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Short communication

First detection of *Leishmania* kDNA in canine cerumen samples by qPCR

Silvia Belinchón-Lorenzo[a,*], Juan Carlos Parejo[b], Virginia Iniesta[a], Javier Fernández-Cotrina[a], Rubén Muñoz-Madrid[a], Isabel Monroy[a], Victoria Baz[a], Adela Gómez-Luque[a], Francisco Javier Serrano-Aguilera[a], José Luis Barneto[a], Luis Carlos Gómez-Nieto[a]

[a] *LeishmanCeres Laboratory (GLP Compliance Certificated), Parasitology Unit, Veterinary Faculty, University of Extremadura, Avenida de la Universidad s/n, 10003 Cáceres, Spain*
[b] *Genetics Unit, Veterinary Faculty, University of Extremadura, Avenida de la Universidad s/n, 10003 Cáceres, Spain*

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ABSTRACT

Nowadays, searching for alternative non-invasive methods for molecular diagnosis of canine visceral leishmaniosis is getting increasingly important. We previously described the presence of *Leishmania* kinetoplast DNA (kDNA) in canine hair; in this case we hypothesized whether foreign DNA might be present in cerumen of dogs with leishmaniosis, and be detected by Real time quantitative PCR (qPCR). A population of 38 dogs that lived in *Leishmania* endemic areas was divided in two groups: A (33 dogs with confirmed leishmaniosis by serological techniques) and B (5 healthy dogs). Blood, lymph node, bone marrow and cerumen samples from all animals were tested for the presence of parasite kDNA. Our method was 100% specific, and in dogs from group A, *Leishmania infantum* kDNA was detected and quantified in the 100% of lymph node samples, in 90.9% of cerumen samples, in 88.5% of the bone marrow samples and in 57.6% of the blood samples. The qPCR-cerumen is a new non-invasive method that shows a high potential for the diagnosis of zoonotic visceral leishmaniosis.

1. Introduction

Leishmaniosis is one of the most important parasitic diseases, endemic in more than 88 countries affecting millions of people (Mutiso et al., 2013) and classified inside category 1 by the World Health Organization. Dogs have been implicated as the main reservoir of *Leishmania infantum* (*L. infantum*) due to its close relationship with humans (Dantas-Torres, 2007), and it is known that the disease affects dogs from all continents except Oceania (Dantas-Torres et al., 2012). For these reasons, it is important to design new diagnostic methods to early detect the canine infection.

Currently, the clinical diagnosis of canine leishmaniosis can be performed using different methods: parasite detection through microscope, several immunological tests and also molecular techniques such as conventional and Real Time PCR (qPCR) (Maia and Campino, 2008).

There is a controversy about which is the best sample for PCR analysis in canine leishmaniosis. Lymph node, bone marrow and skin biopsies provide high sensitivity results (Solano-Gallego et al., 2001; De Almeida Ferreira et al., 2012). Blood samples have lower sensitivity (Solano-Gallego et al., 2011), although the collection is easier (Manna et al., 2004). However, all these samples are obtained by invasive methods that cause animal distress and owner reluctance, so it is important to test non-invasive samples as an alternative. In this way, recent evidences suggest the potential use of conjunctival, oral, nasal, ear and vulvar swabs for sampling, taking cells from the mucosa or from other anatomical regions (De Almeida Ferreira et al., 2012; Ferreira et al., 2013; Hernández et al., 2015). Moreover, it has been previously reported that hair is a suitable sample for the molecular diagnosis of leishmaniosis in dogs, mice and wild mammals (Belinchón-Lorenzo et al., 2013; Iniesta et al., 2013; Muñoz-Madrid et al., 2013), as it does not cause any harm or stress to the animals, and the sensitivity of the technique is similar to that obtained using blood samples.

In this paper we have focused on the sampling of cerumen, since it has been described the presence of high parasite load in the skin

* Corresponding author.
E-mail addresses: sibelo@unex.es, leishmanceres@gmail.com, leishmanceres@unex.es (S. Belinchón-Lorenzo).

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of the canine ear compared to the skin of other corporal zones in dogs with leishmaniosis (Travi et al., 2001; Xavier et al., 2006).

Therefore, our objectives were to evaluate canine cerumen as a new non-invasive sample suitable to detect *L. infantum* kDNA by qPCR in dogs with leishmaniosis and compare the obtained results with those from the invasive samples generally employed for diagnosis: bone marrow, lymph node and blood.

2. Materials and methods

2.1. Animals

A population of 38 dogs from a leishmaniosis endemic area was selected based on the results for SLA ELISA and IFAT techniques, performed as described elsewhere (Belinchón-Lorenzo et al., 2013), and divided in two groups:

– Dogs with confirmed leishmaniosis (group A; n = 33). The majority of the dogs showed at least one of the typical clinical signs of the disease: lymphadenopathy, ocular (blepharitis, keratoconjunctivitis, etc.), cutaneous (alopecia, desquamation, ulcers, etc.) and general signs (apathy, weight loss, weakness, hyperthermia, etc.). Only one dog was asymptomatic.

– Healthy dogs (group B; n = 5). They did not present any clinical sign.

2.2. Sampling

Blood and cerumen samples from all dogs, 37 lymph node samples and 31 bone marrow samples were collected. Blood samples were obtained by fine-needle aspiration from cephalic vein and deposited in EDTA coated tubes. Lymph node biopsies were obtained by fine-needle aspiration and homogenized in 1 ml of sterile PBS. Bone marrow biopsies were obtained by sternal puncture and aspiration and included in EDTA coated tubes. Finally, a double tipped sterile cotton swab (previously treated 40 min with UV rays) was inserted into the ear of the dogs and swabbed against the surface of the vertical ear canal to obtain cerumen samples, cut in halves and introduced in a screw cap sterile microtube. Once processed, all samples were stored at −80 °C until DNA extraction.

2.3. DNA extraction

One cotton tip with cerumen sample per dog was incubated in 250 μl of lysis buffer (10 mM TrisCl, 0.1 M EDTA, 0.5% SDS + 20 μg/ml pancreatic RNase) for two hours at 37 °C. Proteinase K was added to a final minimum concentration of 100 μg/ml and incubated at 56 °C overnight. For DNA extraction, 200 μl of supernatant obtained from processed cotton swabs, 100 μl of whole blood, 100 μl of homogenized lymph node biopsies and 50 μl of bone marrow were used. DNA from all samples was obtained using the UltraClean BloodSpin kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. qPCR assays

Reactions were carried out in 96 wells PCR plates in a final volume of 20 μl (4 μl of DNA + 16 μl of Reaction Mix), containing 20 μM of each primer, 10 μM of TaqMan Probe, and the iTaq Universal Probes Supermix (Biorad Laboratories, Hercules, CA). Primers, probe and the thermal cycling profile used are described elsewhere (Francino et al., 2006). Each amplification run contained positive and negative controls. All qPCR analyses were performed in a Step One Plus Real Time PCR System (Applied Biosystems Laboratories, Foster City, CA, USA). A standard curve was carried out (Rutledge and Côte, 2003) using DNA extracted from six quantities of *L. infantum* parasites (MCAN/ES/1996/BCN150, zymodeme MON-1) ranging from 50,000 to 0.5 (dilution factor x10). The threshold cycle (Ct) corresponding to the Y-intercept of each analysis (that is, the expected Ct value for the estimated quantity of 1 parasite) was used as cut-off, being positive those samples whose Ct values were ≤Y-intercept value of each assay.

2.5. Statistical analyses

Statistical analysis was performed using R version 3.2.3 (R Core Team, 2015). Relationships among diagnostic results were estimated by the non-parametric coefficient rho of Spearman (r) since data did not meet assumptions of normality. The p-values were adjusted by the method of Benjamini and Hochberg (1995) in order to control the false discovery rate due to the multiple comparisons. Corrected p-values < 0.05 were considered statistically significant.

3. Results

Table 1 shows the antibody levels (IFAT and ELISA), presence or absence of clinical signs and qPCR results of dogs from group A (with confirmed leishmaniosis) and B (healthy).

Dogs from group A were positive for both serological techniques with different intensity, as it is shown in Table 1. The percentages of appearance of the typical clinical signs of leishmaniosis in this group were: 81.8% for lymphadenopathy, 78.8% for cutaneous signs, 42.4% for ocular signs and 15.2% for systemic clinical signs.

All dogs from group B were negative by both IFAT and ELISA methods and did not show any clinical sign (Table 1).

Respect to the qPCR technique, the reliability of the results obtained was assessed by the R^2 coefficient data (from 0.996 to 0.999), slope (from −3.38 to −3.20) and the high efficiency reached (between 97.83% and 105.42%).

Regarding qPCR sensitivity, we could detect *Leishmania* kDNA in cerumen of 30 out of 33 dogs from group A (90.9%), higher to that obtained for bone marrow (88.5%, 23+/26). The highest sensitivity (100%, 32+/32) was obtained in lymph node and the lowest in blood samples (57.6%, 19+/33).

There were 12 dogs with negative result for blood-qPCR and with positive result for cerumen-qPCR, whereas there was only one dog (no. 31) positive to blood-qPCR and negative to cerumen-qPCR. The proportion of cases detected in cerumen was not significantly different from lymph node and bone marrow, but it was respect to blood samples ($p < 0.01$).

The highest quantities of parasites were obtained in bone marrow and lymph node (Table 1). Although the parasite load in cerumen samples was, in general, nearest to that obtained in blood and significantly different than lymph node and bone marrow, is worth to note that the quantity of kDNA detected in cerumen showed a better correlation with the parasite load present in target organs. Thus, most of dogs with a number of estimated parasites less than 10^3 in bone marrow and lymph node showed low amount of kDNA in cerumen (less than 20 parasites per swab) and the highest kDNA concentrations observed in bone marrow and/or lymph node corresponded to the highest quantities detected in cerumen.

On the other hand, the specificity of qPCR assays was the 100% applied to all kind of samples taken from healthy dogs of the group B (Table 1).

The statistical analysis showed a significant correlation between the antibody titers determined by both immunological tests and the concentration of *Leishmania* kDNA obtained by qPCR in all the samples ($p < 0.05$). Likewise, the presence of clinical signs and qPCR analyses were significantly correlated $p < 0.01$); being the lymphadenopathy highly correlated with the lymph node qPCR ($r = 0.65$; $p < 0.01$).

Table 1
Summary of clinical signs, immunological and qPCR results of dogs from group A (with leishmaniosis) and B (healthy).

GROUP	ANIMAL No.	CLINICAL SIGNS[a]	IMMUNOLOGICAL ANALYSES		qPCR ANALYSES[d] (Estimated mean quantity of parasites)			
			SLA IgG2 ELISA[b] (O.D.)	IFAT[c] (Titre)	Bone marrow	Lymph node	Blood	Cerumen
A	1	O,C	0.53	Jan-80	N	2.48	N	19.58
	2	–	0.534	Jan-80	553.44	5.87	N	6.44
	3	LN,C	0.946	1/320	476.94	684.96	N	15.57
	4	C	0.766	1/320	5.14	1.91	N	10.69
	5	LN,C	0.858	1/640	N	57.56	N	30.2
	6	LN,C	0.611	1/320	105.14	9.26×10^3	N	39.67
	7	LN,C	0.798	1/320	2.94	4.02	N	7.6
	8	C	0.937	>1/640	N	1.39	N	1.9
	9	LN,C	0.997	>1/640	2.52	6.23	N	9.01
	10	LN,C	0.929	1/320	3.08×10^6	1.70×10^5	260.12	538.13
	11	LN,C	1.063	>1/640	3.23×10^5	648.1	18.9	381.48
	12	LN,C,O	0.712	1/320	9.90×10^4	7.83×10^4	16.29	65.62
	13	LN,C,O	1.003	>1/640	3.30×10^6	1.50×10^6	528.03	510.06
	14	LN,C,O	0.987	1/640	1.81×10^4	8.45×10^4	17.69	19.5
	15	LN,C	1.063	>1/640	3.77×10^5	9.02×10^4	92.3	411.12
	16	LN,C,O	1.019	>1/640	4.53×10^5	6.65×10^3	N	455.38
	17	LN,C,O	0.95	1/640	822.28	948.22	N	339.69
	18	LN,C,O	0.977	>1/640	8.55×10^4	6.52×10^4	14.17	317.7
	19	G,LN,C,O	1.042	1/640	N.D.	3.81×10^3	2.41	13.15
	20	LN	0.984	Jan-80	N.D.	N.D.	N	5.33
	21	G,LN,C,O	0.983	1/640	N.D.	1.51×10^4	27.33	121.27
	22	LN,C,O	0.969	1/640	N.D.	1.49×10^3	25.63	2.03
	23	LN	0.949	1/640	4.42×10^5	9.98×10^3	17.88	5.18×10^3
	24	LN,C	0.957	1/640	1.98×10^5	4.59×10^3	30.49	48.65
	25	C,O	0.938	1/640	7.67×10^3	1.53×10^3	60.64	26.46
	26	G,C,LN	0.91	>1/640	5.53×10^5	1.64×10^5	2.24×10^4	84.81
	27	C,LN,O	0.735	1/640	2.89×10^7	1.86×10^6	1.09×10^3	360.42
	28	G,LN,O	0.922	1/640	N.D.	7.28×10^5	258.68	35.26
	29	LN	0.767	1/320	209.02	523.94	3.5	1.77
	30	C	0.878	>1/640	2.04×10^4	1.52×10^4	29.6	51.75
	31	G, LN	0.918	>1/640	5.32×10^4	1.51×10^4	318.15	N
	32	LN,C,O	0.951	1/640	N.D.	1.60×10^3	N	N
	33	LN	0.839	>1/640	N.D.	2.22×10^3	N	N
	% Sensitivity (X positives / Y total samples analyzed)				88.5% (23/26)	100% (32/32)	57.6% (19/33)	90.9% (30/33)
B	34	–	N (0.347)	N (< 1/40)	N	N	N	N
	35	–	N (0.352)	N (< 1/40)	N	N	N	N
	36	–	N (0.323)	N (< 1/40)	N	N	N	N
	37	–	N (0.206)	N (< 1/40)	N	N	N	N
	38	–	N (0.337)	N (< 1/40)	N	N	N	N
	% Specificity (X negatives / Y total samples analyzed)				100% (5/5)	100% (5/5)	100% (5/5)	100% (5/5)

N: negative, N.D.: Not determined (absence of sample).
[a] Clinical signs: O (ocular signs: blepharitis, keratoconjunctivitis, etc.), G (general signs: apathy, weight loss, weakness, hyperthermia, etc.), LN (lymphadenopathy), C (cutaneous signs: alopecia, desquamation, ulcers, etc.).
[b] SLA IgG2 ELISA: positive O.D. ≥ 0.497 (set as the mean of O.D. values plus 3SD of a population of 30 healthy dogs).
[c] IFAT: positive titre ≥ 1/80.
[d] qPCR: Positive result is considered when Ct ≤ Y-intercept value of each assay (data not shown). Estimated quantity of parasites detected per 100 μl (bone marrow/blood/diluted lymph node biopsy) and per one cotton swab with cerumen sample.

It is important to highlight that the results obtained with cerumen-qPCR method were strongly correlated with those obtained with the qPCR analyses performed with bone marrow ($r = 0.82$, $p < 0.01$), lymph node ($r = 0.70$, $p < 0.01$) and in a lesser extent with the blood results ($r = 0.54$, $p < 0.01$). Significant coefficients ($p < 0.01$) were also found between the cerumen-qPCR and the presence of clinical signs ($r = 0.53$) and between this technique and the antibody titers tested by ELISA and IFAT ($r = 0.50–0.45$).

4. Discussion

The results obtained in this work showed that *Leishmania* kDNA can be isolated, detected and quantified by qPCR in cerumen samples obtained with cotton swabs from dogs with leishmaniosis. As far as we know, this is the first report of the presence of *Leishmania* kDNA in canine cerumen samples.

Cerumen has been investigated as a medium for the biological monitoring of xenobiotics (Guy et al., 1999), even pesticides and metals have been detected in human cerumen (Krishnan and Que Hee, 1992) and also Hepatitis B virus DNA (Parizad et al., 2011).

Because of the composition of the cerumen (desquamated epithelial cells along with the secretions from the sebaceous and ceruminous glands) (Cole, 2009), we suggest that *Leishmania* kDNA would be incorporated to this sample by transepidermal elimination. This phenomenon has been described for *Leishmania* parasites in all epidermal layers, hair follicles and eccrine sweat glands (Perrin et al., 1995; Espinel et al., 2004; Karram et al., 2012). However, it cannot be excluded the incorporation of *Leishmania* kDNA to cerumen by migration from the bloodstream, because ear and the ear canal itself receive blood from a high number of arteries (Steven-Sparks, 2012).

The cerumen-qPCR method showed a sensitivity similar to that obtained using lymph node samples, and higher to that obtained using bone marrow samples. According to many authors, blood was the sample with the lowest sensitivity (Leite et al., 2010; De Almeida Ferreira et al., 2012; Lombardo et al., 2012): 12 dogs that were negative in blood had positive results in cerumen-qPCR and, on the contrary, only one dog was positive to blood and negative to cerumen.

Although the parasite load obtained in cerumen is low, we consider that it is enough to ensure the diagnosis of the disease in most cases, as the results were confirmed with the rest of the methods performed.

Despite the DNA extracted from cerumen showed low yield (data not shown), we could detect *Leishmania* kDNA in cerumen of 30 out of 33 dogs with leishmaniosis, regardless of the presence of clinical lesions in the aural zone (as some dogs only showed lymphadenopathy), even in the asymptomatic dog. We think that the target used (kDNA) and the clinical condition of the canine population have improved the sensitivity of the technique, but it would be necessary to perform future studies to accurately determinate the chronobiology of the *Leishmania* kDNA incorporation to cerumen during the course of the disease.

Finally, the use of cerumen for molecular analyses presents many advantages. The collection of this sample is harmless and easy, and there is no need of specific conditions for transport and storage (though we recommend to freeze samples when a long term storage is needed). So, we propose the cerumen as a new sample to take into account for the molecular diagnosis of canine leishmaniosis.

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