Molecular characterization of Leishmania infantum *strains by kinetoplast DNA RFLP-PCR*

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Summary
Multilocus enzyme electrophoresis is the tool most frequently used to classify *Leishmania* spp., although it is time consuming and, sometimes, a not enough discriminative method. In the present study a kinetoplast DNA polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was used to characterize 16 zymodeme MON-1 *Leishmania infantum* strains: 15 were from dogs housed in public kennels of 7 geographical areas in the Emilia-Romagna region, Northern Italy, 1 was the *L. infantum* reference strain MHOM/TN/1980/IPT1. Six enzymatic patterns were observed. Kinetoplast DNA RFLP-PCR confirmed to have a good discriminatory power within the same zymodeme and proved to be useful for comparing few strains or discriminating between relapse and reinfection in the same host. We therefore recommend it use for discriminating between relapse and reinfection in the same host rather than supporting large-scale epidemiological studies.

Caratterizzazione molecolare di ceppi di Leishmania infantum *mediante kDNA RFLP-PCR*

Parole chiave
Cane,
Epidemiologia,
kDNA RFLP-PCR,
Leishmania infantum,
Tipizzazione molecolare.

Riassunto
Il metodo più frequentemente utilizzato per la classificazione di *Leishmania* spp. è ancora rappresentato dalla caratterizzazione isoenzimatica sebbene sia un metodo laborioso e in alcuni casi non sufficientemente discriminante. Nel presente studio abbiamo utilizzato una reazione di amplificazione del DNA kinetoplastico seguita da digestione enzimatica, al fine di caratterizzare 15 ceppi di *Leishmania infantum* MON-1, isolati da altrettanti cani ospitati in canili pubblici di 7 differenti aree geografiche del nord Italia. Il ceppo di referenza MHOM/TN/1980/IPT1 di *L. infantum* è stato incluso nello studio. A conferma del potere discriminante di questa metodica, sono stati osservati 6 differenti profili enzimatici, anche all'interno di uno stesso zimodema. A nostro avviso, kDNA RFLP-PCR può pertanto trovare un'applicazione utile per discriminare i casi di reinfezioni da quelli dovuti a ricadute, piuttosto che in studi epidemiologici su larga scala.

In the Mediterranean area, *Leishmania infantum* is the causative agent of both cutaneous and visceral human forms of leishmaniasis, with the main domestic reservoir in the dog (WHO 2010).

The taxonomic tool most frequently used to classify *Leishmania* spp. is still the multilocus enzyme electrophoresis (MLEE), which includes the strains having the same enzymatic profile into groups called zymodemes (Rioux *et al.* 1990). However, this technique is time consuming, as it requires the

isolation and cultivation of the parasite and it can only be performed in specialised laboratories. Moreover, nucleotide substitutions and subsequent changes in the amino acid composition are highlighted only when they lead to different electrophoretic mobility (Oshaghi *et al.* 2009). Considering that MON-1 zymodeme is the most widespread in the Mediterranean area, including Italy (Gradoni *et al.* 1984), MLEE may not be sufficient to discriminate between relapse and reinfection in the same host (Ferroglio *et al.* 2006).

To overcome this limit several molecular methods have been developed to identify leishmania parasite at strain level: polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) of antigen-encoding genes (*gp63* and *cpb*) (Mauricio *et al.* 2001, Quispe Tintaya *et al.* 2004), random amplification of polymorphic DNA (Hide *et al.* 2001, Zemanova *et al.* 2004), DNA-fingerprinting (Naserredin *et al.* 2005), microsatellite analysis (Kuhls *et al.* 2008), and kinetoplast DNA (kDNA) RFLP-PCR (Morales *et al.* 2001, Chicarro *et al.* 2002). Recently, a study investigated the number and genetic variability of Kinetoplast minicircles in *L. infantum* MHOM/TN/1980/IPT1 strain by a new SYBR green-based real-time PCR (Ceccarelli *et al.* 2014).

Kinetoplast DNA contains tens of maxicircles and thousands of minicircles (Wesley and Simpson 1973). Each minicircle is about 800 base pair (bp) in size, composed by a conserved region of 200 bp and a variable region of 600 bp, which has been used to analyse the genetic polymorphism in *L. infantum* strains (Morales *et al.* 2001, Cortes *et al.* 2006, Ferroglio *et al.* 2006, Nasereddin *et al.* 2009, Cortes *et al.* 2010).

The aim of this study was to characterize *L. infantum* isolates from dogs living in different geographical areas in the Emilia-Romagna region (Northern Italy) by a kDNA RFLP-PCR.

A total of 16 *L. infantum* samples were analysed. Fifteen isolates were obtained from bone marrow (n. 4) or lymph node (n. 11) of 15 dogs housed in kennels in 7 areas in the Emilia-Romagna region, Northern Italy (Table I). The dogs were serologically tested within a regional surveillance program on canine leishmaniasis and resulted positive by Immunofluorescence Antibody Technique (IFAT) showing an antibody titre ≥160, (Santi *et al.* 2014). MHOM/TN/1980/IPT1 *L. infantum* reference strain was also tested. The isolates, as well as the reference strain, were previously identified as *L. infantum* MON-1 group, by molecular method according to Haramboulos and colleagues (Haramboulos *et al.* 2008). Parasites were cultivated in the biphasic Evans'modified Tobie's medium at 23°C. Promastigotes were collected during the exponential growth phase by centrifugation and washed 5 times by phosphate-buffered saline. DNA was extracted using a commercial kit (DNeasy Blood & Tissue Kit, Qiagen, Hilden, Germany).

All the samples were tested by a kDNARFLP-PCR, following the procedure indicated by Morale and colleagues (Morales *et al.* 2001): primers LINR4 (5'-GGGGTTGGTGTAAAATAG-3') (Noyes *et al.* 1998) and DBY (5'-CCAGGTTCCCGCCCCGGAG-3') were used to amplify the entire kDNA minicircle sequence of 800 bp. The reaction was carried out in a 50 µl final volume containing 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 200 µM of each deoxynucleoside triphosphate, 10 pmol of each primer, 1.25 U of Taq DNA polymerase (Qiagen, Hilden, Germany) and 5 µl of the DNA sample. The conditions of the PCR were as follows: initial denaturation at 94°C (5 minutes), 40 cycles each consisting of denaturation at 94°C (1 minute), annealing at 60°C (1 minute), extension at 72°C (1 minute) and a final elongation step of 10 minutes at 72°C. For each PCR reaction, a positive and a negative control were included; PCR products were visualised after electrophoresis on a 1% agarose gel, using the ethidium bromide staining.

Amplicons were digested by using *RsaI* restriction enzyme (Botilde *et al.* 2006, Nasereddin *et al.* 2009), as follows: after purification of the PCR products (QIAquick PCR purification kit, Roche, Mannheim, Germany), 16 µl of each amplicon were mixed with 2 µl of enzyme and 2 µl 10X Buffer Tango (Promega, Madison, Wisconsin, USA) and digested for 2 hours at 37°C. After 2 hour electrophoresis on 3% agarose gel, the RFLP profiles were visualised with ethidium bromide staining. A DNA marker (Bench Top 100 bp DNA Ladder, Promega, Wisconsin, USA) was included in each gel to determine approximately the fragment sizes. All the samples were digested twice, to verify the reproducibility of the method.

Kinetoplast DNA amplification produced the expected product of 800 bp in all samples.

Table I. Leishmania infantum *samples analysed in this study.* The isolates were obtained from bone marrow (n. 4) or lymph node (n. 11) of 15 dogs housed in kennels in 7 areas in the Emilia-Romagna region, Northern Italy.

Provinces/ District	Number of strains	Number of strains	
		Bone marrow	Lymph node
Bologna	1	0	1
Forlì-Cesena	5	1	4
Modena	2	0	2
Parma	1	0	1
Ravenna	2	0	2
Reggio-Emilia	2	2	0
Piacenza	2	1	1

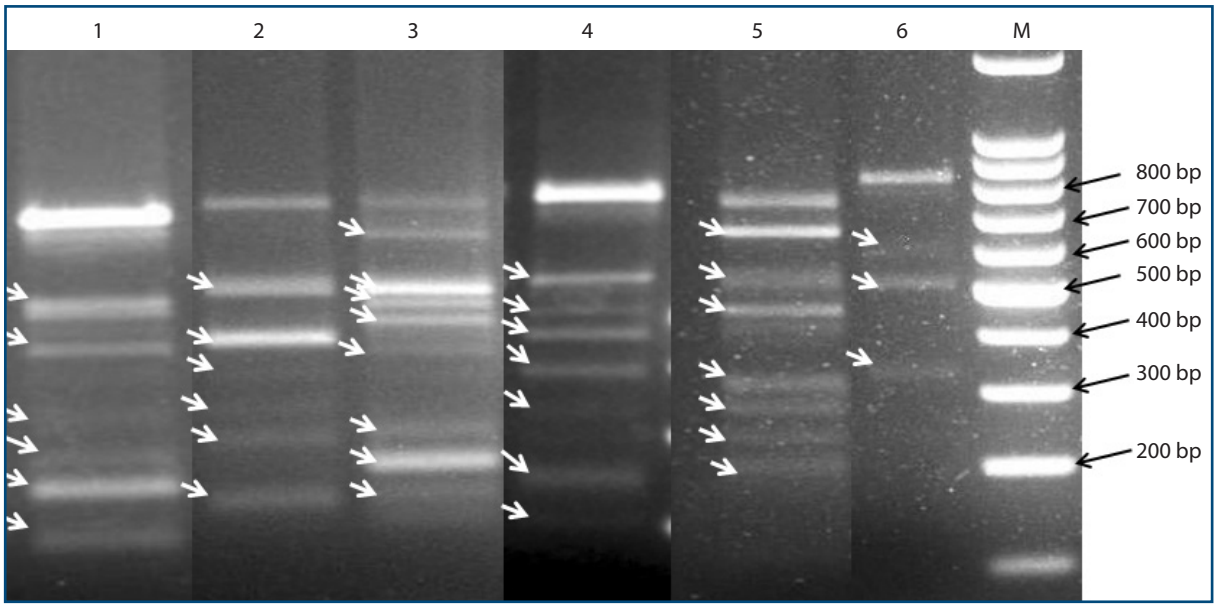

Figure 1. *Different enzymatic patterns obtained analysing the kDNA by a protocol of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) using* RsaI.

Digestion of the amplicons generated 6 (1-6) RFLP patterns, showing a range of 4-9 bands ≥ 800 bp. Because bands > 700 bp can be undigested amplicons (Alonso *et al.* 2010), only ≤ 700 bp bands were considered (Figure 1). The obtained patterns did not change through different digestions of the same sample, but for some samples the intensity of the bands from 2 enzymatic digestions was not constant. Digestion patterns, with respect to the geographical origin of the isolates, are reported in Table II.

In the Mediterranean area, the zymodeme MON-1 is considered responsible for the majority of the leishmaniasis cases. Few studies have molecularly typed *L. infantum* strains circulating in Italy. Ferroglio and colleagues (Ferroglio *et al.* 2006) analysed 28 samples from dogs and humans by kDNA PCR-RFLP, showing 13 RFLP patterns. The samples were from traditionally and newly endemic areas of North-Western Italy and some patterns were specific to certain areas. Reale and colleagues (Reale *et al.* 2010) characterised 51 *L. infantum* strains from dogs in Sicily by multilocus microsatellite analysis and highlighted 2 zymodemes and various genotypes within each zymodeme.

In the present study, *L. infantum* isolates from dogs resident in public kennels of the Emilia-Romagna region were molecularly typed by kDNA PCR-RFLP, using only 1 restriction enzyme.

Six *L. infantum* patterns within MON-1 zymodeme group were shown circulating in a limited geographical area. Despite the origin of sampled housed dogs was very difficult to assess, none of them was reported to originate from a different region. Unfortunately, the number of the strains per area was too little to allow epidemiological suggestions, with the exception of the Forlì-Cesena area, where 5/5 isolates showed the same RFLP pattern. The digestion pattern of the reference strain appeared markedly different with respect to the pattern of other strains. This aspect could be related to the different geographic origin of the reference strain, which is a WHO international reference strain from Tunisia (Hide *et al.* 2001, Ochsenreither *et al.* 2006).

The pattern of the reference strain did not change through different digestions, showing a stability of the enzymatic profile after prolonged culture *in vitro*. A greater number of isolates should be analysed to assess a correlation between geographical area and enzymatic pattern. Clinical data, when available, did not highlight any correlation with the enzymatic profiles. The 5 strains from Forlì-Cesena

Table II. *DNA polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) patterns of 16* Leishmania infantum *strains according to the dog provenence.*

RFLP pattern	N. fragments	N. samples showing the same RFLP pattern	Provenence
1	6	7	Forlì-Cesena/ Piacenza
2	6	1	Parma
3	8	3	Bologna/Ravenna
4	7	2	Reggio Emilia
5	7	2	Modena
6	3	1	IPT1

area showed the same digestion pattern, but 2 were from asymptomatic dogs and 3 from dogs showing clinical signs of leishmaniasis, such as onicogryphosis, lymphadenomegaly, dermatitis.

Kinetoplast DNA PCR-RFLP has been shown to have a good discriminatory power (Botilde *et al.* 2006, Cortes *et al.* 2006). However, we agree with Botilde and colleagues (Botilde *et al.* 2006) and note that the interpretation of the RFLP patterns as well as the inter-laboratory comparison could be relatively difficult. In our opinion, kDNA PCR-RFLP could be successfully used to compare few field strains or different samples from the same subject to discriminate between relapse and reinfection (Morales *et al.* 2002), whereas its use to large-scale epidemiological studies requires high resolution equipment and standard operating procedures.

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