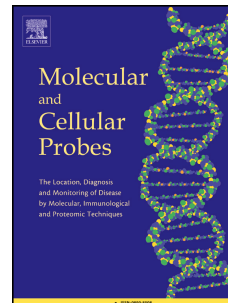


Accepted Manuscript

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PII: S0890-8508(15)30041-4

DOI: [10.1016/j.mcp.2015.09.008](https://doi.org/10.1016/j.mcp.2015.09.008)

Reference: YMCPR 1171

To appear in: *Molecular and Cellular Probes*

Received Date: 4 September 2015

Revised Date: 10 September 2015

Accepted Date: 24 September 2015

Please cite this article as: Müller N, Hentrich B, Frey CF, Welle M, Quantitative PCR for the diagnosis of cutaneous leishmaniasis from formalin-fixed and paraffin-embedded skin sections, *Molecular and Cellular Probes* (2015), doi: 10.1016/j.mcp.2015.09.008.

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

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22 ABSTRACT

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

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33 *Keywords:*34 *Leishmania* spp.

35 Cutaneous leishmaniasis

36 Diagnosis

37 Histology

38 Quantitative real-time PCR

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

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

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

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

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

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

80 Formalin-fixed and paraffin-embedded tissues were sectioned (2 x 4 μm and 1 x 10 μm) and
81 mounted on glass slides. The 4 μm -thick sections were stained with haematoxylin and eosin (HE) for
82 histological examination and then subjected to immunohistochemistry using an established protocol
83 [5].

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

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

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

102 LightCycler™ and corresponding standard software (v.3.5.3). This quantitative PCR amplified a
103 ribosomal ITS-1 sequence tract that is common to a large variety of species of *Leishmania* [6,15,16].
104 Primers L5.8S and LITSR [15] used for the PCR-amplification of the ITS1 region had been utilised
105 previously in conventional PCR assays, allowing sensitive and specific detection as well as genetic
106 characterisation of numerous *Leishmania* species, including *L. siamensis* [5,6,15]. The detection of
107 DNA amplification products was achieved by hybridisation of an ITS-1-specific 5'-LC-Red 640 labelled
108 LITS1-5LC (5'-ATGGATGACTTGGCTTCCTATTTTCGT-3') detection probe and a 3'-fluorescein
109 labelled LITS-1-3FL (5'-AACGGCTCACATAACGTGTGCG-3') anchor probe (TIB MOLBIOL, Munich,
110 Germany). PCR amplification was performed in the LightCycler DNA Master Hybridisation Probes™
111 Kit (Roche Diagnostics) containing "Hot-Start" *Taq* DNA polymerase and dUTP instead of dTTP (see
112 below). PCR mixes (10 µl volume) were supplemented with MgCl₂ to a final concentration of 3 µM and
113 contained 0.5 µM of each primer plus 0.3 µM of each probe. Any potential carry-over contamination
114 was removed by adding 0.125 units of UDG (Roche Diagnostics) [17]. For UDG-mediated
115 decontamination prior to PCR, the reaction mixture was initially incubated for 10 min at 40 °C. This
116 incubation was followed by an incubation at 95°C for 15 min to inactivate UDG, denature the DNA and
117 activate "Hot-Start" *Taq* DNA polymerase. Subsequent amplification was conducted for 50 cycles
118 (denaturation: 95 °C, 20 s; "touch down" annealing: 60 °C to 53 °C including temperature steps of 1 °C
119 per cycle, 20 s; quantitative assessment of fluorescence signal using the 'single' mode with the
120 channel setting F2/1: 63 °C, 0 s; extension: 72 °C, 20 s; ramp rate for all cycle steps was 20 °C) using
121 1 µl of individual, undiluted DNA samples. As external standards, samples containing *L. infantum*
122 (strain IPZ-229-1-89; [11]) DNA equating to 10'000, 1'000, 100, 10, 1, and 0.1 parasite(s) were
123 included (data not shown). When tested using serial 1:10 dilutions of DNA from in vitro-cultured *L.*
124 *infantum* promastigotes, the real-time *Leishmania*-ITS1-PCR had an extremely high analytical
125 sensitivity in that could detect ≤ 0.1 of *L. infantum* genome equivalents per amplification reaction (not
126 shown).

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

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

134 A histological evaluation of all canine and equine cases was conducted prior to PCR analyses (Fig.
135 1). In all HE-stained sections, cellular infiltrates composed of macrophages, plasma cells and
136 lymphocytes were present, such that cutaneous leishmaniasis was amongst differential diagnoses
137 based on histology findings (Fig. 1, panel a). Amastigotes within the macrophages and occasionally in
138 between collagen fibers thus confirmed leishmaniasis, and were identified in all sections that produced
139 a moderate to high amount of *Leishmania* DNA by PCR (cases 7 and 15; Fig. 1, panel a). Amastigotes
140 were not detected, or were questionable, in cases with a low intensity of infection (case 9; Fig. 1,
141 panel a). Immunohistochemical analysis of tissues from cases 9, 7 and 15 that had previously been
142 scored by quantitative *Leishmania*-ITS1-PCR as weak (0.016 amastigotes per μg tissue), moderate
143 (72.5 amastigotes per μg tissue), and strong (305 amastigotes per μg tissue; see Table 1) positive
144 (cf. Fig. 1, panel b). Here, unambiguous microscopic detection of intracellular *Leishmania* amastigotes
145 in HE-stained sections was restricted to high- and medium-positive sections. In sections with low
146 numbers of amastigotes, however, immunohistochemistry was necessary to achieve the detection of
147 parasitised tissue by careful microscopic inspection of the entire section, as demonstrated, for
148 example, for case 9. Here, *Leishmania*-ITS1-PCR-negative canine and equine sections were
149 characterised by a lympho-histiocytic infiltrate consistent with leishmaniasis cases and were included
150 as reference samples to avoid misinterpretation of nuclear debris as *Leishmania* amastigotes.

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

162

164 Acknowledgements

165

166 The authors thank L. Lobsiger (formerly at the Institute of Parasitology, Berne, Switzerland) and W.
167 von Bomhard (Specialty Practice for Veterinary Pathology, Munich, Germany) for providing us with
168 skin biopsy material from cases of cutaneous leishmaniasis. *L. infantum* strain IPZ-229-1-89 was
169 kindly provided by F. Grimm, Institute of Parasitology, Zurich, Switzerland. This study was supported
170 by Departmental funds from the Vetsuisse Faculty, Berne, Switzerland.

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226 **Fig. 1.** Haematoxylin and eosin-stained (panel a) and immunostained (panel b) skin biopsy sections
227 from two dogs and one horse with histological findings that were consistent with cutaneous
228 leishmaniasis (lympho-histiocytic and plasmacytic infiltrate). Arrows indicate areas of parasitised
229 tissue. Cases 9, 7 and 15 were scored by quantitative *Leishmania*-ITS1-PCR as weak-, moderate- or
230 strong-positive for *Leishmania* (see Table 1). In addition, *Leishmania*-negative sections characterised
231 by lympho-histiocytic and/or plasmacytic infiltrates of an etiology other than leishmaniasis were
232 chosen as negative controls (neg.). Equine case 15 and the corresponding negative control are
233 indicated in italics. Magnifications: 400x (images of panels a and b) and 1000x (enlarged inserts
234 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.

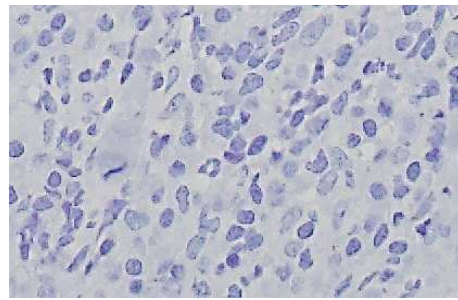
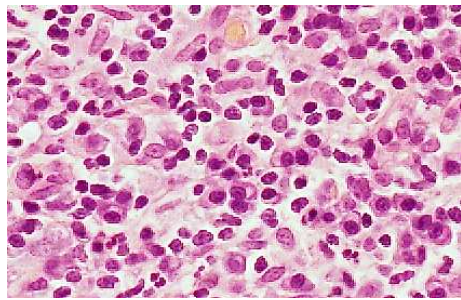
^a Sample no.	^b Total no. amastigotes	^c Tissue (in μg)	^d No. amastigotes per μg tissue
1	0.7	41.7	0.017
2	3344	20.5	163
3	4.3	1.7	2.5
4	12.2	0.7	17.4
5	0.7	35.1	0.020
6	4.6	46.4	0.099
7	2744	37.8	72.5
8	1.6	1.5	1.1
9	0.3	18.4	0.016
10	863	4.9	176.2
11	55.3	1.3	42.5
12	68.2	0.5	136
13	8695	42.4	205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

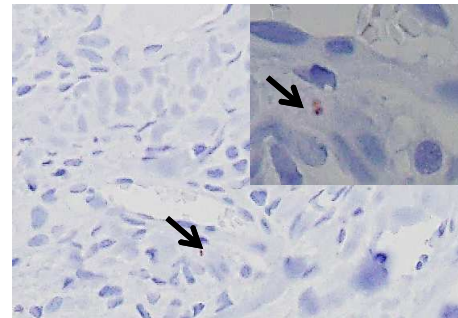
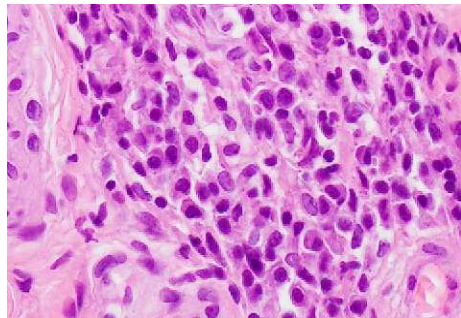
^b Total number of *Leishmania* sp. amastigotes in samples as assessed by quantitative *Leishmania*-ITS1-PCR

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

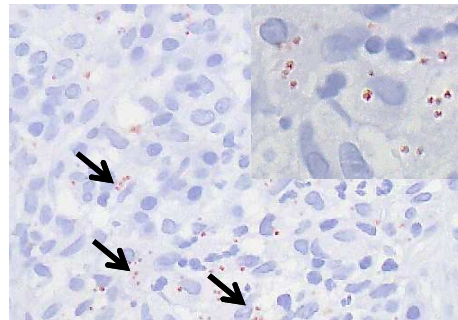
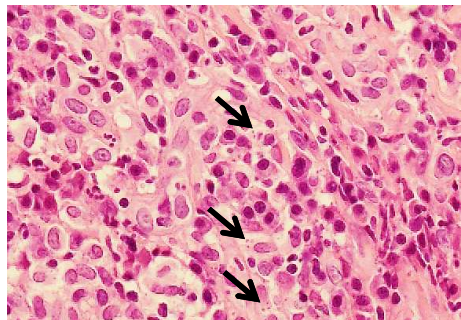
^d Density of *Leishmania* sp. amastigotes in samples



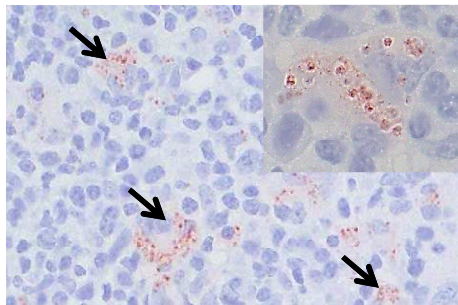
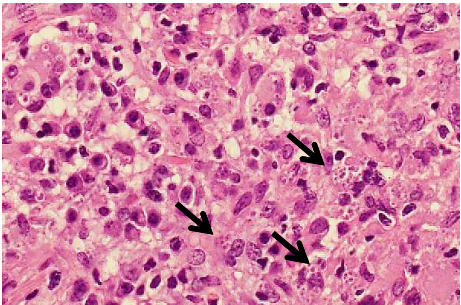
neg.



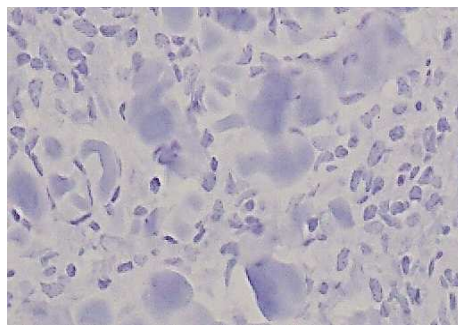
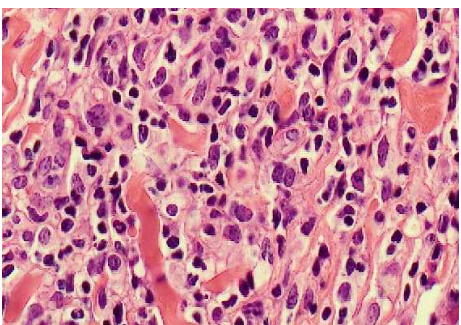
case 9



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.