Detection of a broad range of *Leishmania* species and determination of parasite load of infected mouse by real-time PCR targeting the arginine permease gene *AAP3*

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ABSTRACT

Leishmaniasis is one of the world's most neglected infectious diseases, affecting around 12 million people and more than 350 million at risk of infection. The clinical picture varies from self-healing cutaneous lesions to severe visceral infections, but still no commercial vaccines for humans are available and the currently used drugs have unpleasant side effects. Here we report a real-time PCR assay targeting the arginine permease gene *AAP3* that can be applied for all the nine different species of the *Leishmania* genus tested; 4 Old World species and 5 New World species, from both *L.* (*Leishmania*) and *L.* (*Viannia*) subgenera. No cross-reaction was seen with *Trypanosoma cruzi*, *Trypanosoma brucei*, human or mouse genomic DNA. The assay has a high sensitivity, with a limit of detection of 10 fg DNA for *L.* (*L.*) *major* and *L.* (*L.*) *donovani*, and 100 fg DNA for *L.* (*V.*) *braziliensis*, and can be used for both qualitative and quantitative purposes. This AAP3-Assay, run in duplex with a host specific gene-assay, was also successfully used for quantification of parasite load of footpads from *L.* (*L.*) *major*-infected mice. It can therefore be a valuable tool in applications like monitoring effects of drugs, the selection of vaccine candidates and in screening patients, including asymptomatic carriers.

1. Introduction

Leishmaniasis is a vector-borne disease with different clinical pictures caused by protozoa of the genus *Leishmania*. It is one of the world's most neglected infectious diseases and the second cause of parasite related deaths after malaria (Mathers et al., 2007). Spread of the *Leishmania* parasite is caused by the bite of infected sand flies. Worldwide more than 350 million people in 98 countries or territories are at risk (World Health Organization, 2010). The estimated incidence of new cases each year is 0.2–0.4 million for visceral leishmaniasis and 0.7–1.2 million for cutaneous leishmaniasis, causing 20 000 to 40 000 deaths annually, and these data are probably underestimates (Alvar et al., 2012). In some affected areas both domestic and sylvatic animals seem to be important reservoirs of the parasite, contributing to promote human infections (Quinnell

and Courtenay, 2009). Available drugs for treating the disease can be characterized as limited, expensive and often with unpleasant side effects. Moreover, there has been an emergence of drug resistance (Sundar and Chakravarty, 2013). No commercial vaccines are currently available for preventing leishmaniasis in humans. Therefore, in order to control the disease, there is a need for development of new drugs, vaccines and more specific and sensitive diagnostic methods. Assays for quantification of the parasite in the host tissues are essential for development and testing of prophylactic and therapeutic regimes.

Polymerase chain reaction (PCR) and its variations represent highly sensitive and specific methods for *Leishmania* DNA detection. PCR has shown to be superior to other methods like microscopy and various immunologic tests, reducing time from sampling to test result, optimizing sensitivity and specificity and reducing subjective evaluation (Aviles et al., 1999; Bensoussan et al., 2006; Srivastava et al., 2011a; Wall et al., 2012). Real-time PCR is advantageous over conventional PCR because it is faster, less labor-intensive, reduces risk of contamination, and by using probes the sensitivity and specificity can be increased (Dymond, 2013; Mohammadiha et al., 2013; Yang and Rothman, 2004)

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Furthermore, by using standard curves real-time PCR can be used for quantification. Exploring the *Leishmania* parasites and the clinical manifestations they cause, quantitative real-time PCR (qPCR) can be useful for detection and species identification, but it also has a wider potential, like monitoring the effect of drug activity and measuring the protection as part of vaccine development. Due to the high sensitivity, qPCR can be used for screening of patients and detection of asymptomatic carriers, and thereby addressing gaps in the understanding of infection with *Leishmania* (Francino et al., 2006; Mary et al., 2006; Pourabbas et al., 2013).

There are many publications of different variants of PCRs for *Leishmania*, using different molecular targets, but most protocols target a single species (Francino et al., 2006; Srivastava et al., 2011b), a group of closely related species (Harris et al., 1998; Odiwuor et al., 2011), or they are not quantitative (Berzunza-Cruz et al., 2009; de Almeida et al., 2011; Harris et al., 1998; Odiwuor et al., 2011; Srivastava et al., 2011b), and some also show low sensitivity (Wortmann et al., 2005). There are only few publications of real-time PCR assays that target all or nearly all of the approximately 20 different *Leishmania* species found in humans (Castilho et al., 2008; Tupperwar et al., 2008; Wortmann et al., 2001). Many protocols target multicopy genes (Bossolasco et al., 2003; Francino et al., 2006; Talmi-Frank et al., 2010) and some protocols for multicopy genes also use SYBRGreen (de Monbrison et al., 2007). Multicopy genes are often preferred to enhance sensitivity, and thus are advantageous for detection, but due to potential variations and instability in copy number of the same gene both between and within species (Weirather et al., 2011) they can be challenging and confounding for quantification using standard curves. SYBR-Green has the disadvantages of more unspecific binding, hence a probe, which is more specific, is to be preferred. However, it can be challenging to design a qPCR with a specific probe that target a DNA sequence universal to all species of the *Leishmania* genus.

L-Arginine is an essential amino acid for *Leishmania*, for which metabolism depends on arginine supply from external sources, as no evidence for endogenous synthesis has been reported. The arginine transporter *Leishmania* arginine permease AAP3 is encoded on chromosome 31 in *Leishmania* (*L.*) *major* and other *Leishmania* species, and on chromosome 30 in *Leishmania* (*L.*) *mexicana* (which

due to chromosome fusion events is the equivalent of chromosome 31 in *L.* (*L.*) *major* (Britto et al., 1998)). The AAP3 gene is identified in several different *Leishmania* species (Shaked-Mishan et al., 2006). Likely more *AAP3* sequences will be published along with the increasing number of sequencing projects.

The aim of the study was to develop a quantitative PCR method that could be applied for infectivity studies in mice, focusing on *L.* (*L.*) *major*, as murine models are widely used in *Leishmania* research, with the benefit that it could also be used for other *Leishmania* species from both the Old- and New World groups. We developed a PCR-Assay targeting the arginine transporter gene *AAP3* and included DNA from cell pellets or cultured promastigotes from nine different *Leishmania* species, as well as different negative controls, to validate the assay. This AAP3-Assay, run in duplex with a host specific gene-assay, was then used for quantification of parasite load of footpads from *L.* (*L.*) *major*-infected mice.

2. Materials and methods

2.1. Strains used in this study

Leishmania strains used in this study are given in Table 1. *Leishmania* promastigotes were grown at 26 °C in RPMI 1640 medium (Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% heat inactivated fetal calf serum (Gibco®, Life Technologies – Invitrogen, Carlsbad, CA, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin, or in Schneider's Insect Medium (Sigma–Aldrich) supplemented with 20% heat inactivated fetal calf serum and 1% sterile-filtered human urine. Parasites in culture were washed and counted by flow cytometry using reference beads (Flow-Count Fluorospheres®, Beckman Coulter, Brea, CA, USA) after staining with Vybrant® DyeCycle™ Green Stain (Molecular probes®, Eugene, OR, USA).

L. (*L.*) *mexicana* MHOM/BZ/82/BEL21 was a kind gift from Centro de Investigaciones Regionales 'Dr. Hideyo Noguchi', Universidad Autónoma de Yucatán, Mérida, Yucatán, México. Cell pellets from *Leishmania* strains as indicated in Table 1, as well as from *Trypanosoma brucei* and *T. cruzi*, were kindly donated from Dr. Silvia

Table 1
Leishmania strains used in this study, and their C_q-value when 100 pg of DNA was used as template in the AAP3-Assay.

Species[a]	International code or other reference	Origin	Source[b]	C_q[c]
L. (*L.*) *aethiopica*	MHOM/ET/91/Kassaye	Ethiopia	SMI	23.4
L. (*V.*) *amazonensis*	MHOM/BR/73/M2269, LEM 0690	Brazil	CNRL	23.7
L. (*V.*) *braziliensis*	MHOM/BR/87/LTB12MAR87, LEM 2839	Brazil	CNRL	25.0
L. (*V.*) *braziliensis*	MHOM/BR/75/M2904, LEM 2249	Brazil	CNRL	25.1
L. (*V.*) *braziliensis*	SMI 2094	Unknown	SMI	25.3
L. (*L.*) *donovani*	MHOM/CY/2006/CH33, LEM 5298	Cyprus	CNRL	23.8
L. (*L.*) *donovani*	MHOM/IN/80/DD8, LEM 0703	India	CNRL	23.7
L. (*L.*) *donovani*	MHOM/ET/67/HU3	Ethiopia	SMI	23.7
L. (*V.*) *guyanensis*	MHOM/GF/94/22319, LEM 2763	French Guiana	CNRL	27.6
L. (*L.*) *major*	MHOM/AF/2006/LEM5344, LEM 5344	Afghanistan	CNRL	23.9
L. (*L.*) *major*	MHOM/MA/2004/LEM4905, LEM 4905	Morocco	CNRL	24.2
L. (*L.*) *major*	MHOM/TN/2006/LPN296, LEM 5373	Tunisia	CNRL	23.9
L. (*L.*) *major*	MHOM/IL/80/FRIEDLIN, LEM 3150	Israel	CNRL	23.4
L. (*L.*) *major*-np	MRHO/SU/59/LV39	USSR	CNRL	23.6
L. (*L.*) *major*-p	MRHO/SU/59/LV39	USSR	CNRL	24.0
L. (*L.*) *mexicana*	MHOM/MX/96/NAN01, LEM 3816	Mexico	CNRL	23.8
L. (*L.*) *mexicana*	MHOM/MX/93/CRE47, LEM 2695	Mexico	CNRL	23.5
L. (*L.*) *mexicana*-np	MHOM/BZ/82/BEL21	Belize	CIR	24.3
L. (*L.*) *mexicana*-p	MHOM/BZ/82/BEL21	Belize	CIR	24.4
L. (*V.*) *naiffi*	MHOM/GF/97/CRE88, LEM 3426	French Guiana	CNRL	24.0
L. (*L.*) *tropica*	MHOM/SU/74/K27, LEM 0419	USSR	CNRL	24.2
L. (*L.*) *tropica*	MHOM/MA/2000/INHW10, LEM 5277	Morocco	CNRL	24.1

[a] np: not been propagated in mouse; p: propagated in mouse.
[b] SMI: Public Health Agency of Sweden, Sweden; CNRL: Centre National de Référence des Leishmanioses, Montpellier, France; CIR: Centro de Investigaciones Regionales, Universidad Autónoma de Yucatán, Mérida, Yucatán.
[c] C_q-value from the AAP3-assay using 100 pg DNA as template.

Botero-Kleiven and Dr. Leigh Davidsson at the Public Health Agency of Sweden, Sweden, and were kept in ethanol during transport to our laboratory and until DNA extraction.

2.2. Detection of the AAP3 gene in Leishmania species

2.2.1. DNA extraction

Cultured promastigotes from the stationary phase and cell pellets in ethanol were washed with Dulbecco's phosphate-buffered saline (10 mM Phosphate, 137 mM Sodium chloride, 2.7 mM Potassium chloride, pH 7.4) (DPBS) before subjected to DNA extraction using QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, except that Proteinase K was used instead of protease. DNA was eluted in 100 μl of Buffer AE (Qiagen). In all DNA extractions a negative control of DPBS was included to monitor for contamination. DNA concentration and quality was determined by a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All DNA samples were stored at −20 °C.

2.2.2. Real-time PCR

Primers and hydrolysis probe (TaqMan® MGB probe) targeting a 74 bp region of the *L. (L.) major* gene encoding the arginine transporter AAP3 were designed by the Custom TaqMan® Assay Design Tool from Applied Biosystems (Applied Biosystems, Warrington, UK). The sequences of the primers and probe were 5′-GGCGGC-GGTATTATCTCGAT-3′ (Forward), 5′-ACCACGAGGTAGATGACAGA-CA-3′ (Reverse) and FAM 5′-ATGTCGGGCATCATC-3′ NFQ (probe). In silico analysis of specificity of the assay was performed using the Vector NTI software (Life Technologies). A global BLAST search was undertaken by the 74 bp region wherein the primers and probe bind. In addition, complete genome sequences of some relevant bacteria (like *Mycobacteria*, *Pseudomonas*, *Streptococcus*, *Staphylococcus*) were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and investigated for the extent of binding (BLAST) of the primers and probe.

Each PCR test was performed in triplicate in a 20 μl reaction mixture. The reaction mixture included: 1× TaqMan Universal Master Mix II with UNG (Applied Biosystems), 1× Custom TaqMan® Gene Expression Assay Mix with primers and probe targeting the *Leishmania AAP3* gene, 1× TaqMan® Copy Number Reference Assay, Mouse, Tfrc, water and 100 pg of DNA sample. The PCR was run as a duplex assay after comparing the results of singleplex AAP3-Assay and the duplex with the Tfrc-Assay (results not shown).

The qPCR was performed with an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems), with cycling conditions as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min each. All samples were run on MicroAmp® Optical 96-well Reaction Plates (Applied Biosystems) sealed with MicroAmp® Optical Adhesive Film (Applied Biosystems). Each run included multiple no-template controls. Human DNA, *T. brucei* and *T. cruzi* was used as negative controls to check for cross-reactivity. The human DNA for control was provided by Christel G. Haanshuus, National Centre for Tropical Infectious Diseases, Department of Medicine, Haukeland University Hospital, Bergen, Norway.

Tenfold dilution series of *L. (L.) major* DNA (range 12 ng–1.2×10^{-7} ng) were prepared for creating standard curves and estimating assay performance for the AAP3-Assay. Besides quantification, dilution series and standard curves were used for estimation of PCR efficiency, limit of quantification (LOQ) and limit of detection (LOD). LOQ and LOD were also estimated for *L. (L.) donovani* and *L. (V.) braziliensis*. The dilution corresponding to the LOQ was the highest dilution used for the standard curve. For estimating repeatability and reproducibility, replicates of a DNA sample from the footpads of BALB/c mice infected with *L. (L.)*

major parasites were used. DNA was quantified using the Absolute Quantification Assay. Thresholds were set automatically. Amplicons were run on a 2% agarose gel (SeaKem™, Lonza, Rockland, ME, USA) with 1X GelRed™ (Biotium, Hayward, CA, USA) to check for the correct size. Replicates with quantification cycle (C_q)-value differing by more than 0.3 were omitted.

A unidirectional workflow pre- to post-qPCR was enforced, and preparation of qPCR reaction mixture, DNA preparations and qPCR were carried out in facilities physically separate from each other.

2.3. Detection of the AAP3 gene in L. (L.) major-infected mice

2.3.1. Mouse tissue samples

The left footpads of female BALB/c mice were inoculated with 10 μl of 10^6 ml^{-1} *L. (L.) major* parasites in stationary phase. After swelling and lesions had developed, 56 days post inoculation, mice were euthanized after first using Isoba vet. 100% (Intervet/Schering- Plough Animal Health, Intervet Denmark A/S, Denmark) for anesthesia. Footpads of control mice (2 animals) and infected mice (8 animals), and liver for providing control DNA, were harvested and stored at −80 °C.

2.3.2. Ethical clearance

The animal experiments were approved by the National Animal Research Authority in Norway and carried out at the Laboratory Animal Facility (AAALAC-accredited) at the University of Bergen, Bergen, Norway.

2.3.3. DNA extraction from mice tissue

Mouse footpads were subjected for DNA extraction by a phenol-chloroform based protocol from Instituto Oswaldo Cruz, Rio de Janeiro, Brazil, available at http://clioc.fiocruz.br/documents/mmp.pdf (Leishmaniasis Epidemiology Network South America, 2009). DNA pellet was dissolved in 200 μl TE-buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and incubated at room temperature overnight. Mouse livers were incubated over night at 56 °C in Buffer ATL (Qiagen) with 2 mg/ml Proteinase K (Qiagen), before subjected to DNA extraction using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA was eluted in 100 μl of Buffer AE (Qiagen).

In all DNA extractions a negative control of DPBS was included to monitor for contamination. DNA concentration and quality was determined by a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). All DNA samples were stored at −20 °C.

2.3.4. Quantitative real-time PCR

In order to normalize the parasite load for amount of mouse tissue DNA, and hence overcome the possible quantification errors due to different cutting points when harvesting the footpads, the qPCR was run as a duplex-assay with the AAP3-Assay and the TaqMan® Copy Number Reference Assay, Mouse, Tfrc (Applied Biosystems, Foster City, CA, USA), as a reference assay. This premade reaction mixture consists of primers and a VIC® dye-labeled TAMRA™ probe which detects the single-copy transferrin receptor gene (*Tfrc*) in the mouse genome.

Each qPCR test was performed in triplicate in a 20 μl reaction mixture. The reaction mixture was as in Section 2.2.2, except that 100 ng of DNA sample was used as template. Initial dilution experiments showed that with 100 ng of DNA the background level, or inhibition, was negligible.

Fivefold dilution series of BALB/c mouse DNA (range 1050 ng–0.33 ng) were prepared for creating standard curve and estimating assay performance for the Tfrc-Assay. For estimating repeatability and reproducibility, replicates of a DNA sample from the footpads of BALB/c mice infected with *L. (L.)*

major parasites were used. DNA was quantified using the Absolute Quantification Assay. Thresholds were set automatically. The parasite load in tissue samples was given as the ratio between *Leishmania* DNA and genomic mouse DNA in the 100 ng DNA applied to each PCR reaction. Since the amount of DNA cannot be reliably estimated outside the linear area of the standard curve, the DNA quantity for samples with *Leishmania* DNA less than the LOQ was set to be equal to or less than the LOQ. Parallels with C_q-value differing by more than 0.3 were omitted.

3. Results

3.1. Specificity of the AAP3-Assay and the Tfrc-Assay

The primers and probe for the AAP3-Assay were designed for *L.* (*L.*) *major*. We also experimentally tested the ability of these oligonucleotides to detect other different *Leishmania* species, representing nine different species, either isolated from cell pellets or cultured promastigotes. *L.* (*L.*) *aethiopica*, *L.* (*V.*) *amazonensis*, *L.* (*V.*) *braziliensis*, *L.* (*L.*) *donovani*, *L.* (*V.*) *guyanensis*, *L.* (*L.*) *major*, *L.* (*L.*) *mexicana*, *L.* (*V.*) *naiffi* and *L.* (*L.*) *tropica* could all be amplified with the AAP3-Assay, as seen from Table 1. The PCR resulted in amplicons of the correct size, 74 bp, as visualized by gel electrophoresis (results not shown). Neither the AAP3-Assay nor the Tfrc-Assay could amplify *T. brucei* and *T. cruzi*, or genomic DNA from humans or mice (AAP3-Assay only).

The global BLAST search with the 74 bp region (wherein the primers and the probes bind) gave only hits indicating generation of positive signals of the assay when targeting DNA from *Leishmania* species. Likewise, investigation of the bacterial genomic sequences did not reveal strong binding of any combinations of applied primers in a way that would generate amplification of any segment to which the probe would bind, and thereby giving rise to a positive signal.

3.2. Sensitivity of the AAP3-Assay

LOD and LOQ were estimated for *L.* (*V.*) *braziliensis*, *L.* (*L.*) *donovani* and *L.* (*L.*) *major* using serial dilutions of DNA purified from in vitro cultivated parasites. With the applied method used for DNA extraction, 100 fg corresponded to 2 parasites (p). For *L.* (*V.*) *braziliensis*, *L.* (*L.*) *donovani* and *L.* (*L.*) *major* the LODs, given in fg with number of parasites with our extraction method in brackets, were: ≥100 fg (2 p), ≥10 fg (0.2 p) and ≥10 fg (0.2 p), respectively. The LOQs for *L.* (*V.*) *braziliensis*, *L.* (*L.*) *donovani* and *L.* (*L.*) *major* were ≥1000 fg (20 p), ≥10 fg (0.2 p) and ≥100 fg (2 p), respectively.

3.3. Assay performance

From the DNA dilution series parameters of assay performance other than sensitivity was calculated. From repeated runs the mean slopes of the AAP3-Assay and Tfrc-Assay were −3.205 (range −2.994 to −3.305) and -3.193 (range −3.083 to −3.313), respectively. Efficiency, as determined from the slope and using the formula $E = 10^{-1/\text{slope}} - 1$, was 105.1% for the AAP3-Assay and 105.7% for the Tfrc-Assay. The correlation coefficient, R^2, was 0.999 (range 0.996–0.999) for the AAP3-Assay and 0.997 (range 0.996–0.999) for the Tfrc-Assay, and the Y-intercept was 19.48 (range 18.26–20.85) for the AAP3-Assay and 31.83 (range 31.43–32.11) for the Tfrc-Assay. A DNA sample from the footpads of BALB/c mice infected with *L.* (*L.*) *major* parasites was used for estimating repeatability and reproducibility. The intra-assay coefficient of variation (CV) for the AAP3-Assay, calculated from DNA quantity of replicates, was for each of three separate runs 0.0908, 0.0901 and 0.0882, respectively. For the Tfrc-Assay the intra-assay CV was for each of two separate runs 0.0179 and 0.021. Inter-assay
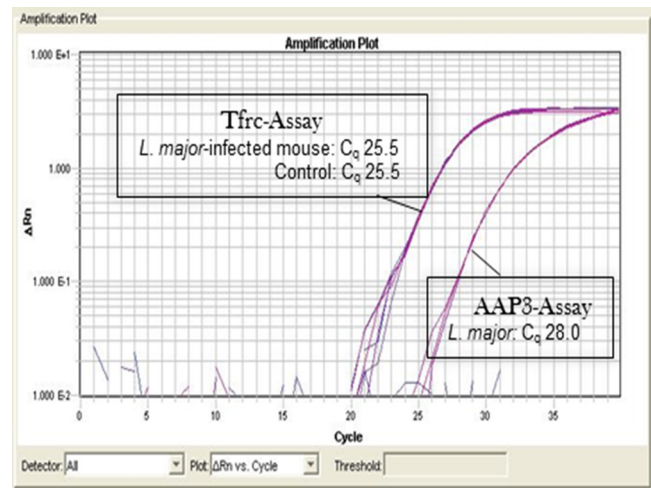

Fig. 1. Amplification plot for the AAP3-Assay and the Tfrc-Assay. Amplification curves for DNA isolated from footpad of uninfected Control mouse (Control) and mouse infected with *L.* (*L.*) *major*.

CV, calculated from DNA quantity of separate runs, was 0.052 for the AAP3-Assay and 0.059 for the Tfrc-Assay. The ratio in 100 ng DNA between quantity of *L.* (*L.*) *major* DNA, as measured by the AAP3-Assay, and mouse DNA, as measured by the Tfrc-Assay, was 3.22×10^{-4} and 3.25×10^{-4} for the same sample on two separate runs.

No amplification of any of the no-template controls was detected.

3.4. Estimation of L. (L.) major DNA in mice footpads

For analyzing and quantification of parasite load of footpads from *L.* (*L.*) *major*-infected mice, the AAP3-Assay was run in duplex with a host specific gene-assay; the Tfrc-Assay, which detects mouse DNA. *Leishmania* DNA was successfully amplified from all the infected mice, but not from the control mice. Fig. 1 shows amplification plots for the AAP3-Assay and the Tfrc-Assay when analyzing a sample of *Leishmania*-infected tissue, together with plots for the Tfrc-Assay when analyzing a control sample of uninfected tissue. As the mice DNA constitute the majority of the DNA in the infected tissue, all plots from the Tfrc-Assay make up a concurrent curve. Values for the AAP3-Assay triplicates and for the triplicates of the Tfrc-Assay are given in Table 2. In addition, DNA isolated from the footpad of one of the other *L.* (*L.*) *major*-infected mice was analyzed, where we made two different dilutions of the DNA to confirm that the same ratio between *Leishmania* DNA and mouse DNA would still be obtained. For 148.5 ng sample the ratio was 1.99×10^{-4} and using 74.3 ng of the same sample the ratio was 1.93×10^{-4}.

4. Discussion

Microscopy has traditionally been the cheapest and easiest method for detecting and counting, *Leishmania* parasites. However, real-time PCR is superior regarding sensitivity, specificity, capacity and has in addition short time of analysis and minimized subjectivity of laboratory staff. We have developed a qPCR assay able to detect as little as 10 fg of DNA, and to quantify down to the limit of 10 fg (*L.* (*L.*) *donovani*). The AAP3-Assay is effective in detecting several different species, and possibly the species range for our assay is broader than we have tested for. Regarding specificity, both experimental and bioinformatic analyzes show no homology of the target sequence to non-*Leishmania* sequences.

Table 2
Quantification of *L.* (*L.*) *major* DNA in footpad from experimental infected mouse.

Sample[a]	$C_{q\,AAP3\text{-}Assay}$[b,c]	Qty LeishDNA[d]	$C_{q\,Tfrc\text{-}Assay}$[b]	Qty mouseDNA[e]	Qty LeishDNA/Qty mouseDNA
L. (*L.*) *major*-1	28.08	2.3×10^{-3}	25.55	90.03	2.55×10^{-5}
L. (*L.*) *major*-2	27.96	2.5×10^{-3}	25.46	96.18	2.60×10^{-5}
L. (*L.*) *major*-3	28.03	2.4×10^{-3}	25.46	95.99	2.50×10^{-5}
Control 1-1	Undet.	–	25.46	95.79	–
Control 1-2	Undet.	–	25.51	92.54	–
Control 1-3	Undet.	–	25.49	93.84	–

[a] Control 1, uninfected mice; results are shown for all triplicates for each sample.
[b] C_q, quantification cycle.
[c] Undet., Undetermined.
[d] Qty LeishDNA, Quantity *Leishmania* DNA in ng detected by the AAP3-Assay per 100 ng DNA.
[e] Qty mouseDNA, Quantity mouse DNA in ng detected by the Tfrc-Assay per 100 ng DNA.

With the applied method used for DNA extraction, 100 fg corresponded to 2 parasites. Assuming 80 fg of *Leishmania* DNA is equivalent to one parasite, then 100 fg corresponds to 1.2 parasites, indicating that the sensitivity of the AAP3-Assay actually is better than experienced, and can be further improved by optimizing DNA extraction.

Regarding sensitivity in terms of DNA quantity, the LOD of our assay equals that of some of the publications targeting both rRNA- and kDNA minicircle genes (Berzunza-Cruz et al., 2009; Nicolas et al., 2002; Prina et al., 2007; Gomes et al., 2012; Talmi-Frank et al., 2010; Wortmann et al., 2001), which are genes with high copy numbers, but there are also publications targeting multicopy genes showing higher sensitivity (Francino et al., 2006). However, when using multicopy genes for quantification there might be a need for a species specific standard curve as number of gene copies vary between species (Weirather et al., 2011), thus making those assays little suitable for a universal quantitative *Leishmania*-assay. Like the AAP3-Assay, the assay of (Wortmann et al., 2001), targeting the *Leishmania 16S rRNA*, was also able to amplify a wide range of *Leishmania* strains at the genus level. However, the sensitivity was only determined for *L.* (*L.*) *mexicana*, the C_q-values both within and between species had a great range, though they not clearly specify if the same amount of DNA was used, and parameters for assay performance, like efficiency, LOQ, reproducibility and repeatability was not estimated, or at least not given in the text.

The chromosome that hosts the *AAP3* gene is supernumerary; tetrasomic for some species, like *L.* (*L.*) *major*, *L.* (*L.*) *infantum* and *L.* (*L.*) *donovani*, and hexasomic for *L.* (*V.*) *braziliensis* (Rogers et al., 2011). In *L.* (*L.*) *major*, *L.* (*L.*) *infantum*, *L.* (*V.*) *amazonensis* and *L.* (*L.*) *donovani* there are two identical copies of *AAP3* (Castilho-Martins et al., 2011; Shaked-Mishan et al., 2006), whereas in *L.* (*L.*) *mexicana* there is no reported equivalent gene duplication. We have included 22 *Leishmania* strains, representing 9 different species. The C_q-values for these strains using 100 pg DNA as template in the AAP3-Assay are shown in Table 1. There is very little intra-species variation in C_q-value, but some inter-species variation, mainly between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis* against the others species included in our study. This might indicate that ploidy between strains and species does not largely affect the results of the assay, but this should be investigated further. Tupperwar et al. (2008) describes a PCR assay targeting the *Leishmania GP63* able to amplify several different species, but compared to the AAP3-Assay there is a markedly difference in the C_q-valuable between species when the same amount of template was used. Whether there is a need for a species specific, or even strain specific, standard curve for the *AAP3*-assay depends on the specific application and the required accuracy, and has yet to be more thoroughly evaluated. In contrast to other multicopy genes like the kDNA minicircle and ITS1, with copy numbers around 10 000 and 200 respectively, and as seen from Table 1, it is less likely, and for several species probably species group-specific standard curve will be sufficient.

A way of avoiding a species specific standard curve could be to use the DNA concentrations for the dilutions of the standard curve instead of the number of parasites, and then using species related conversion factors.

In this study we developed a qPCR assay that can be applied on a broad range of species of the *Leishmania* genus, from both the Old- and New World groups, including both *L.* (*Leishmania*) spp. and *L.* (*Viannia*) spp., and which in addition can be used to analyze DNA from both cell cultures and mouse tissue, and most likely DNA from several other kinds of sample materials. By using a standard curve one can determine the amount of target DNA in a sample, and from that also calculate the number of parasites. However, when isolating DNA from infected tissues the amount of DNA will be dependent on the size of the tissue sample and the recovery of DNA at the different steps of the isolation protocol. The use of host reference gene can circumvent this problem. When applied to *Leishmania* infected mouse, the AAP3- and Tfrc-Assays give a ratio between *Leishmania* DNA and mouse DNA. This ratio, which is independent of the amount of total DNA applied for the analysis, is reflecting parasite load in the tissue and hence differences can be detected. In a study by Tupperwar et al. (2008), they normalized the parasite number to the total DNA isolated from the mice tissue samples, and finally reported the parasite number per milligram of original tissue, not using a host reference gene. This reported result could then be prone to differences in recovery of DNA during the extraction process. Nicolas et al. (2002) describe a real-time PCR assay which successfully detects four different *Leishmania* species from a wide range of mouse infected tissues. However, the study does not include a method, like using a host reference gene, for estimating and normalization of the parasite burden of the tissue. They also target the high copy number minicircle kDNA, which might have drawbacks, as mentioned above. A limitation of our method is that it not necessarily discriminates between live and dead parasites. However, the levels of *Leishmania* DNA detected will still indicate the parasite load at the time of sampling.

In summary, we have developed a very sensitive method for detection of *Leishmania* that can be applied for several different species of the parasite. In combination with an assay for quantification of host DNA, it is possible to measure the load of *Leishmania* in infected tissues. With its high sensitivity the method has potential as a tool for diagnostic purposes, including detecting asymptomatic infections. Due to the quantification possibility, the method can be used to monitor the progress of infection, which will be a valuable tool in testing new drugs and in vaccine development.

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