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Application of qPCR method to hair and cerumen samples for the diagnosis of canine leishmaniosis in Araçatuba, Brazil

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ABSTRACT

Visceral leishmaniosis (VL) remains a serious public health problem in Brazil. Dogs are the main hosts of the parasite, developing canine leishmaniosis (CanL), hence the importance of an accurate diagnosis of the animals. Recently, the application of qPCR method to non-invasive samples obtained from dogs with CanL has shown high sensitivity. Thus, we analyzed by qPCR blood, hair (from healthy zones and cutaneous lesions) and cerumen of 16 dogs with confirmed leishmaniosis from Araçatuba, a Brazilian endemic area. Cerumen-qPCR showed the highest sensitivity (87.5%), followed by hair (lesions: 78.57%, healthy skin: 62.5%), and blood (68.75%). We also analyzed blood, hair and cerumen of 5 healthy dogs from a non-endemic area, obtaining 100% of specificity in all samples. The use of cerumen and hair for qPCR analysis provides high reliability, taking into account the sensitivity and total specificity of the method. The non-invasive sampling procedure without the need of specific conditions of storage and transport support the usefulness of hair and cerumen for the diagnosis of CanL.

1. Introduction

Leishmaniases are diseases caused by protozoan parasites from the genus *Leishmania* transmitted by phlebotomine sandflies (Akhoundi et al., 2016). The visceral form of leishmaniosis (VL) is found worldwide, occurring over 90% of cases in six countries: Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan (WHO, 2017). In Brazil, VL cases are present both in rural and urban areas, being affected large cities such as Rio de Janeiro, Belo Horizonte, Araçatuba, Santarém, Corumbá, Teresina, Natal, São Luís, Fortaleza, Três Lagoas, Campo Grande and Palmas (Ministério da Saúde of Brazil, 2006).

Dogs are the main domestic reservoir of the parasite, developing canine leishmaniosis (CanL), and can present from subclinical to generalized disorders that sometimes overlap with other diseases, which difficult the diagnosis (Gomes et al., 2008). To confirm CanL, the Brazilian Zoonosis Control Centre perform immunological methods (Leite et al., 2010), but it is necessary to combine different techniques for a more accurate diagnosis. In this context, the use of non-invasive samples for the molecular diagnosis of CanL has become increasingly important nowadays, as it has shown high sensitivity results (Leite et al., 2010). In Brazil, previous studies have evaluated conjunctival swabs, obtaining high sensitivity and specificity in symptomatic, asymptomatic and vaccinated dogs (Strauss-Ayali et al., 2004; Leite et al., 2010; Leite et al., 2011). In addition, studies conducted in Spain and Brazil have proved the efficacy of hair from dogs with CanL for the molecular diagnosis of the disease (Belinchón-Lorenzo et al., 2013; De Sousa Gonçalves et al., 2016). Finally, it has been also demonstrated that cerumen is a good source of *Leishmania* kinetoplast DNA (kDNA) (Belinchón-Lorenzo et al., 2016).

Therefore, the aim of this study was to assess the combined use of hair and cerumen samples by Real Time quantitative PCR (qPCR) of dogs from Araçatuba, a Brazilian endemic area, comparing these results with blood-qPCR and serology, in order to emphasize the importance of these non-invasive samples for the molecular diagnosis of CanL.

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2. Material and methods

2.1. Animals

We stablished two different groups:

- Group A ($n = 16$): Dogs with confirmed leishmaniosis, 12 male and 4 female, ranging from 2 to 6 years old were selected from the Veterinary Teaching Hospital of UNESP, São Paulo State University and from the Zoonosis Control Center (Araçatuba, São Paulo State, Brazil). CanL diagnosis was performed by serology (DPP and ELISA, Bio- Manguinhos/Fiocruz, Manguinhos, RJ, Brazil) and afterwards confirmed using popliteal lymph node fine-needle aspiration. In compliance with the current state law (SUCEN of São Paulo, 2006), dogs were euthanized with the owners' permission. All dogs except one showed at least one of the typical clinical signs of the disease.
- Group B ($n = 5$): Healthy dogs were selected from veterinary clinics from Londrina (Paraná State, Brazil), a leishmaniosis non-endemic area. Dogs did not present any clinical signs, and were assessed as negative by parasitological and serological methods.

2.2. Sampling

Dogs from group A were anesthetized with pentobarbital (Hypnol®). Peripheral blood samples (4 ml) were obtained from the cephalic vein and collected in EDTA tubes. 100 µl of blood were placed in Whatman paper. Then, animals were euthanized with potassium chloride. Hair samples were extracted with tweezers, from healthy body zones (all dogs) and from areas surrounding cutaneous lesions when present (14 dogs). Cerumen was obtained inserting double tipped sterile cotton swabs into the dogs' ears and swabbing against the surface of the vertical ear canal. Samples of healthy dogs from group B (blood, cerumen and hair) were obtained *in vivo* without anesthesia. All samples were stored at room temperature.

2.3. SLA ELISA

Whatman papers containing blood were washed in 1 ml of sterile PBS and stirred 24 h at 37 °C. Supernatant was diluted 1/50 and tested in duplicate. SLA ELISA assays were performed as described (Belinchón-Lorenzo et al., 2013). Cut-off value was set as mean O.D. values plus 3SD obtained from the 5 control dogs. Individual results are expressed as the mean O.D. of both duplicates.

2.4. DNA extraction and qPCR analysis

Half cotton swab with cerumen, Whatman papers with blood, and 10–15 hairs/sample were incubated at 56 °C overnight in 250 µl of lysis buffer (10 mM TrisCl, 0.1 M EDTA, 0.5% SDS + 20 µg/ml pancreatic RNase + proteinase K 100 µg/ml). DNA was extracted using 200 µl of supernatant with UltraClean BloodSpin kit (MoBio Laboratories, Carlsbad, CA, USA). The qPCR assays were performed following published protocols (Belinchón-Lorenzo et al., 2016). Samples were considered positive when the estimated quantity of parasites obtained was ≥1.

3. Results

Table 1 shows clinical status, antibody levels and qPCR results of the studied population. All dogs from group A (excepting number 2), showed an advanced status of the disease with the presence of the typical clinical signs. The whole group showed positive antibody response by SLA-ELISA, assessed as low, medium and high. Regarding qPCR, kDNA was detected in 14 of 16 dogs, 9 showed positive results in all samples and the rest were positive to at least two samples. Dogs 8 and 11 did not present kDNA in any sample, despite both animals had clinical signs and showed high and low antibody response, respectively.

Blood-qPCR reached the lowest sensitivity, 68.75% (11 + /16), although the mean estimated quantity of parasites (333.64) was the highest within all samples, due to the great parasite load detected in dogs 2 and 13. In contrast, cerumen-qPCR showed the highest sensitivity, 87.5% (14 + /16), with a mean estimated quantity of parasites of 180.82. Regarding hair-qPCR results, sensitivity was higher in hair from lesions (78.57%) than in healthy hair (62.5%), although mean quantities of kDNA obtained were similar (104.14 and 98.5, respectively). We could not obtain hair from skin lesions from dogs 2 (asymptomatic) and 3 (only had onychogryphosis as cutaneous sign).

On the other hand, the specificity of qPCR assays was the 100% in all samples from healthy dogs of the group B.

4. Discussion

Epidemiological control of VL in Brazil involves systematic treatment of human cases, insecticide treatment (Ferreira et al., 2008) and the elimination of dogs with CanL, previously confirmed by immunological analysis. However, serological techniques may present some problems like cross-reactivity with other parasitic diseases and low sensitivity and specificity in early or asymptomatic infections (De Andrade and Melo, 2014). Thus, for a more accurate diagnosis it is necessary to perform molecular techniques, especially qPCR, which provides a highly sensitive and specific detection and quantification of the parasitic DNA (Reithinger and Dujardin, 2007). Recently, the association between qPCR and non-invasive samples for the diagnosis of CanL has shown high sensitivity results, even when compared with samples obtained by an invasive way (Leite et al., 2010; Belinchón-Lorenzo et al., 2013). In Brazil, non-invasive samples have been evaluated, such as swabs from eye conjunctiva, from oral and nasal mucosa (De Andrade and Melo, 2014) and hair (De Sousa Gonçalves et al., 2016), obtaining high sensitivities. Taking these into account, we have analyzed by qPCR hair (from healthy and injured areas) and cerumen for the diagnosis of CanL in dogs from Araçatuba, a Brazilian endemic area.

Regarding cerumen samples, our sensitivity results (87.50%) are in agreement with those achieved in Spain (90.90%) from a population of 33 dogs (Belinchón-Lorenzo et al., 2016).

Concerning hair, our sensitivity results of hair from different healthy zones (62.50%) are consistent with the published by Belinchón-Lorenzo et al. in 2013 (69.24%, hair from ears), but lower than the 80% (dorsal hair) reached in Brazil (De Sousa Gonçalves et al., 2016). We detected kDNA in hair from different healthy body areas (cervical dorsal, abdominal lateral and head), even from the asymptomatic animal, but further research should be done regarding larger populations of dogs with and without clinical signs. It is worth to note that in the current study sensitivity increased using hair obtained from cutaneous lesions (78.57%), as desquamations or ulcers. A similar finding has been already described using a murine model of cutaneous leishmaniosis (Iniesta et al., 2013).

Despite hair and cerumen are a good source of kDNA for the diagnosis of CanL, we did not detect *Leishmania* kDNA in any sample from dogs 8 and 11. As both animals presented antibody response and clinical signs, a mild or low parasite load could explain their negative PCR results, being kDNA only detectable in lymphoid organs such as lymph nodes, since animals were confirmed as positive using popliteal lymph node fine-needle aspiration.

As samples suitable for qPCR, hair and cerumen have many advantages. The collection is fast, harmless, and they do not need specific conditions for transport and storage, being useful for large-scale field studies. Moreover, it has been demonstrated that *L. infantum* kDNA remains stable within hair after the exposure to different environmental conditions (Muñoz-Madrid et al., 2013).

Finally, we also performed serological and molecular analyses of dried blood spots in Whatman paper stored at room temperature.

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

Group	Animal no.	Clinical signs[a]	Origin of hair		Immunological analyses	qPCR analyses[c] (Estimated mean quantity of parasites/sample)			
			Healthy	Lesion	SLA IgG2 ELISA[b] (O.D.)	Blood	Cerumen	Hair	
								Healthy	Lesion
A	1	G,C,O	Cervical dorsal	Abdominal lateral	0.435(+)	8.13	181.15	3.07	10.9
	2	–	Abdominal dorsal	–	0.346(+)	1714.87	7.48	2.19	ND
	3	G,C,O,LN	Abdominal dorsal	–	0.443(+)	411.3	470.66	207.75	ND
	4	G,C,O	Head	Head	0.674(++)	38.49	85.29	2.68	55.21
	5	C,O,LN	Head	Ear	0.991(+++)	1.35	1193.48	12.37	268.4
	6	G,C,O,LN	Head	Ear	1.475(+++)	419.13	36.18	162.24	138.78
	7	C,LN	Head	Right leg	1.107(+++)	N	1.22	N	24.71
	8	G,C	Head	Ear	1.301(+++)	N	N	N	N
	9	G,C,LN	Head	Ear	1.361(+++)	1.25	1.87	N	12.5
	10	C,O	Head	Ear	0.779(++)	N	1.88	N	24.6
	11	C,O	Head	Ear	0.512(+)	N	N	N	N
	12	G,C,O,LN	Head	Head	1.254(+++)	19.24	59.99	14.1	36.69
	13	G,C	Head	Head	1.035(+++)	1009.88	1.24	N	N
	14	C	Head	Ear	1.102(+++)	N	16.28	300.43	85.67
	15	G,C,LN	Head	Ear	0.687(++)	19.97	5.31	4.46	2.72
	16	C,O,LN	Head	Head	1.277(+++)	26.48	469.49	275.84	485.34
	% Sensitivity (X positives/Y total samples analyzed)					68.75% (11/16)	87.50% (14/16)	62.50% (10/16)	78.57% (11/14)
	Mean estimated quantity of parasites					333.64	180.82	98.51	104.14
B	17	–	Head	–	0.178	N	N	N	ND
	18	–	Head	–	0.122	N	N	N	ND
	19	–	Head	–	0.115	N	N	N	ND
	20	–	Head	–	0.108	N	N	N	ND
	21	–	Head	–	0.114	N	N	N	ND
	% Specificity (X negatives/Y total samples analyzed)					100% (5/5)	100% (5/5)	100% (5/5)	ND

N: negative, ND: Not determined (absence of sample).

[a] Clinical signs: O (ocular signs: conjunctivitis, ocular discharge), G (general signs: cachexia, temporalis muscle atrophy), LN (generalized lymphadenopathy), C (cutaneous signs: generalized alopecia, seborrhea, onychogriphosis, hypotrichosis, nasal hyperkeratosis).

[b] SLA IgG2 ELISA: stablished cut-off positive O.D. ≥ 0.214 (set as the mean of O.D. values plus 3SD of the healthy dogs). Low positive (+): O.D. ≤ 0.5; medium positive (++): 0.5 > O.D. ≤ 0.8; high positive (+++): O.D. > 0.8.

[c] qPCR: Positive result was considered when the estimated quantity of parasites obtained was ≥1. Values obtained per diluted blood disk, 10–15 hairs and half cotton swab with cerumen sample.

Despite all positive dogs showed antibody responses, the sensitivity reached in blood-qPCR was the lowest (68.75%), in accordance with previous findings (Leite et al., 2010; De Almeida Ferreira et al., 2012). Thus, molecular analyses of cerumen and hair provide a more accurate diagnosis of CanL compared to blood.

5. Conclusion

Our results support the use of hair (especially from cutaneous lesions if they are present) and cerumen for the molecular diagnosis of CanL, being appropriate to confirm the results given by the serological analysis commonly used in Brazilian control programs.

Ethical statement

The study was approved by the institutional Ethics and Animal Welfare Committee (CEEA - Comissão de Ética e Experimentação Animal, UNESP, process #2014–00876).

Conflict of interest

The authors have no conflict of interest.

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