

In Silico Identification of B-Cell Epitopes of *Leishmania infantum* Recombinant Histone Shared with Human Sera Stably Living in Area Where *Leishmania* Species Does Perpetuate

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Abstract

Visceral leishmaniasis (VL) can initially be misdiagnosed because its presentation is similar to many autoimmune diseases such as systemic lupus erythematosus (SLE), autoimmune hepatitis and dermatomyositis. Furthermore, serum antibodies from VL patients have been shown to strongly react against proteins that are conserved between the causative agent, *L. infantum*, and humans themselves. Some of these proteins, like histone, have also been described as immunogenic in several auto-immune syndromes, and the detection of antibodies against them is considered to be indicative of immune system disorders.

The potential overlap of autoimmune diseases and VL presents a situation of confounding diagnoses if cross-reactive tests are used. To explore this possibility, we screened sera from three Tunisian populations for the presence, and relative quantity, of antibodies against a panel of *L. infantum* antigens comprising crude extract or recombinant molecules, with special attention being given to evolutionarily conserved histones. Our data indicate that antibodies in many of the SLE at-risk individuals recognized crude soluble *Leishmania* antigen (SLA). This compromised the specificity of SLA-based ELISA, providing many results falsely indicating *L. infantum* infection. Examination of the crude *Leishmania* histone (CLH) mixture, which is expected to contain nucleosomal *Leishmania* histones H2A, H2B and H4, as well as recombinant versions of these *Leishmania* histones, suggested these to be a source of the cross-reactivity. For the purposes of diagnosing VL, it is therefore important to note that the rK39 antigen was found to be more specific and not conflicting with autoimmune presentations. *In silico* prediction data validate and indicate that the human histones are immunologically cross-reactive with *Leishmania* histones.

Keywords: Systemic lupus erythematosus; Associated autoantigen panels; Autoantibodies; *Leishmania* histones; Autoimmune diseases

Introduction

Visceral leishmaniasis (VL) is a severe, life-threatening neglected tropical disease caused by infection with the vector-borne parasites *Leishmania donovani* and *L. infantum*. The vast majority of cases are reported in developing countries and VL is considered to be a disease of poverty [1]. In Europe, Central Asia and North Africa and West, *L. infantum* species are responsible for VL. *L. infantum* infection may be asymptomatic (subclinical), or may emerge to cause VL [2]. The disease occurs mainly in young people and in immuno-compromised individuals [3,4]. VL diagnosis can be attained directly through the visualization of parasites by microscopic examination in spleen or bone marrow cell aspirates, and complemented by detection of *L. infantum* DNA through polymerase chain reaction (PCR)-based assays in the blood [5]. Alternatively, diagnosis can be reached by detection of parasite-specific antibodies in the enzyme-linked immunosorbent assay (ELISA)-based [5,6]. While some *L. infantum* antigenic proteins have emerged as signatures of asymptomatic parasitism, others such as the recombinant kinesin 39 (rK39) have emerged as an indicator of active disease. Indeed, rK39 has proven to be a very sensitive and specific antigen for the sero-diagnosis of VL [7-10].

In our previous studies, in addition to soluble *Leishmania* antigens (SLA), we identified evolutionarily conserved *Leishmania* histones as having potential to complement antibody-based tests for both human and canine VL. These proteins were identified using sera from VL-affected dogs [11-14], with immunoscreening identifying antibodies binding *L. infantum* histone H2A, as well as H2B and H4 [14] that belong to the nucleosomal core of *L. infantum*. In addition, antibodies against recombinant (r)H2A and rH2B were detected in the sera of many Tunisian VL patients [8,15]. VL patients presenting with clinical and laboratory features that mimic autoimmune hepatitis, primary biliary cirrhosis [16,17] and systemic lupus erythematosus (SLE), could therefore be mistakenly misdiagnosed. Like rheumatic disease *Leishmania*-infected patients have higher level of circulating immune complexes and autoantibodies like rheumatoid factor and hypergammaglobulinemia and higher titers of antinuclear antibodies (ANA) observed in systemic autoimmune diseases in particular SLE [18-20].

Apparently healthy subjects at risk of developing autoimmune diseases reside in areas within Tunisia where *L. infantum* and blood-feeding phlebotomine sandflies are endemic, thereby also presenting risk for infection and development of VL [21]. To explore this, we screened sera from three Tunisian populations for the presence, and relative quantity, of antibodies against a panel of *L. infantum* antigens comprising crude extract or recombinant molecules, with special attention being given to evolutionarily conserved histones.

Patients and Methods

Serum samples

All analyses were conducted in compliance with the current laws of Tunisia, the country where the study was conducted. The study was retrospective, so no other requirements were required relating to the subjects from whom the samples originated. The ANA positive sera analyzed in this work are part of clinical practice-based laboratory specimens of the Laboratory of Clinical Immunology, Pasteur Institute of Tunis, Tunisia.

Three panels of sera were screened, with all samples provided from Tunisian subjects. The first panel (n=42) served as a control group (1) and was prepared from healthy pregnant women living in North Tunisia, an area where *L. infantum* is endemic. These sera were initially collected for the purpose of defining their status with respect to colonization (or not) by *T. gondii* through the presence of *Toxoplasma gondii*-binding antibodies. All of these sera had ANA titers <1/80 and did not possess extractable nuclear antigens (ENA)-binding autoantibodies as determined by indirect immunofluorescence on Hep-2 cells (DiaSorin Inc., USA).

A second panel (n=42) served as an auxiliary control group (2) formed from samples of individuals from both VL-endemic and non-endemic regions. It was kindly provided by national blood transfusion center (NBTC) and was prepared from voluntary, healthy adults blood donors.

Sera in panel also had ANA titers <1/80 and did not possess extractable nuclear antigens (ENA)-binding autoantibodies as determined by indirect immunofluorescence on Hep-2 cells. The third panel (n=80) was prepared from healthy adults considered at risk of developing Systemic Lupus Erythematosus (SLE) episodes. Each sample in this panel was assessed by the Clinical Immunology Laboratory of Institut Pasteur de Tunis for the presence of ANA-binding antibodies. Each presented ANA titers ≥ 80 by indirect immunofluorescence on Hep-2 cells.

Antigens

Soluble *Leishmania* Antigen/SLA was prepared from *L. infantum* promastigotes after ultrasonic treatment, as described previously [22,23]. Crude *Leishmania* Histones (CLH) were extracted according to a standard approach of histone protein isolation [24] with some modifications [23,24]. rK39, a recombinant product of the 39 amino acid repeats found in a kinesin-like gene of viscerotropic *Leishmania* species [25], was kindly provided by Dr. Steven G. Reed, infectious disease research institute (IDRI), Seattle, WA. *L. infantum/chagasi* derived recombinant proteins rH2A, rH2B, and rH4 were kindly provided by Dr. Manuel Soto Manuel Centro de Biología Molecular "Severo Ochoa", Facultad de Ciencias, Universidad Autónoma de Madrid, Madrid, Spain [11-14].

Detection of antigen-specific antibodies by enzyme linked immunosorbent assay (ELISA)

Antibodies against CLH, SLA and rK39 were detected by ELISA, as described previously [23]. Antibodies against rH2A, rH2B and rH4 were detected as follows. ELISA were optimized for antigen coating concentration (rH2A [10 µg/ml], rH2B [5 µg/ml] and rH4 [5 µg/ml]), each serum was diluted 1/100 and antibodies detected with horseradish peroxidase-conjugated anti-human IgG diluted 1/10,000.

The optical density (OD) of each well was read at dual wavelengths (492 nm and 620 nm) by ELISA plate reader (Anthos 2020, Biochrom Ltd., United Kingdom). For each serum, the mean OD value was calculated from duplicate wells. The cut off threshold values for positive responses against each antigen were defined as the mean OD plus two standard deviations of the OD obtained with sera from control group (1). OD thresholds values for SLA, rK39 and CLH were set to 0.912, 0.394 and 0.339, respectively. The cut off values for rH2A, rH2B, and rH4 were 0.559, 0.653, and 0.251, respectively.

Detection of subjects at risk of developing SLE

Serum ANA levels were measured using ANA screen 9 kit (Euroimmun, Lübeck, De) using the manufacturer's suggested cut-off to define positive responses [1]. Then, antibodies recognizing Extracted Nuclear Autoantigens (ENA) (consisting of Sm, RNP, SS-A, SS-B, Scl-70, RNP, Jo-1, centromeres, and ribosomal P proteins) were detected with the ENA Profile 1 Euroline (Euroimmun, Lübeck, De).

Mammalian histones-binding antibodies were screened with an anti-histones ELISA (IgG) (Euroimmun, Lübeck, De), with values >20 IU/mL considered as positive. These assays allow detecting antibodies that bind the following antigens: PCNA, PM-Scl, ribosomal P proteins, nRNP/Sm. The cutoff was ≥ 20 RU/ml. Native double-stranded DNA (dsDNA) binding antibodies were detected by indirect immunofluorescence using *Crithidia luciliae* as substrate (Euroimmun, Lübeck, De). Titers ≥ 20 were considered positive.

Bioinformatics analysis

To identify conserved and divergent amino acid sequences between *Leishmania infantum* histones H2A (access number: CAD11893.1), H2B (access number: CAA73826.1), H3 (access number: CAA54693), and their human homologs we used the BLAST server NCBI (<http://blast.ncbi.nlm.nih.gov>).

Each blast focused on *Homo sapiens* (taxid: 9606). The selected sequences identical to histone proteins of *L. infantum* were histone H2A type 1-A (accession number NP_734466.1), histone H2B type 1-L (accession number NP_003510.1), and histone H4 (accession number NP_003529.1). Protein sequence alignment of human and *Leishmania-chagasi* histones were rendered with T-COFFEE.

(M-Coffee; <http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee>) and EMBOSS Needle EMBL-EBI (http://www.ebi.ac.uk/Tools/psa/emboss_needle/).

Antigenic peptides were predicted with an accuracy of 75% using the method described by Burns [26], with the software available at <http://imed.med.ucm.es/Tools/antigenic.pl> (Complutense University of Madrid). Using this software sequence peptide segment is considered significant if it has a minimum of 8 amino acids.

Statistical analysis

Statistical analysis was performed using Prism Version 5 software (Graph Pad Prism Inc., San Diego, CA). Differences in antibody levels between the control groups were assessed using the Kruskal-Wallis test with Dunn post-test. Differences between two groups were compared with Mann Whitney of t-test (non-parametric tests). A p-value of <0.05 was considered significant.

Results

Circulating antibodies against host antigens

The sera prepared from subjects at risk of developing SLE episodes, as indicated by the presence of ANA-binding autoantibodies (n=80, ANA titers ≥80) were tested for the presence of ENA-binding antibodies. All of the ANA-positive samples had antibodies against one or more ENA, particularly SS-A, SS-B, Ro52, RNP, Anti-Jo, Sm and NUC, as well as mammalian histones (Table 1).

Ten percent (8 of 80) of the ANA-positive sera also had detectable anti-DNA antibodies, while 37.5% (30 of 80) had antibodies that bound mammalian histones. In contrast, no ENA- or dsDNA-binding antibodies were detected in either of the control groups. Together, these data indicate the broad range of antibodies against host antigens in individuals at risk of developing autoimmune disease.

Patient code	ANA	dsDNA	ENA (ND)	Number of cases with absorbance mammalian
	IF			Histones value (>20 IU/mL)
P2, P9, P32, P51, P71, P78	+	+	ND	N=6
P11, P12, P14, P22, P26, P34, P35, P36, P39, P46, P49, P50, P56, P58, P63, P69, P70, P73, P77, P79.	+	-	ND	N=20
P18, P44, P52, P68	+	-	SS-A, RNP, Sm; NUC; SS-A; SS-A, Ro52.	N=4
P1, P3, P4, P5, P6, P7, P8, P10, P15, P16, P19, P20, P23, P24, P25, P27, P28, P29, P31, P33, P37, P38, P40, P41, P45, P47, P48, P55, P59, P60, P61, P64, P65, P66, P67, P74, P75, P76.	+	-	ND	N=38
P17, P30, P42, P43, P54, P62, P72, P80	+	-	SS-A, SS-B, Ro52; SS-A, SS-B, Ro52; SS-A, Anti-Jo, Ro52; SS-A, Ro52; RNP, Ro52; Scl 70; SS-A, SS-B, Ro52 ; SS-A, SS-B+/-, Ro52	N=8
P21	+	+	SS-A, SS-B	N=1
P53, P57	+	+	ND	N=2

IF Indirect immunofluorescence, dsDNA double stranded Deoxyribonucleic acid, Sm Smith, SS-A SS-A antigen, SS-B SS-B antigen, RNP Ribonucleoprotein, Scl-70 Scleroderma 70 kDa antigenic protein, Jo-1 Histidyl-tRNA synthetase, NUC Nucleoproteins, mammalian histones (IU/mL) Right column, values above cutoff /threshold are indicated in bold (cutoff ≥20 IU/mL), ND Not Done.

Table 1: Profile of antibodies against extractable nuclear antigens (ENA) and mammalian histones in samples that were ANA-positive.

Circulating antibodies against *Leishmania* antigens

The potential overlap of autoimmune diseases and VL presents a situation of confounding diagnoses if cross-reactive tests are used. We first explored the possibility that samples from individuals at risk of developing SLE due to the presence of antibodies against human histones had antibodies that also reacted with *Leishmania* histones. To determine the apparent *L. infantum* infection status of our subjects, sera were also evaluated for antibodies against a crude mixture of *L. infantum* antigens (SLA). Results indicate that 40% of SLE patients, but only 1 sample from the VL-endemic region and none from the non-endemic region, possessed antibodies that could bind SLA (Figure 1A).

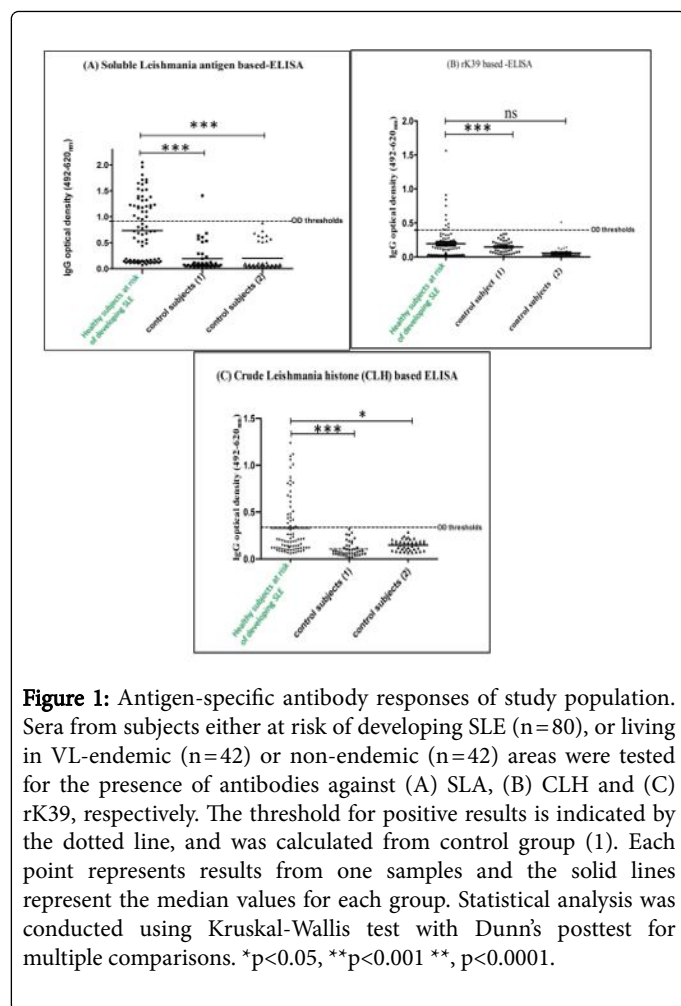
While none of the control samples, be they from VL-endemic or non-endemic regions, reacted with CLH, 30% of the SLE patients presented with positive responses against CLH (Figure 1B). When samples were assessed against the much more refined recombinant rK39 antigen, the proportion of SLE patients indicated to have *L. infantum* infection dropped to 13.75% (Figure 1C). Within the panel of

sera from individuals at risk of SLE, 30% (24 of 80) had detectable antibodies against Crude *Leishmania* Histones (CLH).

Of these 15 anti-CLH positive sera, 9 contained SLA-binding antibodies and 6 contained rK39-binding antibodies (Table 2). Moreover, these 15 sera also relatively high levels of antibodies against mammalian histones, as well as a range of ENA-binding autoantibodies (Table 2). Together, these data support our hypothesis that individuals at risk of developing autoimmune diseases have antibodies that cross-react with *L. infantum* antigens.

Antibody responses against *Leishmania* histones

To further explore this likely cross-reactivity, we investigated responses against recombinantly-expressed H2A, H2B and H4 that were generated using *Leishmania* gene sequences.

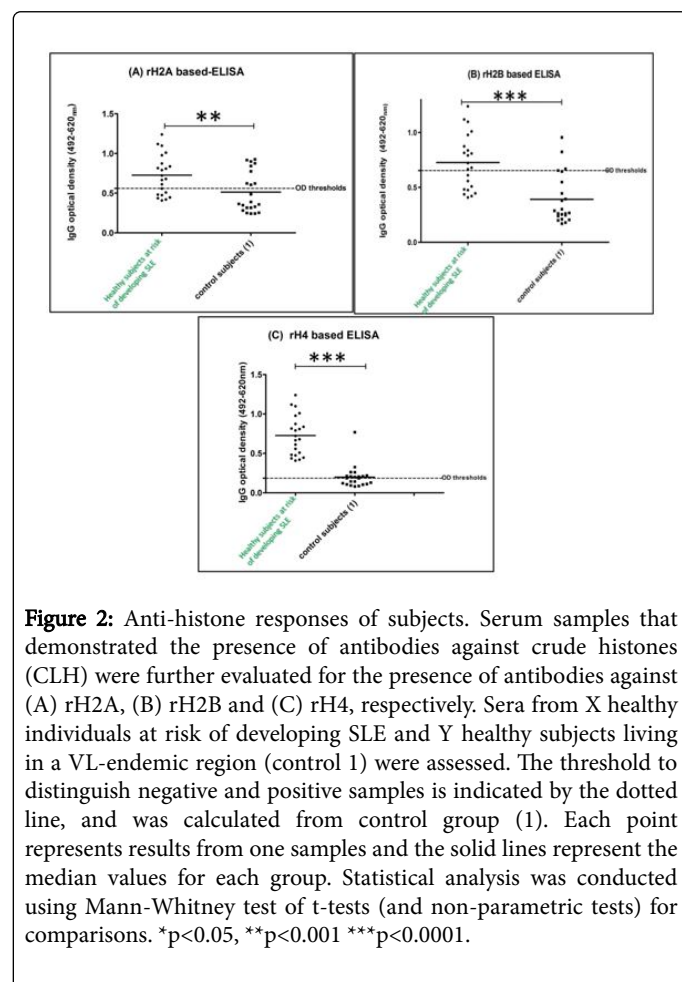


Sample code	Anti-mammalian histones (IU/mL)	CLH ¹	rH2A	rH2B	rH4	SLA	K39
P2	59.038	0.437	0.926	0.646	0.263	1.394	0.03
P9	123.654	0.611	0.876	0.394	0.081	0.802	0.174
P12	238.462	0.666	0.365	0.654	0.187	1.166	0.241
P14	79.423	0.475	0.606	0.445	0.213	0.967	1.561
p22	407.308	0.679	0.776	0.824	0.219	1.227	0.029
p26	106.731	0.446	0.843	0.2	0.209	1.312	0.03
p32	188.462	0.408	0.9	0.211	0.205	1.013	0.91
p35	21.154	0.791	0.244	0.956	0.086	1.716	0.336
p43	407.307	0.509	0.39	0.669	0.093	0.19	0.123
p44	48.269	0.558	0.359	0.302	0.185	1.212	0.753
p46	22.308	0.482	0.243	0.262	0.107	0.161	0.021
p49	72.308	1.119	0.317	0.288	0.129	1.218	0.843
p71	295.769	1.01	0.317	0.18	0.327	0.126	0.129
p73	100.577	0.978	0.282	0.247	0.201	0.103	0.015

p77	120.962	1.098	0.329	0.255	0.769	0.11	0.435
positive threshold 2	20	0.339	0.559	0.653	0.251	0.912	0.319
¹ LeishmaniaLeishmania							
2							

Table 2: Antigen-specific antibody profile of individuals at risk of developing SLE.

Among the 80 sera collected from subjects at risk of developing SLE episodes and containing ANA binding autoantibodies, 22 demonstrated antibodies against the crude histone mixture CLH and these were selected for evaluation. Positive and negative sera were identified, although antibodies against each of the recombinant antigens were detected in a majority of the SLE at-risk group, and at higher levels, relative to healthy controls (Figure 2).



Of the anti-CLH sera evaluated, rH2A yielded a positive response in 63.63% (Figure 2A), rH2B yielded a positive response in 59.09% (Figure 2B) and rH4 yielded a positive response in all (100%) (Figure 2C). Whereas responses against CLH in control subjects (1) were all below OD 0.4 (Figure 1), some subjects from the same group showed stronger responses against single proteins representing only a fraction of CLH (Figure 2). These data validate the in-silico predictions and

indicate that the human histones are immunologically cross-reactive with *Leishmania* histones.

Prediction of antibody-binding epitopes within histones

Genetic analysis indicates that the *L. infantum* H2B, H2A and H4 proteins and their human homologs are highly conserved. To make in silico predictions of the antibody-binding epitopes shared between *L. infantum* H2A (CAD11893.1), H2B (CAA73826.1) and H4 (CAA74210.1) and their respective human counterparts (histone H2A type 1-A, NP_734466.1; histone H2B type 1-L, NP_003510.1 and histone H4, NP_003529.1), blasts were conducted against human proteins (Homo sapiens, taxid: 9606).

The conserved region of recombinant H2A, H2B and H4 exhibits 42.1%, 23.8% and 52.9% identity relative to their human histone homologs, respectively. Interestingly, H2A displays 4 segmental epitopes with highly conserved amino acid sequences (residues 17-29, 45-72, 77-90, 96-119) (Figure 3), H2B displays 5 segmental epitopes with highly conserved sequence (residues 8-17, 23-35, 49-56, 61-71, 82-97) (Figure 4), and H4 displays 2 segmental epitopes (residues 16-23 (<8, non-sig), 42-67, 80-89, 43-49 (<8, non-sig) (Figure 5). These predictions suggest cross-reactivity between autoimmune and *L. infantum*-infected samples is likely.

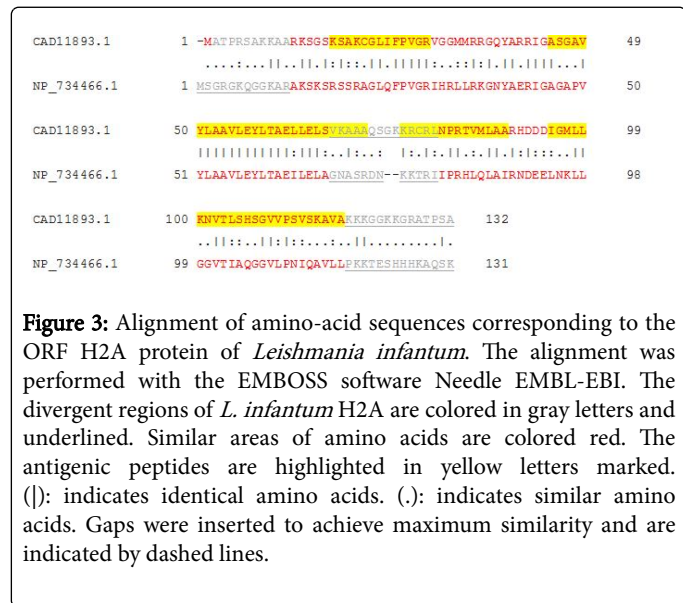


Figure 3: Alignment of amino-acid sequences corresponding to the ORF H2A protein of *Leishmania infantum*. The alignment was performed with the EMBOSS software Needle EMBL-EBI. The divergent regions of *L. infantum* H2A are colored in gray letters and underlined. Similar areas of amino acids are colored red. The antigenic peptides are highlighted in yellow letters marked. (|): indicates identical amino acids. (:): indicates similar amino acids. Gaps were inserted to achieve maximum similarity and are indicated by dashed lines.

Discussion

L. infantum is endemic in northern Tunisia and many Tunisians therefore reside in regions where zoonophilic blood-feeding sand flies and *Leishmania/L. infantum* co-exist. Although the burden of VL in North Africa is mainly in children aged between 1 and 4 years of age, VL can present clinical and laboratory autoimmune manifestations including arthralgia, cutaneous vasculitis, increased titers of rheumatoid factor (RF) and antinuclear antibodies (ANA) [27-29]. VL patients may initially be misdiagnosed as having an autoimmune disease such as SLE or autoimmune hepatitis, leading to the possibility that they could be treated with immunosuppressive drugs with fatal consequences [30].

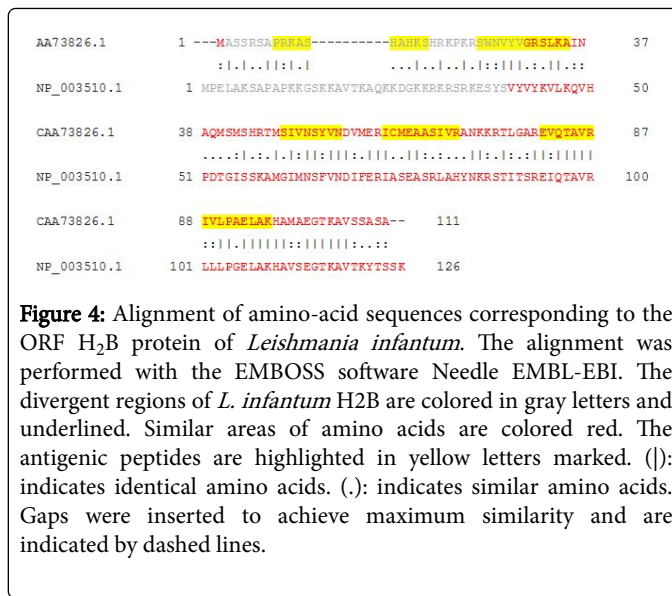


Figure 4: Alignment of amino-acid sequences corresponding to the ORF H₂B protein of *Leishmania infantum*. The alignment was performed with the EMBOSS software Needle EMBL-EBI. The divergent regions of *L. infantum* H2B are colored in gray letters and underlined. Similar areas of amino acids are colored red. The antigenic peptides are highlighted in yellow letters marked. (|): indicates identical amino acids. (:): indicates similar amino acids. Gaps were inserted to achieve maximum similarity and are indicated by dashed lines.

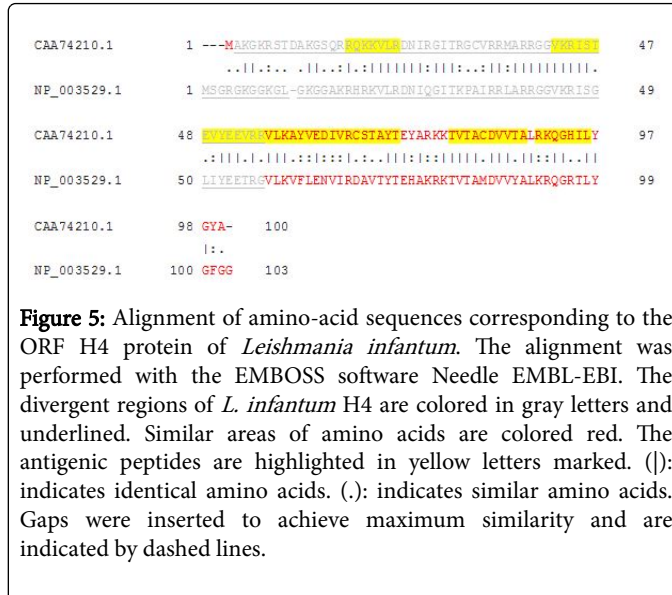


Figure 5: Alignment of amino-acid sequences corresponding to the ORF H4 protein of *Leishmania infantum*. The alignment was performed with the EMBOSS software Needle EMBL-EBI. The divergent regions of *L. infantum* H4 are colored in gray letters and underlined. Similar areas of amino acids are colored red. The antigenic peptides are highlighted in yellow letters marked. (|): indicates identical amino acids. (:): indicates similar amino acids. Gaps were inserted to achieve maximum similarity and are indicated by dashed lines.

Indeed, several reports have described VL patients as being misdiagnosed with an autoimmune disease and then receiving immunosuppressive therapy, particularly anti-tumor necrosis factor agents [31]. In this study, we assessed the likelihood of confounding diagnoses of autoimmune disease and VL/*L. infantum* infection. Serum samples were evaluated for antibody responses against a classical panel of SLE-associated autoantigens and two antigens used for diagnosis of VL (crude soluble *Leishmania* antigen (SLA) and the defined rK39 protein). In addition, we assessed crude mixtures of *Leishmania* histones (CLH) and the related single recombinant histones rH2A, rH2B and rH4 derived from *L. infantum* [27].

Fever, pancytopenia, splenomegaly, hyper-gammaglobulinemia, production of autoantibodies and complement consumption are some of the overlapping features between VL and SLE, presenting a serious problem for differential diagnosis [32]. As with rheumatic disease, VL patients have high levels of circulating immune complexes and autoantibodies such as rheumatoid factor, hyper-gammaglobulinemia and antinuclear antibodies (ANA) observed in systemic autoimmune

diseases in particular SLE. Autoantibodies against histones have also been detected in several autoimmune disorders like inflammatory arthritis, leukocytoclastic vasculitis and autoimmune hepatitis, primary biliary cirrhosis and SLE [33]. While the detection of ANA in human serum is an important screening tool for SLE and indirect immunofluorescence (IIF) is the gold method for ANA testing [34], histones have recently been indicated to be involved in the pathogenesis of SLE. In particular, antibodies specific to H1 and H2B have been detected [35], and our data confirm this.

We previously tested nucleosomal histones which have been extensively employed in ELISA against sera from human and canine VL. In addition, CLH-based ELISA showed an excellent ability to discriminate between VL cases and healthy controls (97.6% sensitivity and 100% specificity). However, humoral response generated in sera from subjects at risk of developing SLE episodes cross-reacts with the parasitic Crude *Leishmania* histone [23,25]. In fact, the native CLH-based ELISA was revealed more accurate than single recombinant histone-based ELISA in some subjects from the same control group prepared from healthy pregnant women living in North Tunisia, an area where *L. infantum* is endemic and was more specific. *Leishmania* histone single protein fractions, specifically those involved in immune response and the production of the corresponding recombinant proteins in Eukaryotic Protein Expression system with *Leishmania tarentolae* could explain this result. In fact, this expression system allows eukaryotic protein folding and posttranslational modifications of target proteins, which may offer more specific and sensitive tools for VL diagnosis.

Recombinant *L. infantum* histones rH2A, rH2B, rH3, and rH4, have shown serodiagnostic potential for both human and canine VL [11-14]. In these studies histones presented a high specificity and sensitivity and were suggested as useful antigens for VL serodiagnosis [15]. The use of histones in the diagnosis of VL in subjects at risk of developing autoimmune complications such as SLE episodes was not defined. Recombinant histone-based ELISA revealed specificity but little sensitivity, especially when recombinant antigens were evaluated for CVL diagnosis (sensitivities of 63.63%, 59.09%, and 100% for H2A, H2B and H4, respectively). All recombinant proteins (H2A, H2B and H4) were recognized by sera from subjects at risk of developing SLE episodes; rH4 presented the best performance in terms of sensitivity and specificity, being superior to H2A and H2B in the diagnosis of VL.

The antigenicity of recombinant histone proteins in general and that of rH4 in particular is not surprising in sera from subjects at risk of developing SLE episodes. Indeed, genomic analysis indicated that the conserved region of recombinant H4 exhibits high identity (52.9%) with human histones and displays 2 segmental epitopes with highly conserved amino acid sequences (residues 42-67, 80-89). Although H2A and H2B were less conserved, identity was still 42.1% to 23.8% relative to human histones, respectively. Four segmental epitopes were indicated in H2B (residues 17-29, 45-72, 77-90, 96-119) and 5 segmental epitopes were indicated in H2A (residues 8-17, 23-35, 49-56, 61-71, 82-97). These *in silico* predictions suggest that anti-histone antibodies in sera from subjects at risk of developing SLE episodes might recognize conserved regions within the parasite histones. Therefore, it is likely that the reaction of 4 sera against rH4, 9 against rH2A and 4 against rH2B is a consequence of cross-reactivity.

Some of VL manifestations are associated with immune responses against *Leishmania* that mimic autoimmune diseases, autoimmune phenomena are common in leishmaniasis and might be due to a

release of host antigens that are released during tissue destruction by these protozoa. Through such mechanism *L. infantum* infection may promote autoreactivity and consequently, production of autoantibodies. Other etio-pathogenic hypotheses are that these manifestations can be related to polyclonal B-cell activation, altered or reduced regulatory and suppressor T cell functions [27,28] or molecular mimicry between *Leishmania* antigens and host antigens [28,29]. Histone proteins H2A, H2B, and H4 play an important role in DNA packaging and, since no cross reactivity of VL sera with mammalian histones was observed, have been reported to induce a humoral response specific for *Leishmania* histones [11-15].

Our data indicate that in the reverse situation, circulating antibodies in many of the SLE at-risk individuals recognized parasite histones. This compromised the specificity of SLA-based ELISA, providing many false-positive results (38). Examination of the CLH mixture which is expected to contain nucleosomal *Leishmania* histones H2A, H2B and H4 suggested these to be a source of the cross-reactivity. Analyses of an additional panel of sera from diagnosed patients for SLE are required/necessary to further verify the conclusions. For the purposes of diagnosing VL, however, it is therefore important to note that the rK39 antigen was found to be more specific than the use of histones.

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