Molecular characterisation, expression and antigenicity analysis of *Leishmania braziliensis* KMP-11.

Carmelo, E.; Martínez, E.; Zurita, A.; Piñero, J.E; Pacheco, R.[1]; del Castillo, A. & Valladares, B.

Departamento de Parasitología, Ecología y Genética, Facultad de Farmacia. Universidad de La Laguna.
[1] Departamento de Microbiología, Universidad San Antonio Abad, Cuzco, Perú.

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Abstract: First described in 1995, Kinetoplastid membrane protein-11 is a protein exclusively found among Trypanosomatid Protozoa. Since then, different research groups have focused on the protein and its codifying genes. In this paper the organisation of *KMP-11* genes the in *Leishmania braziliensis* genome is described. The KMP-11 *locus* in this parasite is consists of three copies of the gene. In order to analyse the humoral immune response of individuals suffering from cutaneous (LC) and mucocutaneous leishmaniasis (LMC), and Chagas' Disease patients, against *L. braziliensis* KMP-11, this protein was purified as a recombinant protein in a prokaryotic expression system. The recombinant LbKMP-11 was used as antigen in ELISA assays, showing that recognition of rLbKMP-11 by LC and LMC patients was very poor, in accordance with the low humoral response elicited in these patients.
Keywords: KMP-11, molecular characterization, antigenicity, leishmaniosis, Chagas' Disease.

Resumen: La *Kinetoplastid Membrane Protein* de 11 KDa o KMP-11 es una proteína exclusiva del grupo de los tripanosomátidos, descrita por primera vez en 1995. Desde ese momento, tanto la proteína como los genes codificantes de la misma han sido objeto de estudio por grupos de investigadores de todo el mundo. En el presente trabajo se describe la organización de los genes *KMP-11* en el genoma de *Leishmania braziliensis* mediante análisis *Southern*, encontrándose que el *locus* génico está compuesto de tres copias del gen organizadas en tandem. Del mismo modo, con el fin de estudiar la respuesta inmune humoral de los enfermos de leishmaniosis cutánea (LC) y mucocutánea (LMC), así como de individuos con la Enfermedad de Chagas (ECh), frente a la KMP-11 de *L. braziliensis*, se purificó en forma de proteína recombinante en un sistema de expresión procariótico. Al utilizar esta proteína recombinante como antígeno por ELISA, encontramos que el reconocimiento de la rLbKMP-11 por parte de los sueros de enfermos con LC y LMC es bajo, de acuerdo a la baja respuesta humoral que presentan estos pacientes.
Palabras Clave: KMP-11, caracterización génica, antigenicidad, leishmaniosis, Enfermedad de Chagas.

1. Introduction.

The Kinetoplastid Membrane Protein of 11 KDa (KMP-11) was first described by Jardim *et al.* (1995 a), as a lipophosphoglycan-associated protein. Since then, the presence of this protein has been confirmed in most of the trypanosomatids: *Trypanosoma brucei rhodesiense* and *T. b. congolense* (Stebeck *et al.*, 1995), *Leishmania infantum* (Berberich *et al.*, 1997), *L. panamensis* (Ramírez *et al.*, 1998), *T. cruzi* (Thomas *et al.*, 2000) and *L. braziliensis* (Carmelo *et al.*, 2000), among others.

Corresponding author: Dr. Basilio Valladares Hernández.
Departamento de Parasitología, Facultad de Farmacia.
Universidad de La Laguna. Avda. Ast. Fco. Sánchez, s/n. 38271, La Laguna, S/C de Tenerife. Spain.
Tlfno: 922 318430 - Fax: 922 318514
e-mail: bvallada@ull.es

The arrangement of *KMP-11* genes in the genome of the trypanosomatids shows two main structures. In *T. brucei* and *T. cruzi*, the *KMP-11* gene *locus* is made up of four copies arranged in a head-to-tail manner (Bridge *et al.*, 1998; Thomas *et al.*, 2000). On the other hand, in *L. donovani*, *L. infantum* and *L. panamensis*, the *KMP-11 locus* is consists of three gene copies with different length intergenic regions of (Jardim *et al.*, 1995 b; Berberich *et al.*, 1997; Ramírez *et al.*, 1998).

Due to its association with LPG and its amphipathic character, KPM-11 was initially considered to be a structural component of the trypanosomatid's plasma membrane. Recent findings using electron microscopy and immunolocalisation have shown that *T. cruzi* KMP-11 is associated with the subpellicular microtubules of the parasite cytoskeleton (Thomas *et al.*, 2000).

KMP-11 protein has been revealed as a B and T cell stimulator during visceral leishmaniosis. This feature was first attributed to the parasite's membrane LPG, but was afterwards found to be caused by the LPG associated protein, KMP-11 (Handman *et al.*, 1986; Mendonça *et al.*, 1991; Jardim *et al.*, 1991; Jensen *et al.*, 1998). Humoral response studies have shown that visceral leishmaniosis (VL) and Chagas' disease (ChD) patients, and some of those having cutaneous (CL) and mucocutaneous leishmaniosis (MCL) display anti-KMP-11 antibodies (Trujillo *et al.*, 1999; Thomas *et al.*, 2001).

Despite the fact that KMP-11 is strongly recognised by VL and ChD patients' sera, its utility for the diagnosis of these diseases is hampered by the cross-reactions between these groups of sera, especially in places where both diseases are prevalent.

Using 16-mer overlapping synthetic peptides, the antigenic determinants of *T. cruzi* KMP-11 have recently been mapped (Thomas *et al.*, 2001). Two antigenic determinants have been described: one of them is located in the central region of the protein, and is recognised by 60 % of the chagasic sera assayed; the other one corresponds to the carboxyl-terminal fragment, and its recognition reaches 100% of the chagasic sera. Interestingly, these results are in contrast with those described by Trujillo *et al.* (1999). These authors found that chagasic sera only recognised the central fragment of the *L. panamensis* KMP-11, and not the carboxyl end.

In this paper, the *L. braziliensis KMP-11* gene (AF142990) is characterised, and the antigenicity of this protein during natural infection with this parasite is studied.

2. Materials and methods.

2.1. Parasites.

Promastigotes of *Leishmania braziliensis* (MOHN/PE/95/LQ-8) were grown at 22°C in RPMI 1640 medium (Gibco Paisley, UK) supplemented with 20% (v/v) heat-inactivated foetal bovine serum.

2.2. Sera.

Fortythree sera from individuals suffering different pathologies: 17 sera from cutaneous leishmaniosis (CL) and 4 with mucocutaneous leishmaniosis (MCL) patients diagnosed by culture and microscopic visualization of parasites, collected by Microbiology Laboratory, Faculty of Biology, San Antonio Abad University of Cuzco (Peru); 8 sera from Peruvian individuals living in the same area as the former, and without history of contact with *Leishmania*; 10 sera from chronic Chagas' disease patients from Brazil, which were diagnosed by ELISA (Enzyme Linked Immuno-Sorbent Assay) and complement fixing; 4 sera from Spanish individuals without antecedents of contact with the parasite and who had never traveled to *Leishmania*-endemic areas. All these sera were assayed by ELISA against a *Leishmania* total protein extract. All the sera from Peruvian (8 serum samples) and Spanish (4 serum samples) individuals without a previous history of contact with *Leishmania* were negative, and were consequently used as controls for statistical purposes in the ELISA assays.

2.3. Southern blot.

L. braziliensis promastigote genomic DNA was isolated according to the protocol described by Carmelo *et al.* (2000). In order to study genomic organisation, 2 mg of genomic DNA was digested with 10 units of different restriction enzymes, electrophoresed in 0.8% agarose gels and transferred to nylon membranes (Nylon membranes positively charged, Roche Diagnostics) as described by Chomczynski (1992).

-Taq I: cuts once inside the coding region, 150 bp downstream the start codon;

-Hinf I: this enzyme cuts 107 pb downstream the start codon;

-Alu I: it has two sites, at nucleotides 31 and 234 pb downstream ATG;

-Rsa I: cuts once inside the coding region (263 pb), and again 372 pb downstream the stop codon;

-Bam HI: this enzyme does not cut inside the *L. braziliensis KMP-11* coding region.

2.4. Radioactive labelling (a-^{32}P) of KMP-11 probe and hybridisation with this probe.

The *KMP-11* probe is a 275 pb BamHI/Hind III fragment from plasmid pQE/LbKMP-11, which covers the *L. braziliensis KMP-11* coding region. This fragment was purified from agarose gels using Qiaex II Gel Extraction Kit (Qiagen) and random primer labelled using 50 mCi (3000 Ci/mmol) [a-^{32}P]dCTP with the Rediprime DNA labelling system (Amersham Pharmacia) as described by the supplier.

Hybridisations were performed in 50% formamide (v/v), 5x SSC (1x SSC 0.15 M NaCl/0.015 M sodium citrate), 0.2 % SDS, 5x Denhart's, 0.01 M Na_2HPO_4/ NaH_2PO_4, 0.1 mg ml^{-1} herring sperm DNA at 42°C overnight. Final post-hybridisation washes were performed at 0.1xSSC/0.1% SDS at 65°C for 30 minutes.

2.5. Determination and analysis of DNA sequences.

Both strands of all the clones in this paper were sequenced by the dideoxy chain termination method using reagents from the ALFexpress Auto Cycle Sequencing Kit and 5'-cyanine labelled primers, on an ALFexpress automated sequencer (Amersham Pharmacia), according to the supplier's descriptions. Analysis of DNA and amino acid sequences was performed using University of Wisconsin Genetics Computer Group and by accessing the GeneBank and EMBL databases of protein and DNA sequences.

2.6. Subcloning of LbKMP-11 gene in pQE30 expression vector.

L. braziliensis KMP-11 coding region was PCR amplified using T7 (5'-TAATACGACTCACTATA-3') and KMP-3 (5'-TCCTTTTCCACCTTCCAA-3') oligonucleotides, and pBS/*KMP-11* plasmid as a template (Carmelo *et al.*, 2000). PCR product was purified using the Qiaex II (Qiagen), subcloned into the *Sma* I-digested pQE30 vector (Qiagen) and then transformed into *E. coli* TOPP 3 competent cells (Stratagene), to give the construction pQE/*LbKMP-11*.The correct in-frame subcloning of the fragment was confirmed by sequencing.

2.7. Induction, solubilisation and purification of rLbKMP-11.

The optimal induction conditions in the prokaryotic expression system *E. coli* Topp 3 must be determined for each recombinant protein. The estimation was performed varying the IPTG concentrations for a given culture.

After induction, the culture was centrifuged at 3500 x g for 10 minutes at 4°C, and the cell pellet was resuspended in lysis buffer: 50 mM Na_2HPO4, 300 mM NaCl, 20 % Glycerol, 0.1 % Tween-20, 5 mM $MgCl_2$ and 1mM PMSF, pH 8. It was then sonicated on ice (12 x 30 s. pulses with 1 min. interval), and centrifuged at 13000 x g for 15 min at 4°C, thus obtaining the soluble extract.

The soluble recombinant protein was purified by Ni^{2+}-NTA-agarose affinity chromatography (Qiagen). After overnight binding to the resin, it was washed twice with the same solubilisation buffer at pH 6 and pH 5. Finally the attached recombinant protein was eluted in a purified form in the same buffer at pH 4.

2.8. ELISA measurements.

The recombinant LbH1 protein was diluted in carbonate/bicarbonate buffer (pH 9.6) to a concentration of 5 mg/ml. The ELISA plates (Immulon 4HBX, Dynex) were sensitized with 100 ml of antigen per well, incubated O/N at 4°C and washed 3 times with 0.05% PBS-Tween-20 (PBS-T) buffer. All the sera were diluted 1:100 in PBS-T with 5% non-fat dried milk, adding 100 ml per well and incubated at 37°C for 1h, after which the washings were repeated. As second antibody, anti-human IgA+IgG+IgM(H+L) conjugated with peroxidase (Jackson ImmunoResearch) was used, diluted 1:2000 in PBS-T and 100 ml added per well. After incubation at 37°C for 1 h and the corresponding washings, it was developed using ortho-phenylenediamide (Sigma) as substrate. The absorbance was read at 450nm. These ELISA trials were performed in triplicate.

3. Results.

3.1. Molecular characterisation of KMP-11 gene locus in Leishmania braziliensis.

Fig. 1A shows hybridisation with *KMP-11* probe with genomic DNA from *L. braziliensis*. The 0.3 Kb Alu I hybridisation band corresponds to most of the KMP-11 coding region from the different gene copies. Both 1 and 0.95 Kb Hinf I bands represent the gene unit, the intergenic and coding regions taken together. This is so because this endonuclease has only one site inside the *L. braziliensis* KMP-11 coding region. The lenght of these two units is not identical, a very common feature among trypanosomatid genes. From this hybridisation pattern it can be concluded that the *KMP-11* gene *locus* in *L. braziliensis* is conformed by three copies of the gene in a head-to-tail tandem array, with an intergenic region of approximately 0.75 Kb, displaying some differences in their nucleotide sequence (Fig. 1B)

3.2. Subcloning, expression and purification of L. braziliensis KMP-11 recombinant protein.

The strategy used for subcloning *L. braziliensis* KMP-11 coding region consisted in amplifying pBS/*KMP-11* plasmid using primers T7 (corresponding to the vector multiple cloning site) and KMP-3. In this way, a 1 Kb fragment was obtained that had the *LbKMP-11* gene at one of the ends. This fragment was *Sma* I digested to yield the coding region, since this endonuclease cut 7 pb upstream the stop codon, and subcloned into *Sma* I-digested pQE30 vector.

Overexpression of rLbKMP-11 in the pQE30/*E. coli* Topp3 expression system was achieved after induction with 1 mM IPTG in LB medium at 37° C for 4 hours. The purification of the recombinant protein was performed by Ni-NTA agarose (Qiagen) affinity chromatography, as described in Materials and Methods. Fig. 2 shows SDS-PAGE electrophoresis of

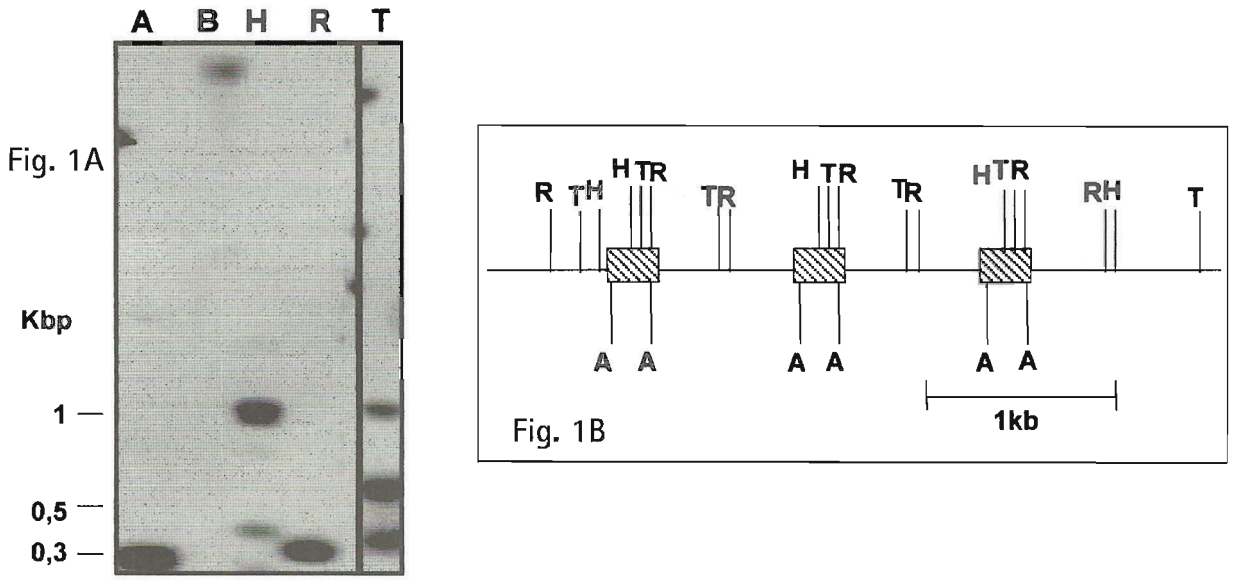

Fig. 1. Molecular characterisation of *L. braziliensis KMP-11* gene *locus*. 1A: hybridisation with *KMP-11* probe of *L. braziliensis* genomic DNA digested with Alu I (A), Bam HI (B), Hinf I (H), Rsa I (R), and Taq I (T). 1B: this drawing represents the proposed genomic organisation of *L. braziliensis* KMP-11 gene *locus*.

the different fractions of purification. It can be observed that purified *L. braziliensis* KMP-11 recombinant protein elutes at pH 4, after extensive column washing at pH 6 and pH 5.

3.3. Evaluation of the humoral immune response against L. braziliensis KMP-11.

After purification, recombinant *L. braziliensis* KMP-11 was quantified and used as an antigen in ELISA assays, as described in Methods.

Regarding the pathology exhibited by the sera, it can be observed that mucocutaneous leishmaniosis sera display the highest reactivity of the sera assayed in terms of mean $O.D._{450nm}$ (Fig. 3), even higher than chagasic and cutaneous leishmaniosis sera.

In order to establish the positivity of these sera against this antigen, a cut-off point was defined as the mean $O.D._{450nm}$ of the control sera assayed plus 3 standard deviations (S.D.). In this way, it was observed that 14.2% of the leishmaniosis sera (3 out of 21) showed $D.O._{450\ nm}$ above the cut-off point, so they can be considered as positive. Out of the chagasic sera assayed, 30% (3 out of 10) showed positive against rKMP-11 from *L. braziliensis*.

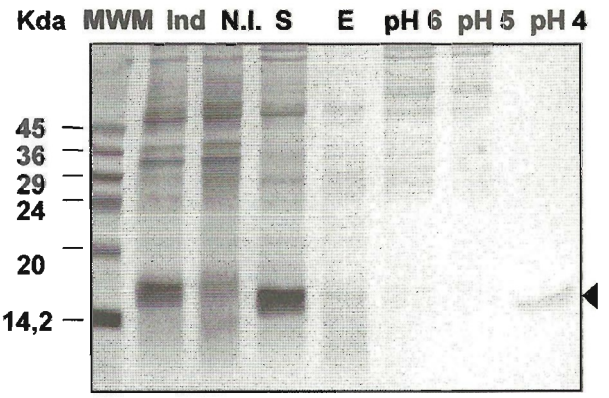

Fig. 2. SDS-PAGE electrophoresis of the fractions from rLbKPM-11 purification. MWM: molecular weight marker: Ind: induced total protein extract; N.I. non-induced control; S: soluble extract; E: column efluent; pH 6: column wash at pH 6; pH 5: column wash at pH 5; pH 4: rLbKMP-11 elution at pH 4.

4. Discussion.

The gene *locus* coding for KMP-11 has previously been described in different Trypanosomatids. In *L. donovani* (Jardim *et al.*, 1995 b), *L. infantum* (Berberich *et al.*, 1997) and *L. panamensis* (Ramírez *et al.*, 1998) the *KMP-11 locus* is conformed by three copies in a head-to-tail arrangement with variable-length intergenic regions. In contrast, regarding the genus *Trypanosoma*, *T. brucei* (Bridge *et al.*, 1998) and *T. cruzi* (Thomas *et al.*, 2000) present four gene copies, with similar intergenic regions.

The arrangement we propose for the *L. braziliensis KMP-11* genes coincides with those found for other *Leishmania* species, since it presents three gene copies arranged in tandem. The main differences are the intergenic regions, since in *L. braziliensis* they are almost identical, but in the rest of the *Leishmania* species where the *KMP-11* genes have been studied, their length is variable.

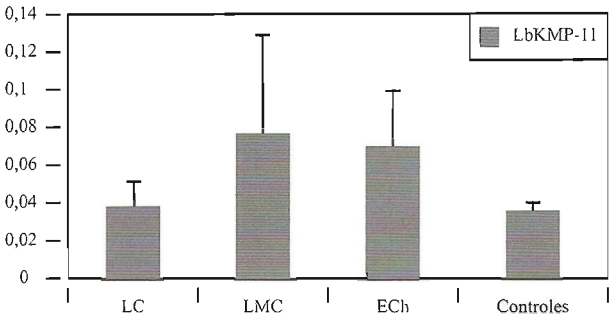

Fig. 3. Mean $O.D._{450\ nm}$ of the sera assayed, regarding the pathology they present.

The KMP-11 protein family is characterised by a high level of sequence conservation. Fig. 4 shows the alignment of KMP-11 proteins from different Trypanosomatids, denoting that the identity of *L. braziliensis* KMP-11 reaches 89% comparing with *L. panamensis* KMP-11, 88% with *L. guyanensis*, 86% against *L. donovani* and 86% regarding *T. cruzi*. This high sequence identity, and the fact that KMP-11 is present in most of the trypanosomatids described so far, has led several authors to analyse the antigenicity of KMP-11 against sera of patients suffering different forms of leishmaniosis and Chagas disease.

In this way, *L. infantum* KMP-11 was recognised by 70% of the sera from individuals with VL caused by *L infantum*, 57% of those with ChD, and 96% of sera from dogs suffering canine VL (Berberich *et al.*, 1997). On the other hand, *T. cruzi* KMP-11 displayed a high recognition when tested against chagasic and VL sera. This recognition of the chagasic sera dropped by 70% when the protein carboxyl end was truncated, but this fall was significantly lower (about 25%) for VL sera, indicating that a prominent antigenic determinant for the chagasic sera is located in this region (Thomas *et al.*, 2001).

In our experiments, *L. braziliensis* KMP-11 is recognised by 14.2 % of the CL and MCL sera caused by *L. braziliensis*, 50% of the MCL sera and 30% of the chagasic sera being positive. Similarly, it can be noted that the mean O.D. of the MCL sera is higher than that shown by CL and ChD sera. This low recognition of recombinant KMP-11 is also found, but not so markedly, in the study by Trujillo *et al.* (1999) with *L. panamensis* KMP-11. In this paper, ELISA assays with recombinant *L. panamensis* KMP-11 resulted in 60% of the MCL, 37% of the CL and 51% of the chagasic sera being positive with an O.D. above the mean of the control sera plus three S.D.

From these results can be concluded that, even though the reactivity of rLbKMP-11 against CL and MCL sera from Cuzco, Peru is lower than that detected by Trujillo *et al.* (1999) with rKMP-11 from *L. panamensis* against Colombian CL and MCL sera, both studies present the same tendency, a considerably drop in reactivity of CL and MCL sera when compared to VL sera.

All the above findings highlight the great differences in the humoral response against KMP-11 from individuals suffering pathologies caused by different trypanosomatids, and this response mostly depends on the disease displayed by the patient. In this context, this humoral response is always higher for VL sera than for CL and MCL sera. This assessment may be explained by the fact that during active VL a suppression of T cell reactivity against *Leishmania* antigens is detected, an increase in IL-4 and IL-10 titres, and an important hypergammaglobulinaemia due to the polyclonal activation of B cells (Kharazmi *et al.*, 1999). All these parameters are related to a marked Th2 cell response and the exacerbation of the disease. In contrast, CL is characterised by a Th1/Th2 balance, resulting in a milder disease the more this balance is inclined

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L.braz        1   MATTYEDFAGKLDRLDEFNKKMQEQNAKFFADKPDDSTLSPEMKE
L.pan         1   MATTYEEFAAKLDRLDEEFNKKMQEQNAKFFADKPDESTLSPEMKE
L.guyan       1   MATTYEEFAAKLDRLDEEFNKKMQEQNAKFFADKPDESTLSPEMKE
L.trop        1   MATTYEEFSAKLDRLDEEFNRKMQEQNAKFFADKPDESTLSPEMKE
T.cruzzi      1   MATTLEEFSAKLDRLDAEFAKKMEEQNKKFFADKPDESTLSPEMKE
                  **** * *  ****** **  ** *** ******** *********

L.braz       51   FEKMIQEHTDKFNKKMRESHSEHFKQKFAELLEQQKNAQYPGK  92
L.pan        51   FERMIKEHTEKFNKKMHEHSEHFKHKFAELLEQQKAAQYPGK  92
L.guyan      51   FERMIKEHTEKFNKKMHEHSEHFKHKFAELLEQQKAAQYPSK  92
L.trop       51   FERMIKEHTEKFNKKMHEHSEHFKQKFAELLEQQKAAQYPSK  92
T.cruzzi     51   FEKMIQEHTDKFNKKMHEHSEHFKAKFAELLEQQKNAQFPGK  92
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Fig. 4. Alingment of KMP-11 proteins from different Trypanosomatids: *L. braziliensis* (AF142990), *L panamensis* (AF219228), *L guyanensis* (AF026139), *L. tropica* (AJ000078) and *T. cruzi* (AJ000077). The asterisks indicate the conserved aminoacids among all the proteins.

towards a Th1 cell response (Kharazmi *et al.*, 1999). During CL, antibody production against parasite antigens is limited (Pedrosa Valli *et al.*, 1999), and this is the reason that has hampered the development of immunologic diagnostic kits for this disease as was possible for VL (Cerdeñosa *et al.*, 1995; Brito *et al.*, 2000). On the other hand, MCL present some common features between VL and CL, like higher anti-leishmania antibody titres than CL sera (Herwaldt, 1999), or a significant cellular response (Pedrosa Valli *et al.*, 1999).

Another characteristic feature of KMP-11 recognition is observed when synthetic peptides derived from its sequence are tested. None of the leishmaniosis sera assayed detects by ELISA any of the KMP-11 peptides, from either *T. cruzi* or *L. panamensis*. On the contrary, these peptides are well recognised by chagasic sera (Trujillo *et al.*, 1999; Thomas *et al.*, 2001). This may indicate that KMP-11 recognition by leishmaniosis sera is mostly conformational, while chagasic sera present both linear and conformational recognition (Thomas *et al.*, 2001). We consider that this difference in the KMP-11 recognition between chagasic and leishmaniosis sera may be related to differences in KMP-11 processing in individuals infected by *T. cruzi* or *Leishmania sp.*

The fact that KMP-11 is a protein exclusively found among the trypanosomatids, and its wide distribution throughout this protozoan group, caused a great stir among numerous researchers in order to unveil its immunological characteristics, in view to develop diagnostic kits that would discriminate between the diseases caused by these parasites. After that, the finding of important cross-reactions for this antigen among leishmaniosis and Chagas disease patients hampered their differentiation, at least until the use of modified forms of this protein (Thomas *et al.*, 2001). Added to the low humoral antigenic response to this protein in MCL and CL patients, this lead us to consider KMP-11 as a protein with a limited interest for leishmaniosis diagnosis.

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