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Detection of *Leishmania infantum* kinetoplast minicircle DNA by Real Time PCR in hair of dogs with leishmaniosis

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ABSTRACT

It is known that hair can accumulate environmental toxics and excrete foreign chemical or biological substances. In this context, we hypothesized that foreign DNA could be found in the hair of an infected organism, and thus, be detected by Real Time PCR in the hair of *Leishmania infantum* naturally infected dogs. A population of 28 dogs living in *Leishmania* endemic areas was divided into two groups: A (13 *Leishmania* infected dogs) and B (15 healthy dogs). Blood, lymph node and ear hair samples from all of them were tested for the presence of parasite kinetoplast DNA (kDNA). For the same purpose, hair of several body areas and hair sections of two infected dogs were also analyzed. Epidermal keratinocytes from an infected animal were also analyzed for reactivity against *Leishmania* antigens by ELISA and for the presence of kDNA. Regarding to dogs from group A, parasite kDNA was detected in the 100% of lymph node samples. The sensitivity of Real Time PCR in ear hair was similar to that obtained in blood (9 positive out of 13 *versus* 8 positive out of 13, respectively). Moreover, the presence of *L. infantum* kDNA was also detected in the hair of all the analyzed body zones, in all hair sections and in epidermal keratinocytes. In infected dogs, parasite kDNA could be detected and quantified from just one single hair, whereas it was not detected in any of the samples of the healthy dogs. This work describes a new method for a reliable and non-invasive diagnosis of canine leishmaniosis using hair samples of infected animals. The data presented also provide some insights for the understanding of the physiology of keratinocytes and the role of hair as a specialized tissue in the kidnapping and removal of foreign DNA.

1. Introduction

The skin is the largest and most visible organ of the body. Together with the hair the skin behaves as a protective barrier against physical, chemical and microbiological damages, reflecting also pathological processes that occur in other organs or tissues (Chuong et al., 2002; Elias, 2007). Other important functions of the skin are the storage and excretion of substances, and it is also involved in processes connected with immune regulation (Scott et al., 2001a).

In leishmaniosis, the infection progresses towards a chronic form of the disease with harmful consequences in absence of an efficient treatment, manifested as dermatological and mucosal lesions (Singh, 2006). It is interesting to notice that high *Leishmania* parasite load has been observed

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in epidermis in up to 40% of biopsies of patients with leishmaniosis (Azadeh et al., 1985). Transepithelial elimination of amastigote forms through pores between cells of epidermis, hair follicles and sweat glands have been also reported (Azadeh and Abdulla, 1994; Perrin et al., 1995). The participation of the skin immune system in different forms of leishmaniosis is largely unknown, but it have been described that some of its constituents are implicated in the control and elimination of infective promastigote forms (Moll, 1993; Maurer et al., 2006) from primary infection until the end of the process. In fact, keratinocytes that represent the 95% of all epidermal cells, play a very important role in innate immunity responses against pathogens due to their capability to express Toll-like receptors (TLRs) and because they are an important source of inflammatory mediators (Pivarcsi et al., 2005; Scott et al., 2001b).

The diagnosis of canine leishmaniosis is currently based on different serological, parasitological and molecular techniques (Francino et al., 2006; Gomes et al., 2007; Rodríguez-Cortés et al., 2007). The use of Real-Time PCR method provides the ability to perform a very sensitive, accurate and reproducible measurements of specific DNA present in a sample. It has been reported its high sensitivity and specificity using many different tissue samples as blood, lymph node and bone marrow – among others – specially when kinetoplast DNA (kDNA), located in the parasite mitochondrion, is targeted (Reale et al., 1999; Francino et al., 2006; Rodríguez-Cortés et al., 2007; Manna et al., 2004). This kDNA is comprised of two units, the maxicircles (present in a number of 30–50 copies/parasite, with 20–40 kb in length) and the minicircles (present in a number of 10,000–20,000 copies/parasite, with 1 kb in length) (Rodgers et al., 1990). The high number of copies of this minicircle and the existence of a conserved region (with 200 base pairs in length, approximately) between species has allowed the use of molecular probes based on it for diagnosis (Rodgers et al., 1990; Weiss, 1995).

As previously described, hair can accumulate environmental toxics and excrete foreign substances. In humans, metal traces, abuse drugs, antibiotics, other drugs, and tobacco residues have been detected in the hair (Agusa et al., 2005; Kim and Kim, 2011; Forman et al., 1992; Musshoff and Madea, 2006; Dorea et al., 1987; Takiguchi et al., 2001; Uematsu, 1993). Hair analyses have been also performed in many animal species for the detection of veterinary drugs in cattle, horses and pigs, of trace metals in cattle and even in seals, and of abuse drugs in rats and mice (Gratacós-Cubarsí et al., 2006, 2007; Patra et al., 2007; Wenzel et al., 1996; Stout et al., 2000). Although various mechanisms have been proposed such as passive diffusion from the bloodstream into the growing hair cells (during their formation), secretion of the apocrine and sebaceous glands (after hair formation), or from the external environment (after hair has emerged from the skin) (Henderson, 1993), little is known about how these substances end up being located in the hair.

In view of these data together, we think it would not be surprising to find foreign DNA in the hair of a parasite infected organism. Thus, the aim of the present work has been to test whether foreign DNA could be kidnapped in the hair of a parasitized organism by using molecular techniques. For this purpose, we have analyzed by Real Time PCR the presence of parasite kDNA in the hair and epidermal keratinocytes of naturally *Leishmania* infected dogs, targeting the conserved region of the *Leishmania infantum* minicircle. We decided to complete this PCR results by analyzing also the presence of antibodies against *Leishmania* antigens in these cells.

The data presented in this paper describe a new method for a reliable and non invasive diagnosis of leishmaniosis. As far as we know, this is the first report of the presence of parasite DNA in the hair of an animal host.

2. Materials and methods

2.1. Animals

The population of 28 dogs used in this study lived in *L. infantum* endemic areas of the Extremadura region (Spain). They were divided into two groups: A (*Leishmania*-infected dogs) and B (healthy dogs). Group A was comprised by 13 animals with positive lymph node PCR result and with anti-soluble *Leishmania* antigen (SLA) antibody response by ELISA. Most of them also presented positive results in blood PCR and IFAT, and showed typical clinical signs of the disease. Group B was constituted by 15 healthy dogs which were *Leishmania* negative as determined by all the techniques described above. They did not present any clinical sign of leishmaniosis.

2.2. Sampling

Blood, lymph node and hair samples from all dogs (and also epidermal keratinocytes from animal A1) were obtained, processed and stored at −80 °C. Blood samples were obtained by fine-needle aspiration according to laboratory standard operating procedures. Two EDTA coated tubes were used to obtain the blood samples. The first one was divided into two aliquots of 200 μl each and stored at −80 °C until DNA extraction. To obtain the sera, the blood from the second tube was centrifuged at 447 × *g* for 10 min at 4 °C and stored at −80 °C. Lymph node biopsies were also obtained by fine-needle aspiration, washed with 500 μl of sterile PBS, and stored in two aliquots of 250 μl at −80 °C for DNA extraction.

Hair samples were extracted with tweezers. Ear hair of all dogs and hair from other corporal zones (head, trunk and extremities) of animals A1 and A6 were obtained in order to quantify the parasite load in different body areas and to determine the minimum amount of hair necessary to detect the presence of *Leishmania* kDNA. To determine the presence of *L. infantum* kDNA along the length of the hair, several hairs were divided in two halves (hair shaft and follicle from animal A6) or in three segments (proximal, central and distal in animal A1). To detect the presence of anti-*Leishmania* antibodies, 30 mg of epidermal keratinocytes were obtained by skin scraping from the ischial area of animal A1.

2.3. Serological analyses

2.3.1. ELISA

This technique was used for the semi-quantitative detection of specific antibodies against the total soluble antigen (SLA) of *L. infantum*. First, 96 wells ELISA plates were coated with 8 μg/ml of SLA antigen (obtained from *L. infantum* promastigotes MCAN/ES/1996/BCN150, zymodeme MON-1). Sera from the 28 dogs were diluted (1/200) and tested in duplicate. Peroxidase-conjugated sheep anti-dog IgG2 (Bethyl Laboratories, Montgomery, TX, USA) was used as secondary antibody. Sera of known reactivity obtained from parasite-free and from *Leishmania* infected dogs (1/200 diluted) were included as negative and positive controls, respectively, and analyzed in quadruplicate. Plates were developed with OPD substrate (with H_2O_2 in citrate buffer). The optical density (O.D.) was read at 492 nm and normalized relative to the positive control mean value (which is considered as O.D. = 1 in each case). The cut-off value was set as the mean of O.D. values plus 3SD of a population of 300 healthy dogs (data not shown). In addition, 100 μl of the supernatant obtained from processed epidermal keratinocytes of dog A1 were analyzed by ELISA titration. To obtain the supernatant, epidermal keratinocytes were sonicated for two cycles of 15 min (187 W) in 1 ml of sterile PBS buffer and then, centrifuged for 3 min at 10,000 × *g*. The supernatant titration was performed ranging in dilution from 1/2 to 1/1280 in sterile PBS, as described above.

2.3.2. IFAT

This technique was used for the determination of the antibody titre ($1/x$) against *L. infantum* antigens. First, the parasites (*L. infantum* promastigotes MCAN/ES/1996/BCN150, zymodeme MON-1) were immobilized on microscope slides. Sera from the 28 dogs were assayed in serial two-fold dilutions from 1/40 to 1/640 in sterile PBS to determine the total IgG levels. The immunofluorescent assay was developed using a rabbit fluorescein-labelled anti-dog IgG (Nordic Immunological Laboratories, Tillburg, The Netherlands) as conjugate. Sera of known reactivity obtained from parasite-free and from *Leishmania* infected dogs were included as negative and positive controls, respectively. The reaction was visualized under fluorescence microscopy. The serum titre was determined by the maximal dilution in which the fluorescence was still detectable. The IFAT was considered positive when the titre was $\geq$1/80.

2.4. Parasitological analyses

2.4.1. DNA extraction

Each one of the hair samples were introduced in screw-cap sterile microtubes and incubated in 250 μl of lysis buffer (10 mM TrisCl, 0.1 EDTA, 0.5% SDS + 20 μg/ml pancreatic RNase) for 2 h at 37 °C. Proteinase K was added to a final concentration of 150 mg/ml and incubated at 56 °C overnight. Then, 200 μl of supernatant were collected and stored at −80 °C. Hairs do not completely dissolve during digestion, according to that is described in specialized articles in which this sample is employed for DNA extraction as forensic techniques (Pfeiffer et al., 2004; Suenaga and Nakamura, 2005). Also, ten hairs from the ischial zone of animal A6 were processed in the same way as above but without the proteinase K treatment. Also, 1 mg of epidermal keratinocytes from animal A1 was processed as indicated above and stored at −80 °C for DNA extraction. The DNA from all samples was obtained using the UltraClean BloodSpin kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions. The detection and quantification of the kDNA from *L. infantum* were performed by Real Time PCR.

The PCR reactions were carried out in 96 wells PCR plates in a final volume of 25 μl (5 μl of DNA + 20 μl of Reaction Mix), containing 20 μM of each primer (Leish 1: 5′-AACTTTTCTGGTCCTCCGGGTAG-3′ and Leish2: 5′-ACCCCCAGTTTCCCGCC-3′), 10 μM of TaqMan Probe (FAM-5′-AAAAATGGGTGCCAGAAAT-3′-non fluorescent quencher-MGB) (Francino et al., 2006), and the Maxima Probe/ROX qPCR Master Mix (Fermentas GmbH Laboratories, St Leon-Rot, Germany). The thermal cycling profile used was one incubation step at 50 °C for 2 min and an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing-extension at 60 °C for 1 min. Each amplification run contained positive controls to test the proper conditions of the reagents (DNA extracted from 55,000 *L. infantum* promastigotes MCAN/ES/1996/BCN150, zymodeme MON-1) and negative controls to detect possible contamination of reagents with target DNA (DNA extracted from a healthy dog blood sample). All PCR analyses were performed in a Step One Plus Real Time PCR System (Applied Biosystems Laboratories, Foster City, CA, USA).

2.4.2. Detection of kinetoplast minicircle DNA from L. infantum

A comparative Ct experiment ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001) using the DNA of the 28 dogs extracted from blood, lymph node and ear hair samples was performed. All samples were tested in duplicate. In order to determine the presence/absence of *Leishmania* kDNA by relative quantification (RQ) we used the TaqMan Gene Expression Assays/Eukariotic 18S rRNA (Applied Biosystems Laboratories, Foster City, CA, USA) as an internal reference of canine genomic DNA and as the calibrator sample the negative control described above. Results were expressed in terms of threshold cycle (Ct). Samples were considered positive when the Ct of each one was lower than 35 (Applied Biosystems Application note, 2006), when the amplification was detected in both replicates and when the $\log_{10}$RQ of each sample was higher than the 5% of the $\log_{10}$RQ of the positive control.

2.4.3. Quantification of kinetoplast minicircle DNA from L. infantum

Once the presence of *Leishmania* kDNA in the hair was detected, in order to know the minimum amount of hair necessary to detect *Leishmania* kDNA and to quantify the parasite load in hairs of various body zones (head, trunk and extremities), in sections of the same hair and in epidermal keratinocytes, a standard curve was carried out (Rutledge and Côte, 2003). To perform

this curve the DNA from six quantities of *Leishmania* parasites (MCAN/ES/1996/BCN150, zymodeme MON-1) ranging from 15,360 to 15 (dilution factor 4×) was extracted and analyzed together with the hair samples in triplicate. Samples were considered positive when the Ct of each one was lower than 35 and when the amplification was detected in all the replicates.

3. Results

As shown in Table 1, most of the analyzed SLA ELISA positive dogs (11 out of 13, Group A) presented the typical signs of canine leishmaniosis (weight loss, hyperkeratosis, lymphadenomegaly, *etc.*), whereas the remaining two dogs were clinically healthy but infected dogs. The Real Time PCR comparative Ct experiment (Table 1) was able to detect the presence of *Leishmania infantum* kDNA in the hair of 9 out of 13 naturally infected dogs (Group A). The sensitivity of the technique in ear hair was similar to that obtained in blood (8 positive out of 13 dogs). The highest sensitivity (100%) was obtained when lymph node samples were analyzed since parasite kDNA was detected in all the infected dogs. As observed in Group A (Table 1), the ELISA assays were more sensitive (100%) than IFAT (10 positive dogs out of 13). The specificity of the Real Time PCR assay either when applied to lymph node, blood and hair samples taken from the healthy dogs (Group B) was confirmed by ELISA and IFAT (Table 1).

Real Time PCR was standardized performing a standard curve experiment (Table 2) in order to quantify the parasite load in hairs from different body zones of *Leishmania* infected dogs (Group A). The limit of detection (LOD) obtained was 0.0079 parasites in the PCR reaction, with a correlation of 0.993. As shown in Table 2, kDNA could be found in the hairs of different body sections (head, trunk and extremities) of dogs A1 and A6. The highest amount of parasite DNA was observed in ischium hair samples of animal A6, even in the case in which the tissue was not treated with proteinase K, a serine protease that digests keratin. However, the quantity of detected kDNA was higher when the same sample was processed with proteinase K.

The standard curve experiment also allows us to quantify the parasite load in the different parts of the hair (Table 2). *L. infantum* kDNA could be detected in all the sections of the same hair as an indication that it is stored along the hair length. The amount of kDNA observed in the hair shaft is larger than in the follicle (animals A1 and A6).

By using the standard curve the minimum amount of hair necessary to detect *Leishmania* kDNA (Table 2) could be also determined. The data shown indicate that there is a direct correlation between the number of ischial hairs (animal A1) and the quantity of parasite DNA. Interestingly, parasite DNA could also be detected and quantified even in a single hair. The individual analysis of ten single ischial hairs from animal A6 (data not shown) showed that most of them (90%) contained kDNA, being the maximum, minimum and average threshold cycles 33.47, 28.25, and 31.21 (SD = 2.19), respectively.

Besides, parasite kDNA was detected and quantified in epidermal keratinocytes of animal A1 (Table 2). In order to determine whether keratinocytes contain parasite kDNA and anti-*Leishmania* antibodies, a supernatant from keratinocytes was obtained as described in Section 2.3.1. The analyses by Real Time PCR showed that keratinocytes contain large amounts of kDNA (Table 2), and also antibodies against *L. infantum*, that were detected by ELISA (Fig. 1) and Western Blot (data not shown).

4. Discussion

Since it has been shown that hair can accumulate many environmental toxics and excrete foreign substances both in humans (Agusa et al., 2005; Kim and Kim, 2011; Forman et al., 1992; Musshoff and Madea, 2006; Dorea et al., 1987; Takiguchi et al., 2001; Uematsu, 1993) and in animals (Gratacós-Cubarsí et al., 2006, 2007; Patra et al., 2007; Wenzel et al., 1996; Stout et al., 2000), and that DNA extraction from hair samples is quite easy (Stolker et al., 2007), we have been tested whether *L. infantum* kDNA could be detected from hair of infected dogs. Thus, the *L. infantum–Canis familiaris* visceral leishmaniosis model was used to examine whether hair is involved

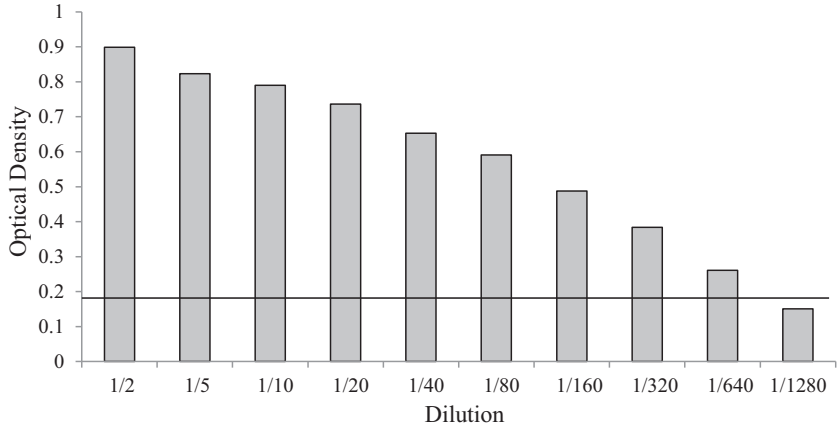

Fig. 1. Analysis by SLA IgG2 ELISA of the epidermal keratinocytes supernatant from animal A1. The results of titration (ranging from 1/2 to 1/1280 dilution) are expressed as the average O.D. obtained from two duplicates. Positive (mean O.D. = 1.061; SD = 0.049) and negative (mean O.D. = 0.148; SD = 0.015) sera controls were employed at 1/200 dilution. The cut-off was established as the mean O.D. values of negative controls + 3SD.

Table 1
Summary of clinical, serological and parasitological evaluation of dogs with (Group A) or without (Group B) canine visceral leishmaniosis caused by *Leishmania infantum*.

Group	Animal	Clinical signs[a]		Serological analyses		Parasitological analyses by Real Time PCR[d]		
		General	Skin	ELISA SLA IgG2[b] Normalized O.D. (assessment)	IFAT[c] 1/*x* (assessment)	Blood Ct (assessment)	Lymph node Ct (assessment)	Ear Hair Ct (assessment)
A (Infected dogs)	1	S, DC, WL, RP, GL	DS, H	1.037 (+)	160 (+)	29.99 (+)	21.38 (+)	29.48 (+)
	2	–	–	0.918 (+)	160 (+)	38.44 (−)	24.75 (+)	39.35 (−)
	3	PL, C	–	0.966 (+)	640 (+)	26.77 (+)	23.23 (+)	29.29 (+)
	4	WL, S, PM, DC, GL, B, C	H, DS, DT, A	1.007 (+)	640 (+)	22.28 (+)	25.26 (+)	30.99 (+)
	5	WL, PM, HY, GL	H	0.926 (+)	320 (+)	22.26 (+)	17.77 (+)	26.16 (+)
	6	HY, GL, E	U	0.982 (+)	640 (+)	32.95 (+)	24.19 (+)	31.17 (+)
	7	GL, C	DS	0.897 (+)	320 (+)	31.06 (+)	21.41 (+)	26.90 (+)
	8	WL, DC, GL, C, B, E	–	0.859 (+)	320 (+)	39.11 (−)	23.24 (+)	36.42 (−)
	9	–	H	0.770 (+)	80 (+)	35.52 (−)	29.84 (+)	29.34 (+)
	10	WL, GL, B	U, H, A	0.889 (+)	320 (+)	34.60 (+)	24.64 (+)	34.16 (+)
	11	E, C	–	0.696 (+)	<40 (−)	37.29 (−)	31.27 (+)	39.38 (−)
	12	–	–	0.651 (+)	<40 (−)	36.36 (−)	31.14 (+)	34.77 (+)
	13	GL	A	0.741 (+)	<40 (−)	33.64 (+)	31.11 (+)	(−)[e]
B (Healthy dogs)	1	–	–	0.318 (−)	<40 (−)	(−)[e]	(−)[e]	36.31 (−)
	2	–	–	0.220 (−)	<40 (−)	37.37 (−)	(−)[e]	35.69 (−)
	3	–	–	0.402 (−)	<40 (−)	35.99 (−)	(−)[e]	(−)[e]
	4	–	–	0.431 (−)	<40 (−)	36 (−)	(−)[e]	36.55 (−)
	5	–	–	0.343 (−)	<40 (−)	36.24 (−)	(−)[e]	35.54 (−)
	6	–	–	0.469 (−)	40 (−)	38.76 (−)	(−)[e]	36.89 (−)
	7	–	–	0.253 (−)	<40 (−)	39.88 (−)	(−)[e]	37.29 (−)
	8	–	–	0.256 (−)	<40 (−)	36.99 (−)	(−)[e]	35.66 (−)
	9	–	–	0.308 (−)	<40 (−)	(−)[e]	(−)[e]	36.28 (−)
	10	–	–	0.473 (−)	<40 (−)	38.12 (−)	(−)[e]	36.55 (−)
	11	–	–	0.319 (−)	<40 (−)	(−)[e]	(−)[e]	(−)[e]
	12	–	–	0.400 (−)	<40 (−)	(−)[e]	36.89 (−)	(−)[e]
	13	–	–	0.250 (−)	<40 (−)	(−)[e]	(−)[e]	(−)[e]
	14	–	–	0.322 (−)	<40 (−)	(−)[e]	(−)[e]	(−)[e]
	15	–	–	0.348 (−)	<40 (−)	(−)[e]	37.71 (−)	(−)[e]

[a] Clinical signs. *General*: weight loss (WL), dull coat (DC), pale mucous membranes (PM), renal pain (RP), hyperthermia (HY), syndrome: anorexia, apathy (S), generalized lymphadenomegaly (GL), partial lymphadenomegaly (PL), conjunctivitis (C), blepharitis (B), episcleritis (E). *Skin*: dermatitis (DT), alopecia (A), ulceration (U), desquamation (DS), hyperkeratosis (H).

[b] ELISA SLA IgG2. Cut-off established: positive result (+): O.D. values >0.640; negative result (−): O.D. values <0.480.

[c] IFAT. Cut-off established: positive result (+) titre ≥1/80; negative result (−) titre <1/80.

[d] Real Time PCR. Cut-off established: positive result (+): Ct values <35; negative result (−): Ct values ≥35.

[e] kDNA not detected at Ct = 40.

Table 2
Real Time PCR results of *Leishmania* kDNA corporal distribution and location in different fragments of the hair and skin.

Animal	kDNA detection and quantification	Body area	Sample	No. of hairs employed	Real Time PCR results	
					Ct[a] (Assessment)	Estimated number of parasites[b]
A1	In different corporal zones	Lumbar	Whole hairs	5 units	32.06 (+)	1.92
		Prescapular	Whole hairs	5 units	31.03 (+)	3.50
		Ischium	Whole hairs	5 units	31.82 (+)	2.14
	In different parts of the hair	Ischium	Hair proximal segments	5 units	30.96 (+)	3.74
			Hair central segments	5 units	32.70 (+)	1.25
			Hair distal segments	5 units	28.73 (+)	15.21
	In different quantities of sample	Ischium	Whole hair	1 unit	34.59 (+)	0.37
			Whole hairs	2 units	33.18 (+)	0.91
			Whole hairs	5 units	31.81 (+)	2.14
			Whole hairs	10 units	29.48 (+)	9.55
	In epidermal keratinocytes	Ischium	Epidermal keratinocites	1 mg	24.89 (+)	170.27
A6	In different corporal zones	Frontal	Whole hairs	10 units	26.79 (+)	53.63
		Hock	Whole hairs	10 units	26.12 (+)	81.44
		Ischium	Whole hairs	10 units	23.98 (+)	301.43
	Without proteinase K treatment	Ischium	Whole hairs	10 units	28.44 (+)	129.22
	In different parts of the hair	Back	Whole hairs	5 units	29.10 (+)	13.13
			Hair shafts	5 units	27.70 (+)	30.50
			Hair follicles	5 units	32.46 (+)	1.67

[a] Cut-off established: positive result (+): Ct values <35; negative result (−): Ct values ≥35.
[b] Each parasite contains about of 10,000–20,000 copies of the minicircle kDNA.

in the recognition, isolation and removal of foreign DNA sequences. We tested if this DNA could be isolated from hair without keratin digestion, as previously stated for endogenous DNA (McNevin et al., 2005). The positive PCR results obtained without digestion with proteinase K suggest that parasite DNA is probably kidnapped among cells (in the extracellular matrix) and that it can be released after simple hair washes. The amount of parasite DNA that hair is able to eliminate and the quantity of hair/kg body removed in dogs every year (60–180 g) (Scott et al., 2001a), suggest that the hair has an important role in the detoxification of the organism.

Nowadays, the clinical diagnosis of canine leishmaniosis is based on different immunological techniques, such as IFAT and ELISA and also on parasite detection through microscope together with molecular techniques like conventional and Real Time PCR (Francino et al., 2006; Gomes et al., 2007; Rodríguez-Cortés et al., 2007). The selection of the best sample for PCR analysis is a very important matter to consider in the veterinarian practice. Lymph node biopsies provide high sensitivity results, but sampling could be difficult in dogs without lymphoadenomegaly (Manna et al., 2004; Maia et al., 2009). Bone marrow aspirates seem to be a suitable alternative, but the collection is invasive and traumatic for animals (De Almeida Ferreira et al., 2012). The PCR analysis of skin biopsies also provide high sensitivity in the medical diagnosis of canine leishmaniosis even when compared with bone marrow (Manna et al., 2004; Rodríguez-Cortés et al., 2007; De Almeida Ferreira et al., 2012), since it has been stated that the main tissue reserve of the parasite in dogs is the skin (Solano-Gallego et al., 2001). On the other hand, many authors suggest that blood is the choice sample because the collection method is simple and slightly invasive, but the sensitivity obtained is lower when compared with the other tissues described above (Manna et al., 2004; Francino et al., 2006). The use of hair for PCR analyses of DNA has many advantages over other methods because collection, transporting and storing is rather easy (Stolker et al., 2007) and the sampling process is non-invasive. We think that hair is a new sample to take in account in the clinical diagnosis of canine leishmaniosis, specially in field studies. We also suggest that this method could be applicable not only in domestic dogs, but also in wild carnivores where the study of leishmaniosis by molecular methods is still scarce (Sobrino et al., 2008). The data presented in this paper describe a new method for a reliable diagnosis of leishmaniosis with a sensitivity similar to that obtained in blood samples even analyzing one single hair, although the use of at least five hairs is recommend to increase the sensitivity of the method.

Since the highest amount of parasite DNA was detected in the hair from the ischial zone of one of the *Leishmania* infected animals, we suggest this corporal zone as one of the choice area for sampling. However, we recommend to take samples at least from other two body regions (head, trunk) for more reliable results.

Future studies using hair samples are necessary to accurately determinate the sensitivity of Real Time PCR technique for the diagnosis of the different forms of canine leishmaniosis. Whether parasite kDNA could be detected only in recent cases of leishmaniosis or in other situation like corpses of human and animal fossils remain to be examined. The detection of parasitic diseases in dead bodies is difficult, being reported only by several archaeological references such as the isolation of *Trypanosoma cruzi* DNA in mummified corpses (Guhl et al., 1999; Guhl, 2005). Thus, we think that multiple applications of hair analysis could be developed to perform forensic and ancient

DNA detection for archeoparasitological studies in animal and human fossils. Hair is a contaminant–resistant tissue higher than bone and teeth samples. This fact is due to the hydrophobic nature of the cuticle proteins and the presence of keratin that avoid the penetration of contaminant extracorporeal DNA in hair (Gilbert et al., 2006). Based on the data presented, we believe that hair could have significant scientific and diagnostic value for the ongoing analysis of a *Leishmania* infection course. Moreover, we think that these data also add some insights to the understanding of the physiology of hair and epidermal keratinocytes and the possible involvement of these cells in the kidnapping and removal of foreign DNA in other infectious, neoplasic or autoimmune diseases.

It is known that the development of dermatitis is very variable among individuals due to the different host immune responses induced by *Leishmania* infection (Tapia et al., 1993). The role, however, that keratinocytes can play in primary and in already established infections is poorly known. As many other types of cells like leukocytes, macrophages and plasmocytes (Tapia et al., 1994) increasing evidences suggest that keratinocytes participate in cutaneous immune responses against pathogens observed in dermal and follicular inflammations that lead to the typical alopecia observed in leishmaniosis. Recently, it has been reported (Pivarcsi et al., 2005) that keratinocytes may play initiating roles by secreting cytokines and participate in the innate and acquired immune response against parasites (Girolomoni et al., 2006; Scott et al., 2001b). Furthermore, it is known that immunoglobulin receptors are present on keratinocytes (Cauza et al., 2005), and that these cells also express at least seven members of the human Toll-like receptors surface family (Pivarcsi et al., 2005). Our finding of anti-*L. infantum* immunoglobulins in keratinocytes suggests that a transepidermal elimination process of these proteins in the course of the disease is taking place. Keratinocytes may also participate in the mechanisms of control, elimination, and repair of damaged ecological niches. The presence of parasite kDNA in hair is probably connected with this metabolic and immunorregulatory function of keratinocytes that act as fixer cells of molecules for its subsequent elimination by the organism. This function may be extended along all the epidermal surfaces, as shown by the presence of parasite DNA sequences in keratinocytes from different body zones in infected animals.

In view of the data obtained we propose two mechanisms for the origin of foreign kDNA in the hair of infected animals, although the precise mechanisms have yet to be determined. One of them would be its direct incorporation in follicular cells, during keratinization process (Bengtsson et al., 2012; Harkey, 1993), due to local inflammation/dermatitis, that we consider the most important. But we do not exclude also the incorporation of this kDNA by migration from the bloodstream.

In conclusion, the large amounts of parasite kDNA sequestered and removed by growing hair suggest that it is a highly specialized tissue for the excretion of foreign organic toxic metabolites. The methodology described in this work could be useful to detect DNA molecules not only in cutaneous and mucocutaneous leishmaniosis forms but also in diseases coursing with skin manifestations, such as tuberculosis, sarcoidosis, histoplasmosis, and schistosomiasis. In all of these diseases, transepithelial elimination of microorganisms has been reported (Goette and Odom, 1986; Welykyj et al., 1991; Wood et al., 1976).

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