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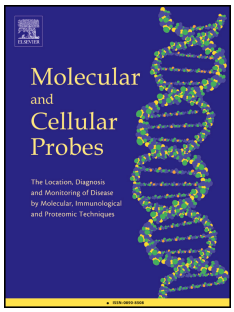

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Norbert Müller, Brigitte Hentrich, Caroline F. Frey, Monika Welle

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Quantitative PCR for the diagnosis of cutaneous leishmaniasis from formalin-fixed and paraffin-embedded skin sections

Norbert Müller[a,*], Brigitte Hentrich[a], Caroline F. Frey[a], Monika Welle[b]

[a] *Institute of Parasitology, Vetsuisse Faculty, University of Berne; Berne, Switzerland*

[b] *Institute of Veterinary Pathology, Vetsuisse Faculty, University of Berne; Berne, Switzerland*

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* *Corresponding author*. Tel: +41 31 631 2474; Fax: +41 31 631 2622

E-mail address: norbert.mueller@vetsuisse.unibe.ch (N. Müller)

ABSTRACT

The present report describes a real-time PCR-based procedure to reliably determine the quantity of *Leishmania* amastigotes in relation to the amount of host tissue in histological skin sections from canine and equine cases of cutaneous leishmaniasis. The novel diagnostic *Leishmania*-PCR has a detection limit of <0.02 amastigotes per µg tissue, which corresponds well to the detection limit of immunohistochemistry and is far beyond that of conventional histology. Our results emphasise the importance of PCR to complement routine histology of cutaneous leishmaniasis cases, particularly in laboratories in which no immunohistochemical assay is available.

Keywords:

Leishmania spp.

Cutaneous leishmaniasis

Diagnosis

Histology

Quantitative real-time PCR

Leishmaniasis is a disease caused by different species of protists of the genus *Leishmania* and is transmitted by sandflies. The species *Leishmania infantum* is an important causative agent of canine leishmaniasis [1-3]. This disease is associated with a wide range of symptoms including skin lesions, which are present in most clinical cases. Histology most commonly reveals a granulomatous band-like infiltrate in the superficial dermis, a granulomatous perifolliculitis and/or interstitial and perivascular dermatitis [4].

A few years ago, some cases of confirmed non-imported equine [5], and one case of bovine [6], cutaneous leishmaniasis were detected in Switzerland and Germany. Here, disease symptoms were mostly temporary, and included nodules of varying size sometimes covered by an eroded or ulcerated epidermis. Histologically, the lesions were characterized by an interstitial to diffuse or a nodular infiltrate composed of macrophages, as well as scattered lymphocytes, plasma cells, multinucleated giant cells and neutrophils. Surprisingly, phylogenetic analyses of sequence data for the first internal transcribed spacer (ITS-1) of nuclear ribosomal DNA from parasites from these cases revealed that all animals were infected with the same "exotic" *Leishmania* species and exhibiting a close phylogenetic relationship to *Leishmania siamensis* (syn. *Leishmania martiniquensis* belonging to the *Leishmania enriettii* complex; [7]) identified previously in a patient from Thailand with visceral leishmaniasis [8].

Conventional diagnosis of cutaneous leishmaniasis relies predominantly on serology and histological examination of skin biopsy samples. Although histology can be facilitated by immunohistochemistry, the intracellular amastigotes are difficult to identify, particularly in sections that contain small numbers of parasites [9,10]. Accordingly, it is not surprising that the sensitive PCR technology has gained increasing importance as an alternative method to substantiate the histological diagnosis of cutaneous leishmaniasis. However, PCR performed on histological slides is often complicated by the fact that DNA extracted from formalin-fixed and paraffin-embedded tissue may be partially degraded and/or contains components that inhibit PCR [10]. In this context, the quality of the DNA extracted from such fixed material and the robustness of a particular amplification procedure determine the diagnostic operating characteristics of a PCR for histological samples.

In the past, several groups [12,13] including ours [5,11] have demonstrated that PCR technology is well suited for the detection of *Leishmania* amastigotes in formalin-fixed and paraffin-embedded skin biopsy samples. However, since none of these studies was able to establish diagnostic sensitivity of

the tests used, we now elaborated a protocol to compare the performance of PCR, routine histology and immunohistochemistry for the diagnosis of cutaneous leishmaniasis.

In order to obtain test samples suitable for the development of our PCR protocol, archival skin biopsy specimens from 9 dogs and 6 horses with confirmed cutaneous leishmaniasis were selected by searching a data-base with information on cases studied between 2007 and 2015 at the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Berne (see Table 1). Using our previous method of PCR-based sequencing of ITS-1, followed by phylogenetic analysis revealed *L. infantum* in canine samples (unpublished data), and *L. siamensis*-related species ([5] and unpublished data) in equine samples.

Formalin-fixed and paraffin-embedded tissues were sectioned (2 x 4 µm and 1 x 10 µm) and mounted on glass slides. The 4 µm-thick sections were stained with haematoxylin and eosin (HE) for histological examination and then subjected to immunohistochemistry using an established protocol [5].

The 10 µm-thick sections of individual samples were subjected to DNA extraction according to Müller et al. [11]. To prepare a defined DNA standard for PCR-based quantification of skin biopsy material, a *Leishmania*-negative skin sample was also subjected to DNA preparation by accurately scraping the tissue off the slide with a disposable sterile scalpel blade. The tissue isolated in this manner was weighed and then used for DNA extraction. From the extracted DNA, we prepared a control sample containing DNA equating to 50 µg of tissue per µl, representing the starting concentration of the alpha-actin-PCR standard dilutions (see below). Prior to quantitative PCR analysis, the quality of all DNA preparations was assessed [11] in order to exclude issues pertaining to DNA degradation and residual PCR inhibitors in samples (not shown).

The quantification of skin tissue on histological sections was performed by real-time PCR using a LightCycler™ 2.0 Instrument (Roche Diagnostics, Basel, Switzerland). Here, PCR primers designed to an alpha-actin gene sequence, which is conserved for mammals, were employed to allow comparative quantification of tissues from different mammalian hosts of *Leishmania* sp. [11,14]. The standard curve from the alpha-actin–PCR included 4 log units within a linear range (undiluted to 1:1000-diluted control sample, containing DNA equating to 50 µg to 0.05 µg of tissue per µl; data not shown) that essentially covered the maximal and minimal concentrations of DNA within the different test samples.

The quantitative assessment of *Leishmania* in DNA prepared from tissue sections was performed using a fluorescence resonance energy transfer (FRET) probe-based real-time PCR employing the

LightCycler™ and corresponding standard software (v.3.5.3). This quantitative PCR amplified a ribosomal ITS-1 sequence tract that is common to a large variety of species of *Leishmania* [6,15,16]. Primers L5.8S and LITSR [15] used for the PCR-amplification of the ITS1 region had been utilised previously in conventional PCR assays, allowing sensitive and specific detection as well as genetic characterisation of numerous *Leishmania* species, including *L. siamensis* [5,6,15]. The detection of DNA amplification products was achieved by hybridisation of an ITS-1-specific 5′-LC-Red 640 labelled LITS1-5LC (5′-ATGGATGACTTGGCTTCCTATTTCGT-3′) detection probe and a 3′-fluorescein labelled LITS-1-3FL (5′-AACGGCTCACATAACGTGTCG-3′) anchor probe (TIB MOLBIOL, Munich, Germany). PCR amplification was performed in the LightCycler DNA Master Hybridisation Probes™ Kit (Roche Diagnostics) containing “Hot-Start” *Taq* DNA polymerase and dUTP instead of dTTP (see below). PCR mixes (10 µl volume) were supplemented with $MgCl_2$ to a final concentration of 3 µM and contained 0.5 µM of each primer plus 0.3 µM of each probe. Any potential carry-over contamination was removed by adding 0.125 units of UDG (Roche Diagnostics) [17]. For UDG-mediated decontamination prior to PCR, the reaction mixture was initially incubated for 10 min at 40 ℃. This incubation was followed by an incubation at 95℃ for 15 min to inactivate UDG, denature the DNA and activate “Hot-Start” *Taq* DNA polymerase. Subsequent amplification was conducted for 50 cycles (denaturation: 95 ℃, 20 s; "touch down" annealing: 60 ℃ to 53 ℃ including temperature steps of 1 ℃ per cycle, 20 s; quantitative assessment of fluorescence signal using the ‘single’ mode with the channel setting F2/1: 63 ℃, 0 s; extension: 72 ℃, 20 s; ramp rate for all cycle steps was 20 ℃) using 1 µl of individual, undiluted DNA samples. As external standards, samples containing *L. infantum* (strain IPZ-229-1-89; [11]) DNA equating to 10'000, 1'000, 100, 10, 1, and 0.1 parasite(s) were included (data not shown). When tested using serial 1:10 dilutions of DNA from in vitro-cultured *L. infantum* promastigotes, the real-time *Leishmania*-ITS1-PCR had an extremely high analytical sensitivity in that could detect $\leq$0.1 of *L. infantum* genome equivalents per amplification reaction (not shown).

The *Leishmania*-ITS1-PCR and the alpha-actin PCR were used to assess the relative amounts of *Leishmania* amastigotes (below referred to as *Leishmania* densities) in formalin-fixed and paraffin-embedded tissues from archival samples (Table 1). These quantitative PCR assays allowed the determination of the ratio between *Leishmania* amastigotes (given as number of cells) and amount of histological tissue material tested (given as µg tissue). Respective analyses revealed a large variety of

Leishmania amastigote densities ranging from 0.016 (canine case 9) to 305 (equine case 15) amastigotes per µg of tissue.

A histological evaluation of all canine and equine cases was conducted prior to PCR analyses (Fig. 1). In all HE-stained sections, cellular infiltrates composed of macrophages, plasma cells and lymphocytes were present, such that cutaneous leishmaniasis was amongst differential diagnoses based on histology findings (Fig. 1, panel a). Amastigotes within the macrophages and occasionally in between collagen fibers thus confirmed leishmaniasis, and were identified in all sections that produced a moderate to high amount of *Leishmania* DNA by PCR (cases 7 and 15; Fig. 1, panel a). Amastigotes were not detected, or were questionable, in cases with a low intensity of infection (case 9; Fig. 1, panel a). Immunohistochemical analysis of tissues from cases 9, 7 and 15 that had previously been scored by quantitative *Leishmania*-ITS1-PCR as weak (0.016 amastogotes per µg tissue), moderate (72.5 amastogotes per µg tissue), and strong (305 amastigotes per µg tissue; see Table 1) positive (cf. Fig. 1, panel b). Here, unambiguous microscopic detection of intracellular *Leishmania* amastigotes in HE-stained sections was restricted to high- and medium-positive sections. In sections with low numbers of amastigotes, however, immunohistochemistry was necessary to achieve the detection of parasitised tissue by careful microscopic inspection of the entire section, as demonstrated, for example, for case 9. Here, *Leishmania*-ITS1-PCR-negative canine and equine sections were characterised by a lympho-histiocytic infiltrate consistent with leishmaniasis cases and were included as reference samples to avoid misinterpretation of nuclear debris as *Leishmania* amastigotes.

In conclusion, we describe a novel real-time PCR which allows a highly sensitive detection of various (if not all) species pf the genus *Leishmania*. This *Leishmania*-ITS1-PCR exhibits an excellent diagnostic sensitivity when applied to the testing of paraffin-embedded skin biopsy samples. The *Leishmania*-ITS1-PCR achieved a very similar diagnostic sensitivity as immunohistochemistry. In contrast to immunohistochemistry, however, the PCR allowed an unambiguous interpretation of the results, even in cutaneous leishmaniasis cases with extremely low numbers of amastigotes. This comparative evaluation of the ITS1-PCR exemplifies the importance of PCR-based technology to complement the histological diagnosis of cutaneous leishmaniasis. The data presented here also emphasise the suitability of the present quantitative PCR assay for retrospective epidemiological and genetic studies, particularly in situations where only archival histological specimens from cutaneous leishmaniasis cases are available.

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Fig. 1. Haematoxylin and eosin-stained (panel a) and immunostained (panel b) skin biopsy sections from two dogs and one horse with histological findings that were consistent with cutaneous leishmaniasis (lympho-histiocytic and plasmacytic infiltrate). Arrows indicate areas of parasitised tissue. Cases 9, 7 and 15 were scored by quantitative *Leishmania*-ITS1-PCR as weak-, moderate- or strong-positive for *Leishmania* (see Table 1). In addition, *Leishmania*-negative sections characterised by lympho-histiocytic and/or plasmacytic infiltrates of an etiology other than leishmaniasis were chosen as negative controls (neg.). Equine case 15 and the corresponding negative control are indicated in italics. Magnifications: 400x (images of panels a and b) and 1000x (enlarged inserts presented for the immuno-stained sections of cases 9, 7 and 15).

Table 1

Quantitative real-time PCR-based determination of *Leishmania* amastigote densities in formalin-fixed and paraffin-embedded skin biopsy samples from dogs and horses.

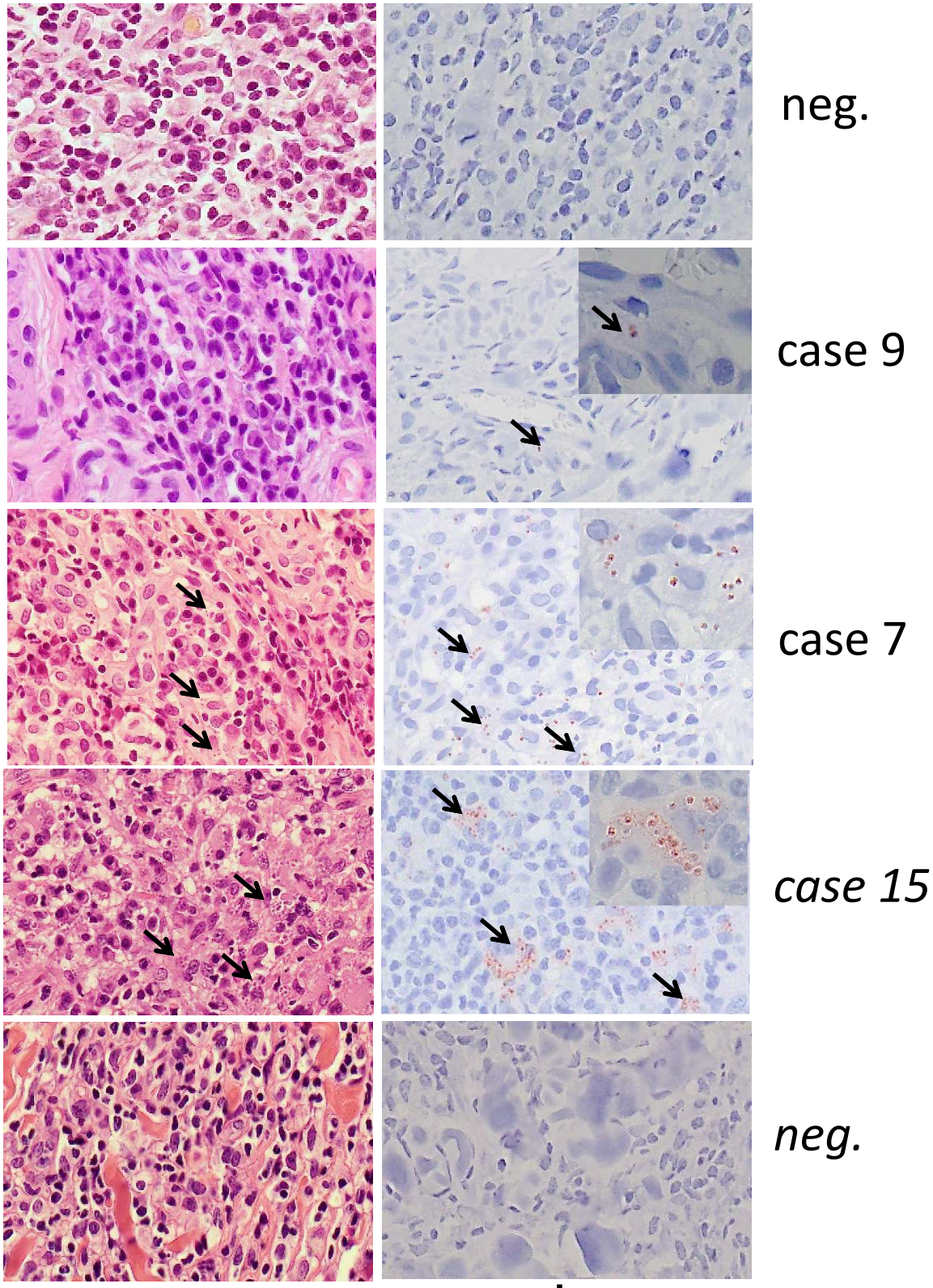
[a]Sample no.	[b]Total no. amastigotes	[c]Tissue (in µg µg)	[d]No. amastigotes per µg tissue
1	0.7	41.7	0.017
2	3344	20.5	163
3	4.3	1.7	2.5
4	12.2	0.7	17.4
5	0.7	35.1	0.020
6	4.6	46.4	0.099
7	2744	37.8	72.5
8	1.6	1.5	1.1
9	0.3	18.4	0.016
10	863	4.9	176.2
11	55.3	1.3	42.5
12	68.2	0.5	136
13	8695	42.4	205
14	21.9	5.2	4.2
15	457	1.5	305

[a] Samples 1-9, canine biopsies positive for *L. infantum;* samples 10-15, positive for *L. siamensis*

[b] Total number of *Leishmania* sp. amastigotes in samples as assessed by quantitative *Leishmania*-ITS1-PCR

[c] Total amount of tissue in samples as assessed by quantitative alpha-actin-PCR

[d] Density of *Leishmania* sp. amastigotes in samples


neg.
case 9
case 7
case 15
neg.
a
b

Highlights

- This report describes a real-time PCR approach for the quantification of *Leishmania* amastigotes in paraffin-embedded skin biopsy samples.
- In contrast to (immuno-) histology, the novel *Leishmania*-PCR allowed to unambiguously diagnose even cases of cutaneous leishmaniasis with an extremely low amastigote load.
- Our results highlighted the importance of PCR supporting routine histology to diagnose cutaneous leishmaniasis.