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A novel A2 allele found in *Leishmania* (*Leishmania*) *infantum chagasi*

Novo alelo do gene A2 descrito em *Leishmania* (*Leishmania*) *infantum chagasi*

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Abstract

Visceral leishmaniasis (VL) is a widely spread zoonotic disease. In Brazil the disease is caused by *Leishmania* (*Leishmania*) *infantum chagasi*. Peridomestic sandflies acquire the etiological agent by feeding on blood of infected reservoir animals, such as dogs or wildlife. The disease is endemic in Brazil and epidemic foci have been reported in densely populated cities all over the country. Many clinical features of *Leishmania* infection are related to the host-parasite relationship, and many candidate virulence factors in parasites that cause VL have been studied such as A2 genes. The A2 gene was first isolated in 1994 and then in 2005 three new alleles were described in *Leishmania* (*Leishmania*) *infantum*. In the present study we amplified by polymerase chain reaction (PCR) and sequenced the A2 gene from the genome of a clonal population of *L.* (*L.*) *infantum chagasi* VL parasites. The *L.* (*L.*) *infantum chagasi* A2 gene was amplified, cloned, and sequenced in. The amplified fragment showed approximately 90% similarity with another A2 allele amplified in *Leishmania* (*Leishmania*) *donovani* and in *L.* (*L.*) *infantum* described in literature. However, nucleotide translation shows differences in protein amino acid sequence, which may be essential to determine the variability of A2 genes in the species of the *L.* (*L.*) *donovani* complex and represents an additional tool to help understanding the role this gene family may have in establishing virulence and immunity in visceral leishmaniasis. This knowledge is important for the development of more accurate diagnostic tests and effective tools for disease control.

Keywords: *Leishmania* (*Leishmania*) *infantum chagasi*, A2 gene, allele, sequencing.

Resumo

A leishmaniose visceral (LV) é uma zoonose amplamente disseminada, causada no Brasil pela *Leishmania* (*Leishmania*) *infantum chagasi*. Flebotomíneos vetores adquirem o agente etiológico, alimentando-se do sangue de animais contaminados, como cachorros ou animais selvagens. A doença é endêmica no Brasil, e focos de epidemia são relatados em cidades densamente povoadas por todo o país. Muitas manifestações clínicas relacionadas à infecção por *Leishmania* estão ligadas à relação parasito-hospedeiro, e vários possíveis fatores de virulência dos parasitas, que causam a LV, são alvos de estudo, tais como os genes A2. O gene A2 foi isolado pela primeira vez em 1994 e, em seguida, em 2005, três novos alelos foram descritos em *Leishmania* (*Leishmania*) *infantum*. No presente estudo, um fragmento do gene A2 de uma população clonal de *L.* (*L.*) *infantum chagasi* foi amplificado por PCR e sua sequência de nucleotídeos determinada. O fragmento mostrou 90% de similaridade com alelos do gene A2 de *Leishmania* (*Leishmania*) *donovani* e de *L.* (*L.*) *infantum*, descritos na literatura. Entretanto, a tradução da sequência de nucleotídeos mostra diferenças na sequência de aminoácidos da proteína, que podem ser essenciais em determinar a variabilidade do gene A2 em espécies do complexo *L.* (*L.*) *donovani* e representa uma ferramenta adicional na compreenssão do papel dessa família de genes na virulência e imunidade da leishmaniose visceral. O conhecimento dessa variação é importante para o desenvolvimento de testes diagnósticos mais precisos e ferramentas mais eficazes no controle da doença.

Palavras-chave: *Leishmania* (*Leishmania*) *infantum chagasi*, gene A2, alelo, sequenciamento.

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Introduction

Visceral leishmaniasis (VL) is widespread in tropical and subtropical areas of Latin America, Europe, Africa and Asia. In the New World, the disease is caused by *L.* (*L.*) *infantum chagasi* being the sandfly *Lutzomyia longipalpis* its main vector (MELO et al., 2009). Peridomestic sandflies acquire the etiological agent *L.* (*L.*) *infantum chagasi* – which some authors consider to be the same species as *L.* (*L.*) *infantum* (MAURÍCIO et al., 1999) –, by feeding on blood of infected reservoir animals such as dogs or wildlife. Infected sandflies represent great danger to surrounding human populations because transmission to non-infected hosts can cause severe disease that may be fatal if not promptly treated (TESH, 1995). In Brazil, canine visceral leishmaniasis (CVL) has become a serious public health problem due to the growing number of urban cases and new foci (SILVA et al., 2001; GONTIJO; MELO, 2004; NASCIMENTO et al., 2008). From the epidemiological standing point, CVL is more important than human disease due to its higher prevalence and greater number of parasites in the skin, which can favors vector infection (TRAVI et al., 2001; VERÇOSA et al., 2008).

Clinical signs in CVL depend on the immune response, which is directly associated to the host's genetic factors. Humoral and cellular immune response are involved, and severity and clinical manifestations depends on a balance between these two systems, parasite tropism and virulence (CIARAMELLA et al., 1997; HONORE et al., 1998; GARIN et al., 2001). Among candidate virulence factors in parasites that cause VL are the A2 genes (ZHANG; MATLASHEWSKI, 1997, 2001).

A2 genes consist of a family of genes that are abundantly transcribed and translated during the amastigote phase in leishmanias from the Donovani complex whereas their messenger RNA and protein are not present in the promastigote phase (CHAREST; MATLASHEWSKI, 1994; GHEDIN et al., 1997; ZHANG; MATLASHEWSKI, 2001; FARAHMAND et al., 2008). The A2 genes of *L.* (*L.*) *donovani* are composed predominantly of a sequence encoding 10 amino acids that may be repeated 40 to 90 times depending on each specific gene (CHAREST; MATLASHEWSKI, 1994; GHOSH; ZHANG; MATLASHEWSKI, 2001).

A2 genes share similarities with the S antigen expressed by *Plasmodium falciparum* (CHAREST; MATLASHEWSKI, 1994), and a possible role for the structure/functionality of A2 genes in the cutaneous or visceral tropism of *Leishmania* parasites in *L. major* and *L.* (*L.*) *infantum* has been studied and suggested (GHEDIN et al., 1997; ZHANG et al., 2003; GARIN et al., 2005). Species that cause Old and New World VL, *L.* (*L.*) *donovani*, *L.* (*L.*) *infantum*, and *L.* (*L.*) *infantum chagasi*, and New World cutaneous leishmaniasis (CL), *L. mexicana* and *L. amazonensis*, have A2 genes in their chromosomes (GHEDIN et al., 1997). In *L. major* A2 is considered a pseudogene, and the introduction of *L.* (*L.*) *donovani* A2 gene in this species allowed its survival in visceral organs of mice (ZHANG et al., 2003). The gene was isolated and expressed for the first time in *L.* (*L.*) *donovani* (CHAREST; MATLASHEWSKI, 1994, ZHANG et al., 1996). In 2005, three new alleles were described in *L.* (*L.*) *infantum* (GARIN et al., 2005). There is evidence showing that A2 are stress response proteins, important to *L.* (*L.*) *donovani* survival in visceral organs (McCALL; MATLASHEWSKI, 2010). Sera from humans and dogs naturally infected with *L.* (*L.*) *infantum chagasi* reacted in a serological assay using A2 recombinant proteins (GHEDIN et al., 1997; CARVALHO et al., 2002).

The present study describes an A2 allele found in a *L.* (*L.*) *infantum chagasi* strain, isolated from a dog with VL treated in the Veterinary Hospital of Universidade Estadual Paulista in the city of Jaboticabal, São Paulo, Southeastern Brazil. We amplified, cloned, and sequenced a 743 bp fragment. The amplified fragment showed approximately 90% similarity with another A2 allele amplified in *L.* (*L.*) *donovani* (CHAREST; MATLASHEWSKI, 1994) and *L.* (*L.*) *infantum* (GARIN et al., 2005).

Material and Methods

1. L. (L.) infantum chagasi strain

A strain of *L.* (*L.*) *infantum chagasi* was isolated from a dog's bone marrow sample from the town of Olympia, São Paulo. The animal was examined at the Veterinary Hospital of FCAV-UNESP, Jaboticabal, São Paulo, with clinical symptoms of CVL and the strain obtained was used to isolate the A2 gene. The parasites were maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Canyon City, USA) at 25 °C and strain characterization was made by polymerase chain reaction (PCR) and restriction fragment length polymorfism (PCR-RFLP) technique (CORTES et al., 2004; ANDRADE et al., 2006).

2. DNA extraction

L. (*L.*) *infantum chagasi* genomic DNA extraction was adapted from a proteinase K extraction previously described (CLER et al., 2006). Briefly, promastigotes from axenic culture were harvested and then resuspended in 350 µL of TE buffer (Tris 10 mM; EDTA 1 mM, pH 8.0) and 20 µL of SDS 10%. Ten µL of proteinase K (10 $\mu g.\mu L^{-1}$) were subsequently added, and microtubes were incubated for 36 hours at 37 °C. After incubation, DNA was extracted with a standard procedure (phenol/chloroform/isoamilic alcohol) (25:24:1) and DNA precipitation was done using NaCl/ethanol procedure (SAMBROOK; FRITSCH; MANIATIS, 2001). The extracted DNA was dissolved in 20 µL of sterile water.

3. PCR

Amplification of *L.* (*L.*) *infantum chagasi* DNA (50 ng) matrix was done using L2/R3 primers(5'-TTGGCAATGCGAGCGTCACAGTC/5'-CAACGCGTACGATAATGCCACA) that correspond to the 5' end position 16301 and 16603 of the reverse complementary strand of the AC010851 sequence, respectively (GARIN et al., 2005). In addition to DNA, PCR was performed in a reaction mixture of 25 µL containing 2.5 µL PCR buffer 10× (200 mM

Tris-HCl, pH 8.4, 500 mM KCl), 3 mM MgCl2, 200 µM each dNTP (dTTP, dATP, dGTP, dCTP, Eppendorf), 0.5 µM of each primer (Invitrogen, Carlsbad, USA) and 1 U recombinant Taq polymerase (Invitrogen, Carlsbad, USA). L2/R3-PCR conditions consisted of denaturation for 3 minutes at 94 °C, followed by 35 amplification cycles at 94 °C for 1 minute, 1 minute at 58 °C, 1 minute at 72 °C, then one cycle at 72 °C for 5 minutes. A total of 5 µL of PCR product was electrophoresed in 1% agarose gel in the presence of ethidium bromide, and visualized under UV light. A 100 bp ladder (Invitrogen, Carlsbad, USA) was used as MW marker.

4. DNA libraries and sequencing

L2/R3-PCR products were purified with GeneClean II (QbioGene, 1001-400, Carlsbad, USA). The insertion of PCR products into pGEM-T Easy Vector Systems (Promega, Madison, USA), transformation of *Escherichia coli* DH10B competent cells, and cloning were performed using a PCR Cloning Kit (Promega, Madison, USA) as described by the manufacturer. One hundred and ninety-two clones were obtained and two A2-containing clones identified by sequencing. The plasmid DNA was extracted by alkaline lysis (SAMBROOK; FRITSCH; MANIATIS, 2001) and sequenced by the Sanger method with M13 forward and M13 reverse (Promega, Madison, USA) primers set using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems, Courtaboeuf, France) on a ABI PRISM 3100 DNA Analyzer (Applied Biosystems, Courtaboeuf, France).

5. Sequence analysis

Sequences obtained were then analyzed by Phred (EWING et al., 1998) and cross-match (www.phrap.org) algorithms to perform base calling and trimming (Q ≥ 20) and to mask cloning vector bases, respectively. After this filtering process the sequences were submitted to BlastN and BlastX (ALTSCHUL et al., 1990) analysis against non-redundant (nr) NCBI database and the *L.* (*L.*) *infantum* v3.0a database (ftp://ftp.sanger.ac.uk/pub4/pathogens/L_infantum/ DATASETS/). The *L.* (*L.*) *infantum chagasi* A2 nucleotide sequence was translated by dna2pep algorithm (http://www.cbs.dtu.dk/services/VirtualRibosome/) and we took the same translation frame that was observed on BlastX best hits.

Global multiple alignments for DNA and protein sequences of the identified *L.* (*L.*) *infantum chagasi* A2 gene, three A2 alleles from *L.* (*L.*) *infantum* (GARIN et al., 2005) and a *L.* (*L.*) *donovani* A2 gene (CHAREST; MATLASHEWSKI, 1994) were performed using ClustalW algorithm (THOMPSON; HIGGINS, 1994) and edited by BoxShade (http://www.ch.embnet.org/software/BOX_form.html). The identity percentages among sequences on global multiple alignments were measured by the *alistat* algorithm included in the HMMER package (DURBIN et al., 1998).

6. GeneBank accession numbers

The accession number for the A2 gene isolated in *L.* (*L.*) *infantum chagasi* described in this study is [GenBank:GQ290460]; the accession numbers for *L.*(*L.*)*infantum* A2 alleles II, III, and IV are [GenBank:AY255807, GenBank: AY255808, and GenBank: AY255809], respectively (GARIN et al., 2005), and the accession number for *L.* (*L.*) *donovani* is [GenBank:S69693] (CHAREST; MATLASHEWSKI, 1994).

Results and Discussion

Direct sequencing and analyses of L2R3 PCR products from crude genomic DNA of *L.* (*L.*) *infantum chagasi* could not be performed. These PCR products resolved in a pattern of multiple bands (Figure 1), and thus A2 sequence was obtained from clone libraries as described in Methods.

An identical A2-gene sequence of 743 nucleotides was isolated in two clones from the genomic library of *L.* (*L.*) *infantum chagasi*. BlastN analysis of this new sequence and other four different VL A2 alleles, against all annotated CDSs from *L.* (*L.*) *infantum* genome version 3.0a, showed gene LinJ22_V3.0670 as the best hit for all five query sequences. Our sequence showed 95% similarity with this gene; alleles isolated from *L.* (*L.*) *infantum* showed 97% (Type II), 91% (Type III), and 92% (Type IV) similarity; and *L.* (*L.*) *donovani* A2 allele showed 87% similarity (Table 1). However, considering only the coding region *L.* (*L.*) *infantum chagasi* has the same stop codon TAA present in *L.* (*L.*) *infantum* (Type II, III, and IV) (Figure 2), the sequence translated to an amino acid sequence has 342bp and is 98,9% identical to *L.* (*L.*) *infantum* (Type II) (Figure 3).

Since the first description of A2 gene, the existence of at least five closely related genes (A2 series) has been investigated (CHAREST; MATLASHEWSKI, 1994; ZHANG; MATLASHEWSKI, 2001). Zhang et al. (1996) described A2 as a multigene family, which expresses different sizes of 10-amino acid repeats. Three different A2-gene alleles in a genetically pure parasite clonal lineage (*L. infantum* MHOM/FR/92/LEM 2385 clone-1) were subsequently isolated, providing additional evidence of the existence of multiple A2 genes (GARIN et al., 2005) and a different A2 sequence is here described in *L.* (*L.*) *infantum chagasi*. Allele types I, II, and III described in the pure clonal lineage

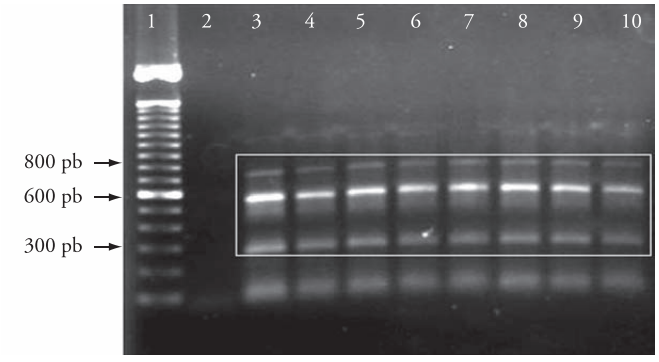

Figure 1. PCR electrophoresis patterns. Electrophoretic pattern of PCR products obtained from *L.* (*L.*) *infantum chagasi* genomic DNA. Each slot corresponds to a single sample amplified using the same PCR mix. The framed area corresponds to the expected sizes for the A2 gene.

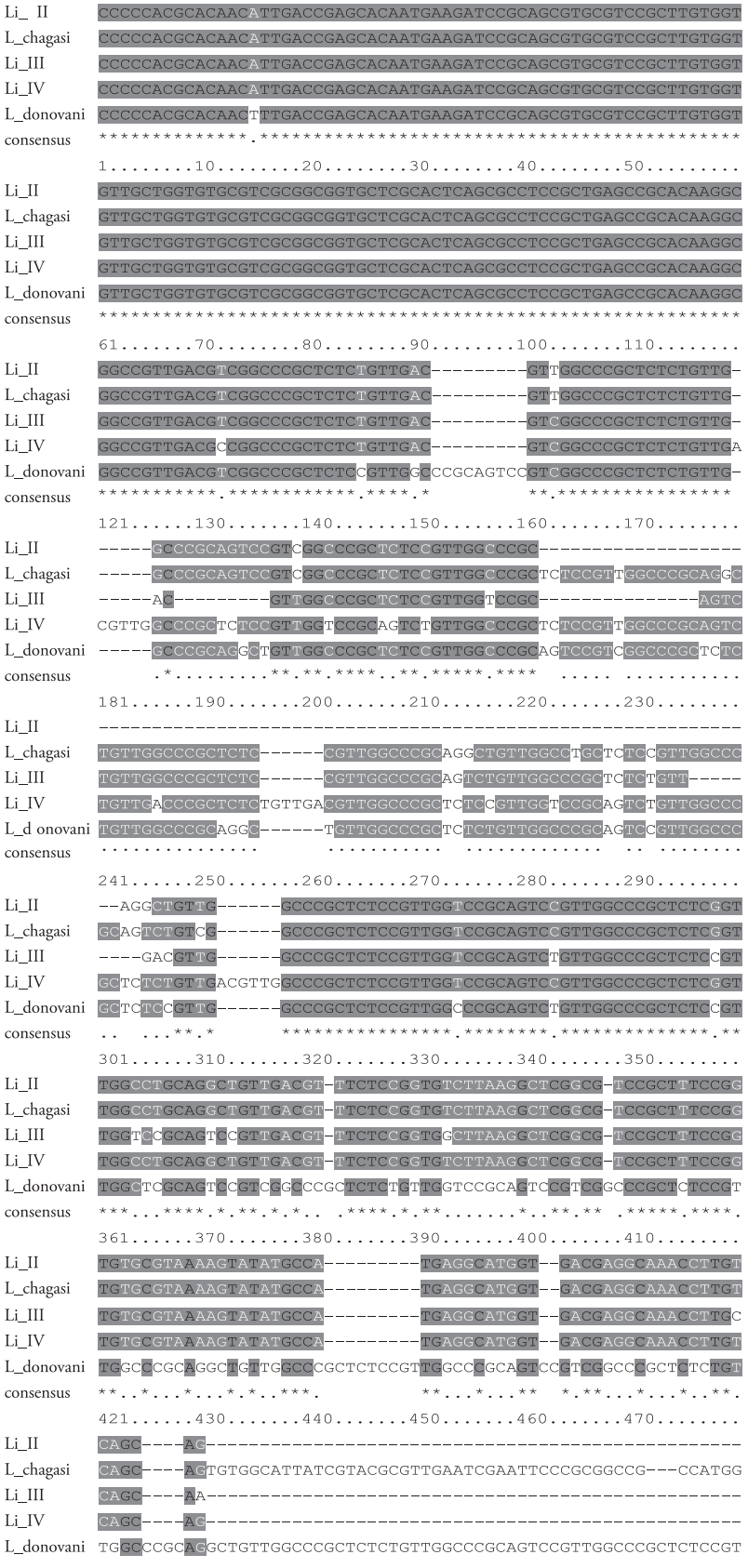

Figure 2. Multiple alignment of A2 nucleotide sequences from *L.* (*L.*) *infantum* (allele types II, III, and IV), *L.* (*L.*) *donovani* and *L.* (*L.*) *infantum chagasi*.

Table 1. BlastN analysis of different A2 alleles. BlastN analysis of five different VL A2 alleles against all annotated CDSs in *L.* (*L.*) *infantum* genome version 3.0a. The LinJ22_V3.0670 gene was the best hit for all five query sequences. Queries Li_II, Li_III, and Li_IV are *L.* (*L.*) *infantum* A2 alleles described by Garin et al. (2005), and query *L. donovani* is the A2 gene from *L.* (*L.*) *donovani* described by Charest and Matlashewski (1994), and query *L. chagasi* is the V allele herein described.

Query id	Subject id	% identity	Alignment length	q. start	q. end	s. start	s. end	e-value	Bit
Li_II	LinJ22_V3.0670	97	202	29	230	1	202	2,00E-97	353
Li_III	LinJ22_V3.0670	91	274	29	302	1	253	1,00E-109	392
Li_IV	LinJ22_V3.0670	92	340	29	368	1	319	1,00E-141	500
L_donovani	LinJ22_V3.0670	87	574	29	590	1	556	1,00E-165	579
L_chagasi	LinJ22_V3.0670	95	194	54	247	1	193	3,00E-85	313

(q.start: start of query sequence; q. end: end of query sequence; s. start: start of sequence; s.end: end of sequence).

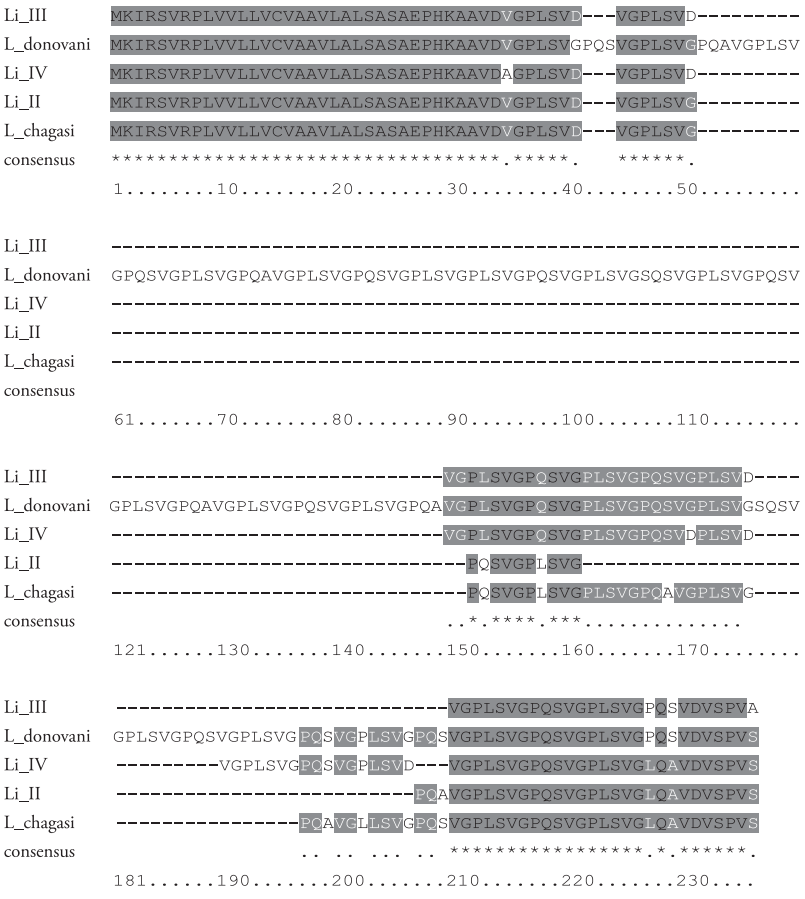

Figure 3. Multiple alignments of A2 amino acid sequences (translated nucleotide sequences by dna2pep algorithm.) from *L.* (*L.*) *infantum* (allele types II, III, and IV), *L.* (*L.*) *donovani* and *L.* (*L.*) *infantum chagasi*.

L. (*L.*) *infantum* MHOM/FR/92/LEM 2385 clone-1 differ only in the number and arrangements of the repeated motifs at the 3' end variable region of the gene (GARIN et al., 2005) and in all cases, only strains from the *L.* (*L.*) *donovani* complex causing VL in Old World were sequenced.

In the present study, we used a clinical isolate of a *L.* (*L.*) *infantum chagasi* infected dog to identify and sequence an A2 gene copy, corroborating previous findings that the A2 family of VL species is a multiple gene family.

The present study evidenced a divergent A2 gene in the *L.* (*L.*) *infantum chagasi* strain when compared to the *L.* (*L.*) *infantum* and *L.* (*L.*) *donovani* A2 alleles first identified. A comparative analysis of the A2 gene isolated from *L.* (*L.*) *donovani*, three A2-gene alleles isolated from *L.* (*L.*) *infantum* (Type II, III, and IV), and the A2 gene isolated from *L.* (*L.*) *infantum chagasi* herein described showed that the 5' ORF is highly preserved (Figure 2) in both nucleotide and amino acid sequences obtained by nucleotide sequence translated by dna2pep algorithm. At the protein level, the sequences aligned had an identity average of 93%, but this analysis showed few differences among the sequences that can cause great changes in protein function. Close to 170 and 200 positions of the alignment ruler (Figure 3) there is an alanine residue in *L.* (*L.*) *infantum chagasi* sequence instead of a serine residue present in the other A2 sequences analyzed.

The *L.* (*L.*) *donovani's* A2 protein shows immunogenicity. Significant protection against *L.* (*L.*) *donovani* and *L. amazonensis* infections associated with both humoral and cellular immune responses has been seen when using A2 as a recombinant protein or a DNA vaccine in BALB/c mice, and more recently in dogs (GHOSH; ZHANG; MATLASHEWSKI, 2001; COELHO et al., 2003; ZANIN et al., 2007; RESENDE et al., 2008; FERNANDES et al., 2008). A recombinant *L.* (*L.*) *tarentolae* expressing the *L.* (*L.*) *donovani*-specific A2 protein used as a live vaccine against *L.* (*L.*) *infantum* infection in BALB/c mice has provided evidence of favorable immune response and significant levels of protective immunity against *L.* (*L.*) *infantum* infections (MIZBANI et al., 2009).

This study evidences that there are differences in nucleotide sequences, and possible in amino acid of A2 protein sequences, which can be essential to establish specific pathogenic pathways in *Leishmania* species. In two positions of the C-terminal region on the A2 protein multiple alignment, *L.* (*L.*) *infantum chagasi* has an alanine residue while the other A2 sequences have a serine residue. This change might cause changes in three-dimensional structure of the protein and consequently in its function. Phenotypic effects of the alteration are more drastic, according to the differences in the chemical nature of side chains of amino acid residues, the substitution of an amino acid hydrophilic by one amino acid with hydrophobic side chain of residue, as here described, can cause great changes in functional activity of the protein (NELSON; COX, 2006). Since multiple A2 sequences of varying lengths were observed in different VL isolates by our group and by others, our results may help to determine the variability of A2 genes in the species of the *L.* (*L.*) *donovani* complex and represents an additional tool to help us understand the role this family of genes may have in establishing virulence in visceral leishmaniasis. The exact function of A2 protein in *Leishmania* is still unknown (AZIZI et al., 2009). Gene silencing of A2 in *L.* (*L.*) *donovani* dramatically decreased its viability and pathogenicity in mammalian macrophages (ZHANG; MATLASHEWSKI, 1997). Overexpression of A2 in *L. major* resulted in visceralization of infection (ZHANG; MATLASHEWSKI, 1997; GARIN et al. 2005) and A2 gene was the only virulence factor that was not found in non-pathogenic parasites to human *L.*(*L.*) *tarentolae* (AZIZI et al., 2009), further supporting A2 gene importance in the pathogenesis of leishmaniasis. In Brazil, visceral leishmaniasis is caused by *L.* (*L.*) *infantum chagasi*, and the evidenced differences among A2 genes in species are important to elucidate the role of this gene in VL, which is essential for the development of more accurate diagnostic tests and effective tools for disease control. Studies with southern blotting and protein expression are being conducted with A2 gene isolated in *L.* (*L.*) *infantum chagasi* to confirm and better understand the changes found.

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