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A duplex real-time PCR assay for the detection and differentiation of Leishmania infantum and Leishmania tarentolae in vectors and potential reservoir hosts

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Abstract

Leishmanioses are vector-borne diseases caused by Leishmania spp., which are transmitted by phlebotomine sand flies (Diptera, Psychodidae). The recent reports in humans of Leishmania tarentolae, which is primarily found in cold-blooded animals, and Leishmania infantum in Sergentomyia minuta spurred us to develop an internal transcribed spacer 1-based duplex quantitative real-time PCR (dqPCR) assay for the detection and differentiation between these Leishmania spp. The specificity of dqPCR was assessed by processing DNA samples from Phlebotomus spp. (n=188) and Se. minuta (n=171) and from tissues (i.e., heart, liver, muscle, lungs, spleen, kidney, eggs) of Podarcis siculus (n=4) and Tarentola mauritanica (n=3). In the absence of naturally infected and/or co-infected lizards, DNA from cultured L. infantum and L. tarentolae were spiked into tissues of lizards and used as controls. The analytical sensitivity of the dqPCR, assessed using 10-fold serial dilutions of DNA from both Leishmania spp. and spiked DNA samples from lizards was 2.3 x 10-7 ng/2 µl for L. infantum and 2.1 x 10-7 ng/2 µl for L. tarentolae. With the spiked DNA samples, the dqPCR detected up to 2.6 x 10-6 ng/2 µl of L. infantum and up to 2.1 x 10-7 ng/2 µl of L. tarentolae. Of 359 phlebotomine sand flies tested, five (3.6%) and two (1.4%) Ph. perniciosus scored positive for L. infantum and L. tarentolae, respectively. Similarly, of 171 Se. minuta, 56 (32.7%) and six (3.5%) scored positive for L. tarentolae and L. infantum, respectively. Co-infection with both Leishmania spp. was detected in two Se. minuta (1.2%). Out of seven reptiles tested, four P. siculus were positive for L. tarentolae. The newly dqPCR herein described may represent an improvement in the diagnosis of L. infantum and L. tarentolae and may assist in identifying the role of lizards as reservoirs and Se. minuta as vector, for these Leishmania spp.

A duplex real-time PCR assay for the detection and differentiation of *Leishmania infantum* and *Leishmania tarentolae* in vectors and potential reservoir hosts

Running Title : dqPCR for the detection and differentiation of*L. infantum* and *L. tarentolae*

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Summary

Leishmanioses are vector-borne diseases caused by *Leishmania*spp., which are transmitted by phlebotomine sand flies (Diptera, Psychodidae). The recent reports in humans of *Leishmania tarentolae* , which is primarily found in cold-blooded animals, and*Leishmania infantum* in *Sergentomyia minuta* spurred us to develop an internal transcribed spacer 1-based duplex quantitative real-time PCR (dqPCR) assay for the detection and differentiation between these *Leishmania* spp. The specificity of dqPCR was assessed by processing DNA samples from *Phlebotomus* spp. (n=188) and *Se. minuta* (n=171) and from tissues (i.e., heart, liver, muscle, lungs, spleen, kidney, eggs) of *Podarcis siculus* (n=4) and *Tarentola mauritanica* (n=3). In the absence of naturally infected and/or co-infected lizards, DNA from cultured *L. infantum* and *L. tarentolae* were spiked into tissues of lizards and used as controls. The analytical sensitivity of the dqPCR, assessed using 10-fold serial dilutions of DNA from both *Leishmania* spp. and spiked DNA samples from lizards was 2.3 x 10^{-7}ng/2 µl for *L. infantum* and 2.1 x 10^{-7} ng/2 µl for *L. tarentolae* . With the spiked DNA samples, the dqPCR detected up to 2.6 x 10^{-6} ng/2 µl of *L. infantum* and up to 2.1 x 10^{-7} ng/2 µl of *L. tarentolae* . Of 359 phlebotomine sand flies tested, five (3.6%) and two (1.4%) *Ph. perniciosus* scored positive for *L. infantum*and *L. tarentolae* , respectively. Similarly, of 171 *Se. minuta* , 56 (32.7%) and six (3.5%) scored positive for *L. tarentolae* and *L. infantum,* respectively. Co-infection with both*Leishmania* spp. was detected in two *Se. minuta* (1.2%). Out of seven reptiles tested, four *P. siculus* were positive for*L. tarentolae* . The newly dqPCR herein described may represent an improvement in the diagnosis of *L. infantum* and *L. tarentolae* and may assist in identifying the role of lizards as reservoirs and *Se. minuta* as vector, for these *Leishmania*spp.

Key words: duplex real-time PCR, ITS1, *Leishmania infantum* , *Leishmania tarentolae* , Lizards, *Sergentomyia minuta*

Introduction

Leishmanioses are vector-borne diseases that cause clinical conditions of varying degrees to different mammal species, including humans. The diseases are caused by *Leishmania* spp. (Kinetoplastida, Trypanosomatidae), which are transmitted by bites of phlebotomine sand flies of the genera *Phlebotomus* in the Old World (Maroli et al., 2013). Among the *circa* 100 sand flies species indicated as proven vectors of *Leishmania* spp. (Maroli et al., 2013), some are highly specific for certain *Leishmania* spp. (e.g., *Phlebotomus sergenti* and *Phlebotomus papatasi* for *Leishmania tropica*and *Leishmania major* , respectively) (Kamhawi, 2006), whereas others support the development of more than one *Leishmania* spp. (i.e., *Phlebotomus arabicus* is susceptible to the development of both *L. major* and *Leishmania infantum*) being considered permissive vectors (Jacobson et al., 2003; Myskova et al., 2007). However, DNA of *Leishmania* spp. may be retrieved in non-competent vectors such as in the case of *L. tropica* and *L. major* in*Phlebotomus perniciosus* and *L. infantum* in*Phlebotomus mascittii* (Vaselek et al., 2017; Gherbi et al., 2020). Meanwhile, phlebotomine sand flies of the genus*Sergentomyia* are known to feed primarily on cold-blooded animals (Lewis, 1987) and are associated to the transmission of*Leishmania* (*Sauroleishmania*) spp. in lizards (Killick-Kendrick et al., 1986; Lewis, 1987; Noyes et al., 1997; Tuon et al., 2008). These reptiles have also been found molecularly positive for some *Leishmania* spp. pathogenic to humans (Pombi et al., 2020). For example, DNA of *L. major, L. tropica* and *Leishmania martiniquensis* were found in different species of *Sergentomyia*(e.g., *Sergentomyia barraudi* , *Sergentomyia darling* ,*Sergentomyia garhami, Sergentomyia clydei* , *Sergentomyia gemmea* , and *Sergentomyia iyengari*) (Mutinga et al., 1994; Berdjane-Brouk et al., 2012; Kanjanopas et al., 2013; Nzelu et al., 2014; Ayari et al., 2016; Siripattanapipong et al., 2018). Similarly, the DNA of *L. infantum* has been detected in several*Sergentomyia* spp., such as *Sergentomyia dubia* ,*Sergentomyia magna* and *Sergentomyia schewtzi* in Africa (Senghor et al., 2016), and *Sergentomyia minuta* from areas endemic for canine leishmaniosis in Europe (Tarallo et al., 2010; Campino et al., 2013; Bravo-Barriga et al., 2016;

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Maia et al., 2015; Latrofa et al., 2018; Gonzales et al., 2020; Pombi et al., 2020).

Sergentomyia spp. female is known to feed mostly on cold-blooded animals, such as lizards, which are, with geckos, the primary hosts of*Leishmania tarentolae* (Telford 1995; Sloboda et al., 2007; Halla et al., 2014). This *Leishmania* species is considered non-pathogenic for mammals, although some strains (e.g., LEM-125) were associated to transient infectious in rodents and humans (Adler, 1962; Breton, 2005; Taylor et al., 2010; Novo et al., 2015). On the contrary, the DNA and/or amastigote forms of pathogenic *Leishmania* spp. (i.e., *L. tropica* , *Leishmania donovani* and *L. infantum*) have been detected in reptiles (Wilson and Southgate, 1979; Simpson and Holtz, 1988; Belova, 1971; Zhang et al., 2019; Chen et al., 2019) . Therefore, in addition to mammals and various other hosts such as black rat, hares, horse, cat, red fox and wild carnivores (Criado-Fornelio et al., 2000; Solano-Gallego et al., 2003; Sobrino et al., 2008; Millán et al., 2011; Gramiccia, 2011; Montoya et al., 2016), reptiles have been suggested as potential reservoirs for different*Leishmania* spp. (Zhang et al., 2016; Mendoza-Roldan et al., 2020).

The recent reports of *L. tarentolae* in human blood and of*L. infantum* in *Se. minuta* (Latrofa et al., 2018; Gonzales et al., 2020; Pombi et al., 2020) spurred us to develop a duplex quantitative real-time PCR (dqPCR), with species-specific probes targeting the internal transcribed spacer 1 (ITS1) region, for the detection and differentiation between *L. infantum* and *L. tarentolae* . This assay may provide a fundamental contribution for understand the potential ecological role of sand flies and reptiles in spreading these two parasites in endemic and non-endemic geographical areas where they live in sympatry with animals and humans.

Materials and Methods

2.1 Samples

To obtain sequences of *Leishmania* spp. for primes and probes designing, promastigotes of *L. infantum* (zymodeme MON-1; 1.6 x 10^7 promastigotes/ml) and *L. tarentolae* (strain RTAR/IT/81/ISS21-G.6c; 1.7 x 10^7 promastigotes/ml) were extracted and their genomic DNA (gDNA) amplified by conventional PCR using primes (L5.8S/LITSR) targeting a fragment of the ITS1 (300-350 bp), as described elsewhere (El Tai et al., 2001). Genomic DNA of*L. major* (MHOM/TM/1973/5ASKH), *L. aethiopica*(MHOM/ET/72/L100), *L. donovani* (MHOM/IN/00/DEVI) and *L. tropica* (MHOM/IL/2005/LRC-L1239) were also used for testing the specificity of the newly developed assay (see below).

2.2 Primers, probes, and dqPCR protocol

Generic primers (*L.i.t.* -ITS1-F 5'-GCAGTAAAAAAAAGGCCG-3';*L.i.t.* -ITS1-R 5'-CGGCTCACATAACGTGTCGCG-3') and two specific hydrolysis TaqMan-MGB probes (*L.t.* -6-FAM-5'-CACGCCGCGTATACAAAAACAC-3'-non-fluorescent quencher-MGB;*L.i.*- VIC-5'-TAACGCACCGCCTATACAAAAGCA-3'-non-fluorescent quencher-MGB (Applied Biosystems; Foster City, CA, USA) targeting 150 bp of the ITS1 of *L. infantum* and *L. tarentolae,* were designed by alignment of the reference *Leishmania* spp. sequences using Primer Express 2.0 (Applied Biosystems, Foster City, CA). Sequences of *L. major* (accession number HG512924), *L. tropica* (accession number HG512927), *L. turanica* (accession number AJ272382) and *Leishmania aethiopica* (accession number HG512923) available from GenBank were included in the alignment. The primer and probe specificity were analysed in silico using the basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). dqPCR reactions were carried out in a final volume of 20 µl, consisting of 10 µl of IQ Supermix (Bio-Rad Laboratories, Hercules CA, USA), 7 µl of Di-Ethyl Pyro-Carbonate (DEPC) treated pyrogen-free DNase/RNase-free water (Invitrogen, Carlsbad, CA, USA), 2 µl of template DNA and 950 nM of each primer, as well as 200 and 300 nM of FAM and VIC TaqMan-MGB probes, respectively. The thermal cycling conditions consisted of a hot start at 95°C for 3 min, and 40 cycles of denaturation (95°C for 10 sec) and annealing-extension (55°C for 30 sec). All assays were carried out in duplicate and a no-template control (NTC) was included in each run. The dqPCR was performed in a CFX96^{TM} Real-Time System (Bio-Rad Laboratories, Inc., Hercules CA, USA) and the increase in the fluorescent signals were registered during the extension step of the reaction and analysed by the CFX Manager^{TM} software, version 3.1 (Bio-Rad).

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2.3 Specificity and sensitivity of the dqPCR

To investigate the analytical specificity of the assay, gDNA of both*L. infantum* and *L. tarentolae* , alone or in combination, as well as gDNA of *L. major* , *L. aethiopica* , *L. donovani* and *L. tropica* were tested. In addition, DNA from*Phlebotomus* spp. (i.e., five *Ph. neglectus* , three*Ph. papatasi* , 140 *Ph. perniciosus* , and 40 *Ph. perfiliewi*) and *Se. minuta* (n=171) previously collected in a*L. infantum* -endemic area (Latrofa et al., 2018; Iatta et al., 2020) (Table 1) and DNA from various types of samples (i.e., heart, liver, muscle, lungs, spleen, kidney, and eggs), from four Italian wall lizards (*Podarcis siculus*) and three Moorish wall geckos (*Tarentola mauritanica*) were tested. In addition, in the absence of naturally infected and/or co-infected lizard samples, DNA from*L. infantum* and *L. tarentolae* were spiked with the DNA from lizard samples and used as controls (Table 2).

The analytical sensitivity of the dqPCR assay was assessed using 10-fold serial dilutions of DNA from *L. infantum* (ranging from 2.7×10^{-1} to 2.7×10^{-8} ng/2 µl of reaction), *L. tarentolae* (ranging from 2.1×10^{-1} to 2.1×10^{-8} ng/2 µl of reaction) and from the spiked DNAs of both *Leishmania* species (ranged from 2.4×10^{-1} to 2.4×10^{-8} ng/2 µl of reaction) and expressed also by threshold cycle values (Cq) and parasite load amount (promastigotes/2 µl of reaction). gDNA was extracted from *Leishmania* spp. cultures and from lizard tissues using the commercial kit DNeasy Blood & Tissue (Qiagen, GmbH, Hilden, Germany), following the manufacturer's instructions. The amount of purified DNA from each *Leishmania*strain (*L. infantum* = 2.6×10^{-1} ng/µl;*L. tarentolae* = 2.1×10^{-1} ng/µl) was determined using Qubit (Applied Biosystems, Foster City, CA, USA).

Results

Fluorescent signals were recorded for DNA of *L. infantum* and*L. tarentolae* , and for the spiked DNA from both *Leishmania*spp. (Figure 1 A, B, C). Though BLASTn analyses did not found sequences identity with other *Leishmania* spp., fluorescence signals were obtained for *L. donovani* (Cq=16.5), *L. tropica* (Cq=16.2) and *L. major* (Cq=17.7) tested with the VIC-*L. infantum*probe, whilst no fluorescence signals were obtained with other*Leishmania* spp., or NTC. The performance of the assay was confirmed by the efficiency values (ranging from 106.5 to 115%) of the standard curves obtained with each *Leishmania* spp. and for spiked DNAs, with R^2 ranging from 0.978 to 1.000 and the slope ranging from -3.009 to -3.178 (Figure 1 A, B, C). The analytical sensitivity of the dqPCR was 2.3×10^{-7} ng/2 µl for*L. infantum* (i.e., 3.3×10^{-3} promastigotes/2 µl, Cq of 37.4) and 2.1×10^{-7} ng/2 µl for *L. tarentolae* (i.e., 3.3×10^{-3} promastigotes/2 µl, Cq of 36.3). With the spiked DNA, the assay detected up to 2.6×10^{-6} ng/2 µl of *L. infantum* (i.e., 3.3×10^{-2} promastigotes/2 µl, Cq of 38.0) and up to 2.1×10^{-7} ng/2 µl for *L. tarentolae* (i.e., 3.3×10^{-3} promastigotes/2 µl, Cq up to 38.6).

Out of 359 phlebotomine sand flies tested, 63 (17.5%) scored positive for *Leishmania* spp. (Table 1) with five (3.6%) and two (1.4%) *Ph. perniciosus* specimens, positive for *L. infantum* and *L. tarentolae* , respectively (Table 1). Out of 171*Se. minuta* tested, 56 (32.7%) scored positive for *L. tarentolae* and six (3.5%) for *L. infantum* . Co-infection with both *Leishmania* spp. was detected in two samples of *Se. minuta* (1.2%). No *Ph. papatasi* , *Ph. neglectus* and*Ph. perfiliewi* scored positive for *Leishmania* spp. (Table 1). The mean DNA load detected in phlebotomine sand flies ranged from 2.2×10^{-5} to 3.0 ng/2 µl for *L. infantum* (mean Cq up to 35.3) and from 2.1×10^{-6} to 4.1 ng/2 µl for*L. tarentolae* (mean Cq up to 35.9) (Table 1).

Out of seven reptiles tested, four *P. siculus* were naturally infected by *L. tarentolae* . In particular, of the five types of lizard tissue analysed, one each of muscle, heart and spleen (Cq values ranging from 34.1 to 36.9, corresponding to $2.3 \times 10^{-1}$and 2.9×10^{-2} promastigotes/2 µl) and two each of kidney and egg samples (Cq value ranging from 20.3 to 37.2, corresponding to 3.5×10^{3} and 3.1×10^{-2} promastigotes/2 µl) scored positive for *L. tarentolae* . All DNA samples from lizard spiked with DNA of *L. infantum* and/or *L. tarentolae* returned positive signals for one or both species (Table 2). Similar mean Cq values were detected for all tissues spiked with each *Leishmania* spp. (ranging from 19.0 to 21.4), with the exclusion of lung samples of *T. mauritanica* , where an increasing Cq values up to 25 were detected (Table 2). A slightly increased Cq values was obtained by testing spiked lizard samples with both *Leishmania* spp. (Table 2).

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Discussion

The dqPCR assay, developed for the simultaneous detection and differentiation of *L. infantum* and *L. tarentolae* , proved to be an efficient and sensitive tool for detecting both parasites, alone or in combination. In particular, the smallest amount of DNA detected (2.1 x 10^{-7} ng/2 µl for *L. tarentolae*and 2.6 x 10^{-6} ng/2 µl for *L. infantum*) corresponded to a low parasite load (3.3 x 10^{-3} and 3.3 x 10^{-3} promastigotes/2 µl per reaction). However, a slight shift in the Cq values was observed for *L. infantum* and*L. tarentole* spiked with DNA samples from lizards. The results obtained may be explained by the copy number (i.e., 200) of the genetic target (ITS1) present in the genome of *Leishmania* spp. (Van der Auwera and Dujardin 2015; Schonian et al., 2011) and by the high genetic variability of this region, which was pivotal for delineating the two species. Indeed, differently from the minicircle kinetoplast DNA, which exhibits a high sensitivity in detecting low amounts of DNA (equivalent to 0.004 parasites) but low specificity (Kuhls et al., 2011; Galluzzi et al., 2018), the ITS1 has been successfully used to resolve taxonomical and phylogenetic relationships among closely related *Leishmania*spp. (Dávila and Momen, 2000; Parvizi and Amirkhani, 2008; Wang et al., 2010; Hajjaran et al., 2013).

The sensitivity of the dqPCR (100%) was also confirmed by detection of DNA of both *Leishmania* spp. in all lizard spiked DNA samples, showing approximately the same Cq values (ranging from 19.0 to 22.1). Similarly, the good performance of the dqPCR was also determined by the values of the slope of standard curves, the efficiency and the coefficient of determination obtained with both *Leishmania* spp. and the lizard-spiked DNA.

The dqPCR assay detected low amounts of *Leishmania* spp. DNA (mean value of 2.2 x10^{-5} for *L. infantum* and 2.1 x 10^{-6} for *L. tarentolae*) in naturally infected phlebotomine sand flies as well as discriminated both species in co-infected *Se. minuta.* Though a low number of phlebotomine sand flies were tested, the finding of the same prevalence of infection for*L. infantum* in *Ph. perniciosus* (3.6%) and in *Se. minuta* (3.5%) and the co-infection for both *Leishmania* spp. provide additional circumstantial evidence on the involvement of*Se. minuta* in the circulation of *L. infantum* in some endemic areas (Maia et al., 2015; Maia and Depaquit, 2016; Latrofa et al., 2018; Pombi et al., 2020). Indeed, the role of *Se. minuta* in spreading *Leishmania* spp. other than *L. tarentolae* has been suggested in different countries of the Mediterranean region such as Italy, Portugal, Tunisia and Turkey (Campino et al., 2013; Jaouadi et al., 2015; Maia et al., 2015; Ayari et al., 2016; Özbel et al., 2016; Latrofa et al., 2018) and also in regions of Africa (Mutinga et al., 1994; Tateng et al., 2018; Nzelu et al., 2014) and Asia (Chusri, et al., 2014; Siripattanapipong et al., 2018). Therefore, given the opportunistic anthropophilic feeding behaviour of this phlebotomine sand fly, susceptible hosts could be infected by *L. infantum* through bites of *Se. minuta* females (Maia et al., 2015; Gonzales et al., 2020; Pombi et al., 2020). In this context, the pathogenic role of*L. tarentolae* detected in humans should not be ruled out. Indeed, some virulence factors, (i.e., GP63, CPB, LPG3 and amastin) present in the pathogenic *L. infantum* and *L. major* is also expressed in *L. tarentolae* (Azizi et al., 2009; Mizbani et al., 2011; Raymond et al., 2012; Novo et al., 2015) . The detection of the DNA of *L. tarentole* in *P. siculus* suggest that more than one lizard species could be infected by this pathogen, besides the previously reported *T. mauritanica* (Pozio et al., 1983). Finally, even if no lizard samples scored positive for *L. infantum* in the present investigation, the limited number of samples tested precludes any further inference on this.

In conclusion, the dqPCR herein described may represent an improvement in the discrimination of *L. infantum* and *L. tarentolae* in order to identify the role of lizards and *Se. minuta* as reservoirs and vector, respectively, for these *Leishmania* spp. Furthermore, this assay will allow large-scale testing of humans (and also dogs, for instance) in areas where *Se. minuta* occurs, enabling a more robust assessment on how frequent these hosts are exposed to *L. tarentolae* .

Authorship

Conceptualization, Maria Stefania Latrofa, Jairo Alfonso Mendoza-Roldan, Domenico Otranto; Methodology, Maria Stefania Latrofa, Ranju Ravindran Santhakumari Manoj; Formal Analysis, Maria Stefania Latrofa, Ranju Ravindran Santhakumari Manoj; Data Curation, Maria Stefania Latrofa, Ranju Ravindran Santha-

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kumari Manoj, Domenico Otranto; Writing – Original Draft Preparation, Maria Stefania Latrofa, Domenico Otranto; Writing – Review & Editing, Maria Stefania Latrofa, Jairo Alfonso Mendoza-Roldan, Ranju Ravindran Santhakumari Manoj, Filipe Dantas-Torres, Domenico Otranto.

Conflicts of Interest

The authors declare no conflict of interest.

Ethical Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to the European Directive 2010/63/EU and the study was approved by the ethical committee of the Department of Veterinary Medicine of the University of Bari, Italy (Prot. Uniba 7/17) and of Ministry authorization (ISPRA Prot 73267; 31/12/2019).

Data Availability Statement

The data that support the findings of this study are available from the corresponding author.

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TABLE 1

Samples of phlebotomine sand flies used to assess the analytical specificity of the duplex quantitative PCR. The mean threshold cycle (Cq), parasite load (Starting Quantity (SQ), value expressed as ng/µl of DNA for

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reaction) and the mean, minimum, maximum and standard deviation (SD) of values of the threshold cycle (Cq) are reported for *Leishmania infantum* and *Leishmania tarentolae* assessed by assay is reported

Pathogen	*Ph. perniciosus*	*Ph. perniciosus*	*Ph. perniciosus*	*Ph. perniciosus*	*Ph. perniciosus*	*Ph. perniciosus*	*Ph. perniciosus*	*Se. minuta*$^{+}$	*Se. minuta*$^{+}$	*Se. minuta*$^{+}$	*Se. minuta*$^{+}$	*Se. minuta*$^{+}$	*Se. minuta*$^{+}$	*Se. minuta*$^{+}$	*Se. minuta*$^{+}$	*Ph. pa at*
	Pos/Tot (%)	Cq Mean	Cq Min-max^{++}	Cq SD	SQ Mean	SQ Min-max	SQ SD	Pos/Tot (%)	Cq Mean	Cq Min-max	Cq SD	SQ Mean	SQ Min-max	SQ SD	Pc (%
L. infantum	5/140 (3.6)	33.3	20.5-37.5	7.2	3	2.1x10^{-6}-3.3x10	9.9	6/171 (3.5)	35.2	33.3-37.6	1.6	2.2x10^{-5}	5.5x10^{-6}-5.6x10^{-5}	1.8x10^{-5}	0/
L. tarentolae	2/140 (1.4)	35.9	35.7-36.1	0.3	2.1x10^{-6}	1.8 x10^{-6}-2.5x10^{-6}	4.9 x10^{-7}	50/171 (29.2)	33.0	18.5-38.9	5.9	4.09	8.8x10^{-7}-7.9x10^{1}	1.52x10^{1}	0/
Total (Pos/Tot %)	7/140 (5)	-	-	-	-	-	-	56*/171 (32.7)	-	-	-	-	-	-	0/

$+$ *Se. minuta* ($n = 2$) scored positive for both *L. infantum* and *L. tarentolae*

++ Pos, positive; Tot, total; Min, minimum; Max, maximum

TABLE 2

DNA from lizard tissues samples spiked with DNA of *Leishmania infantum* and/or *Leishmania tarentolae* tested by duplex quantitative PCR. The mean, minimum, maximum and standard deviation (SD) of values of the threshold cycle (Cq) are reported

Pathogen	*Podarcis siculus* Muscle Cq Mean	*Podarcis siculus* Muscle Cq Min-max^{+}	*Podarcis siculus* Muscle Cq SD	*Podarcis siculus* Lung Cq Mean	*Podarcis siculus* Lung Cq Min-max	*Podarcis siculus* Lung Cq SD	*Podarcis siculus* Heart Cq Mean	*Podarcis siculus* Heart Cq Min-max	*Podarcis siculus* Heart Cq Min-max	*Podarcis siculus* Heart Cq SD	*Podarcis siculus* Spleen Cq Mean	*Podarcis siculus* Spleen Cq Min-max	*Podarcis siculus* Spleen Cq SD	*Podarcis siculus* Kidney Cq Mean	*Podarcis siculus* Kidney Cq Min-max	*Podarcis siculus* Kidney Cq SD	*Podarcis siculus* Eggs Cq Mea
L. infantum	20.6	20.4-21.0	0.3	22.8	22.1-23.7	0.8	21	21.0-21.2	21.0-21.2	0.1	21.2	20.7-21.7	0.4	20.7	20.1-21.2	0.4	21.1
L. tarentolae	19.3	19.1-19.7	0.4	22.8	22.4-23.7	0.7	21.4	19.6-22.4	19.6-22.4	1.6	20.3	19.6-21.4	1.1	19	18.9-19.4	0.2	19.9

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L. infantum+ *L. tarentolae*	21.4 20.2	22.2-21.3 20.0-20.3	1.2 0.1	23.6 23.2	23.2-24.2 22.0-23.9	0.5 1.03	22.3 20.3	22.2-22.5 20.2-20.5	22.2-22.5 20.2-20.5	0.2 0.1	22.3 20.1	22.1-22.8 19.7-20.5	0.4 0.3	22.3 20.2	22.2-22.6 20.0-20.4	0.2 0.1	22.2 20.6
	Tarentola mauritanica	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*	*Tarentola mauritanica*
	Muscle Cq Mean	Muscle Cq Min-max	Muscle Cq SD	Lung Cq Mean	Lung Cq Min-max	Lung Cq SD	Heart Cq Mean	Heart Cq Min-max	Heart Cq SD	Heart Cq SD	Spleen Cq Mean	Spleen Cq Min-max	Spleen Cq SD	Kidney Cq Mean	Kidney Cq Min-max	Kidney Cq SD	Eggs Cq Mea
L. infantum	20.9	21.0-20.9	0.01	25	24.7-25.3	0.3	21.1	21.1-21.2	0.01	0.01	20.9	20.7-21.1	0.2	20.8	20.4-21.1	0.3	-
L. tarentolae	19.1	19.1-19.1	0.01	24.1	23.4-24.5	0.7	20.6	19.1-22.1	2.1	2.1	19.4	18.7-19.8	0.6	19.1	18.7-19.6	0.4	-
L. infantum+ *L. tarentolae*	21.7 20	21.6-21.8 19.9-20.1	0.1 0.1	25 24.4	24.5-25.4 24.1-24.7	0.4 0.3	22 21.7	21.8-22 21.7-21.8	0.1 0.06	0.1 0.06	20 19.9	21.8-22.2 19.7-20.5	0.2 0.3	22.1 20.3	22.0-22.6 20.1-20.4	0.1 0.1	-

+ Pos, positive; Tot, total; Min, minimum; Max, maximum

Figure legends

FIGURE 1 Assessment of the specificity of dqPCR assay in the detection of *Leishmania* spp. DNA. The amplification plot represented by the fluorescent signal, accordingly to relative fluorescence units (RFU) and threshold cycle and standard curves generated from serial dilutions of genomic DNA from *L. tarentolae*(A), *L. infantum* (B) and for spiked DNA of *L. infantum* and*L. tarentolae* (C)

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