solar radiation. The results after exposure to putrefaction are the exception, suggesting that the hair contained in coprolites are not suitable for the search and isolation of parasite DNA because these samples present the ideal conditions for the growth of bacteria that could degrade nucleic acids.

In Spain, only two studies about the detection of *Leishmania* DNA by Real Time PCR in wolves (Sastre et al., 2008) or wild rabbits (Chitimia et al., 2011) have been reported, in which conventional samples (blood, spleen, skin, lymph node or bone marrow) were analyzed. As far as we know, this is the first time that *L. infantum* kDNA is detected by qPCR in hair of foxes, rats and beech martens (and in a hedgehog as a potential parasite reservoir). The low number of positive samples obtained from wolves may be due to their geographical origin, since the north of Spain is considered a non-endemic area of leishmaniosis (Miró et al., 2012).

Since we still do not know whether there is a correlation between the quantity of parasite DNA accumulated in hair and the parasite load in other organic tissues, future studies with higher number of naturally and experimentally infected animals are necessary to determine this correlation, the actual sensitivity of the hair-qPCR method in epidemiological studies, and also the chronobiology of the appearance of this extracorporeal DNA in the hair.

**Table 2**
Summary of assays performed and Real Time PCR results obtained after exposure of positive hair samples to different adverse environmental conditions.

| | Assay | Sample conditions | Ct* (assessment) |
|---|---|---|---|
| Exposure to | Freezing | Control (time 0) | 31.81 (+) |
| | | 10 months at -80 °C | 33.58 (+) |
| | Desiccation | Control (time 0) | 29.48 (+) |
| | | 4 months at 56 °C | 29.83 (+) |
| | None | Control 3 days at room temperature | 28.73 (+) |
| | Faecal contamination (putrefaction) | 1 day in a stool solution (1 g/10 ml) at 37 °C | N.D.** (−) |
| | | 3 days in a stool solution (1 g/10 ml) at 37 °C | 35.69 (−) |
| | | 1 day on a stool sample at 37 °C | N.D.** (−) |
| | UVC rays | 3 days of exposure | 33.95 (+) |
| | Tanning salts treatment | 3 days of exposure | 30.80 (+) |

Cut-off established: positive result (+): Ct values < 35; negative result (−): Ct values ≥35.
* Mean value of the three replicates.
** kDNA not detected at Ct = 40.

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## References

Ashford, R.W., 1996. Leishmaniasis reservoirs and their significance in control. Clin. Dermatol. 14, 523–532.

Belinchón-Lorenzo, S., Iniesta, V., Parejo, J.C., Fernández-Cotrina, J., Muñoz-Madrid, R., Soto, M., Alonso, C., Gómez Nieto, L.C., 2013. Detection of *Leishmania infantum* kinetoplast minicircle DNA by Real Time PCR in hair of dogs with leishmaniosis. Vet. Parasitol. 192 (1–3), 43–50.

Bengston, C.F., Olsen, M.E., Brandt, L.O., Bertelsen, M.F., Willerslev, E., Tabin, D.J., Wilson, A.S., Gilbert, M.I.P., 2012. DNA from keratinous tissue, part I: hair and nail. Ann. Anat. 194, 17–25.

Chitimia, L., Muñoz-García, C.I., Sánchez-Velasco, D., Lizana, V., Del Río, L., Murcia, L., Fisa, R., Riera, C., Giménez-Font, P., Jiménez-Montalbán, P., Martínez-Ramírez, Á., Meseguer-Meseguer, J.M., García-Bacete, I., Sánchez-Isarria, M.A., Sanchis-Monsonís, G., García-Martínez, J.D., Vicente, V., Segovia, M., Berriatua, E., 2011. Cryptic Leishmaniosis by *Leishmania infantum*, a feature of canines only? A study of natural infection in wild rabbits, humans and dogs in southeastern Spain. Vet. Parasitol. 181, 12–16.

Fowler, M.E., 2008. Chemical restraint. In: Restraint and Handling of Wild and Domestic Animals. Wiley-Blackwell, Iowa, USA, pp 227-247.

Garzel, L.M., Hankenson, F.C., Combs, J., Hankenson, K.D., 2010. Use of quantitative polymerase chain reaction analysis to compare quantity and stability of isolated murine DNA. Lab. Anim. 39 (9), 283–289.

Gilbert, M.T.P., Menez, L., Janaway, R.C., Tobin, D.J., Cooper, A., Wilson, A.S., 2006. Resistance of degraded hair shafts to contaminant DNA. Forensic Sci. Int. 156, 208–212.

Gil de Mendonça, P., Harsch, A., Mogl, C., Walther, B., Boje, J., Dimke, C., 2011. Molecular screening of wild dodents for *Leishmania infantum* in Germany. Acta Zool. Bulg. 63 (3), 307–311.

Guerrero-Beltrán, J.A., Barbosa-Cánovas, G.V., 2004. Advantages and limitations on processing foods by UV light. Food Sci. Technol. Int. 10 (3), 0137–211.

Millán, J., Zanet, S., Gomis, M., Trisciuoglio, A., Negre, N., Ferroglio, E., 2011. An investigation into alternative reservoirs of canine leishmaniasis on the endemic island of Mallorca (Spain). Transbound. Emerg. Dis. 58, 352–357.

Miró, G., Checa, R., Montoya, A., Hernández, L., Dado, D., Gálvez, R., 2012. Current situation of *Leishmania infantum* infection in shelter dogs in northern Spain. Parasit. Vector. 5, 60.

Molina, R., Jiménez, M.I., Cruza, I., Iriso, A., Martín-Martín, I., Sevillano, O., Melero, S., Bernal, J., 2012. The hare (*Lepus granatensis*) as potential sylvatic reservoir of *Leishmania infantum* in Spain. Vet. Parasitol. 190, 268–271.

Morillas Márquez, F., Benavides Delgado, I., González Castro, J., Reyes Magaña, A., Valero López, A., 1985. Dêcouverte de *Leishmania sp.* dans des *Rattus rattus* de la province de Granade (Espagne). Ann. Parasitol. Hum. Comp. 60 (6), 768–770.

Amplification efficiency of TaqMan gene expression assays. Applied Biosystems Application note. 2006 http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldument/cms_040377.pdf (accessed 21.03.12).

Portús, M., Gállego, M., Riera, C., Aisa, M.J., Fisa, R., Castillejo, S., 2002. Wild and domestic mammals in the life cycle of *Leishmania infantum* in southwest Europe. A literature review and studies performed in Catalonia (Spain). Rev. Iber. Parasitol. 62, 72–76.

Quinnell, R.J., Courtenay, O., 2009. Transmission, reservoir hosts and control of zoonotic visceral leishmaniasis. Parasitology. 136, 1915–1934.

Reyes, A., Arrivillaga, J., 2009. Fauna Mammalia asociada a los focos de leishmaniasis neotropical. Situación en Venezuela. Bol. Mal. Salud Amb. 49 (1), 35–52.

Sastre, N., Francino, O., Ramírez, O., Enseñat, C., Sánchez, A., Altet, L., 2008. Detection of *Leishmania infantum* in captive wolves from Southwestern Europe. Vet. Parasitol. 158, 117–120.

Sobrino, R., Ferroglio, E., Oleaga, A., Romano, A., Millan, J., Revilla, M., Arnal, M.C., Trisciuoglio, A., Gortázar, C., 2008. Characterization of widespread canine leishmaniasis among wild carnivores from Spain. Vet. Parasitol. 155, 198–203.

Talmi-Frank, D., Jaffe, C.L., Nasereddin, A., Warburg, A., King, R., Svobodova, M., Peleg, O., Baneth, G., 2010a. *Leishmania tropica* in Rock Hyraxes (*Procavia capensis*) in a focus of human cutaneous leishmaniasis. Am. J. Trop. Med. Hyg. 82 (5), 814–818.

Talmi-Frank, D., Kedem-Vaanunu, N., King, R., Bar-Gal, G.B., Edery, N., Jaffe, C.L., Baneth, G., 2010b. *Leishmania tropica* infection in Golden Jackals and Red Foxes, Israel. Emerg. Infect. Dis. 16 (12), 1973–1975.

World Health Organization Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, 2010. WHO technical report series, 949.