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# The 70-kDa heat-shock protein is a major antigenic determinant in human *Trypanosoma cruzi/Leishmania braziliensis braziliensis* mixed infection

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## 1. Summary

Five sera from Bolivian individuals chronically infected by *Trypanosoma cruzi*, and suffering an active *Leishmania braziliensis braziliensis* metastasic mucocutaneous lesion were characterized. They reacted with the *T. cruzi* recombinant antigens that are currently used as Chagas diagnostic reagents, and with several *L. b. braziliensis* proteins as assessed by Western blot. These sera showed an intense reaction with a *T. cruzi* and an *L. b. braziliensis* polypeptide of about 70 kDa. Expression cloning techniques demonstrated that the target of this immunologic reaction was a cross-reactive antigen, the 70-kDa heat-shock protein (HSP 70). High levels of anti-HSP 70 reactivity and positive reactions with all or some of the *T. cruzi* recombinant antigens JL7, JL8, and JL5, defined a serologic pattern that was characteristic of the *T. cruzi/ L. b. braziliensis* mixed infection.

*Key words: Trypanosoma cruzi; Leishmania braziliensis braziliensis*; 70-kDa Heat-shock protein; Recombinant antigen; Diagnosis

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## 2. Introduction

*Trypanosoma cruzi* and *Leishmania braziliensis braziliensis*, two protozoa of the same Trypanosomatidae family, are the etiologic agents of Chagas' disease and mucocutaneous leishmaniasis (espundia), respectively [1, 2]. The most critical consequence of the chronic *T. cruzi* infection is a late-developing myocarditis that, in many cases, evolves without being noticed by the infected individual [1].

In Bolivia, both parasites constitute a serious sanitary problem. Recent social and economic changes in this country have compelled populations to move in search of work from regions where they were domiciled, some of which are endemic for Chagas' disease, to tropical forest regions where mucocutaneous leishmaniasis is endemic. In the case of leishmanial infection, those individuals with an undetected *T. cruzi* infection constitute a particularly vulnerable group, not only due to the possible combined effects of the mixed infection, but also because the antimonial drugs used to cure leishmaniasis have cardiotoxic effects [3].

This study was initiated to obtain a precise molecular characterization of the antigens recognized by sera from individuals concurrently affected by these two related parasites, a necessary step towards the design of an adequate diagnostic test of the double infection.

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## 3. Materials and Methods

The λgt11 cDNA cloning and random priming kits were from Amersham, U.K. The origin of the other reagents has been described [4, 5].

### 3.1. *Human sera*

Sera were obtained from chronically infected chagasic individuals with espundia showing metastatic lesion of the facial mucosae, positive skin tests and positive immunofluorescence (N = 5). The existence of a *T. cruzi* infection was assessed serologically by complement fixation, passive hemagglutination, immunofluorescence and ELISA. Clinical and electrocardiographic evaluation of the patients revealed clear signs of severe chagasic chronic cardiopathy. These patients were clinically characterized at the Instituto Boliviano de Biología de la Altura (I.B.B.A.), La Paz, Bolivia. The sera from patients with Chagas' disease (N = 4), and those from patients with espundia (N = 5) were provided by the Servicio de Cardiología del Hospital Ramos Mejia, Buenos Aires, Argentina, and by the Instituto de Medicina Tropical "Alexander von Humboldt", Lima, Peru, respectively.

### 3.2. *λgt11 cloning and screening*

*T. cruzi* blood stream trypomastigotes of the RA strain were prepared as described by Mortatti and Munk [6]. Poly(A)$^+$ RNA from trypomastigotes was obtained by the method of Chirgwin et al. [7], and checked by agarose gel electrophoresis and ethidium bromide staining. A λgt11 library was constructed from this poly(A)$^+$ RNA according to

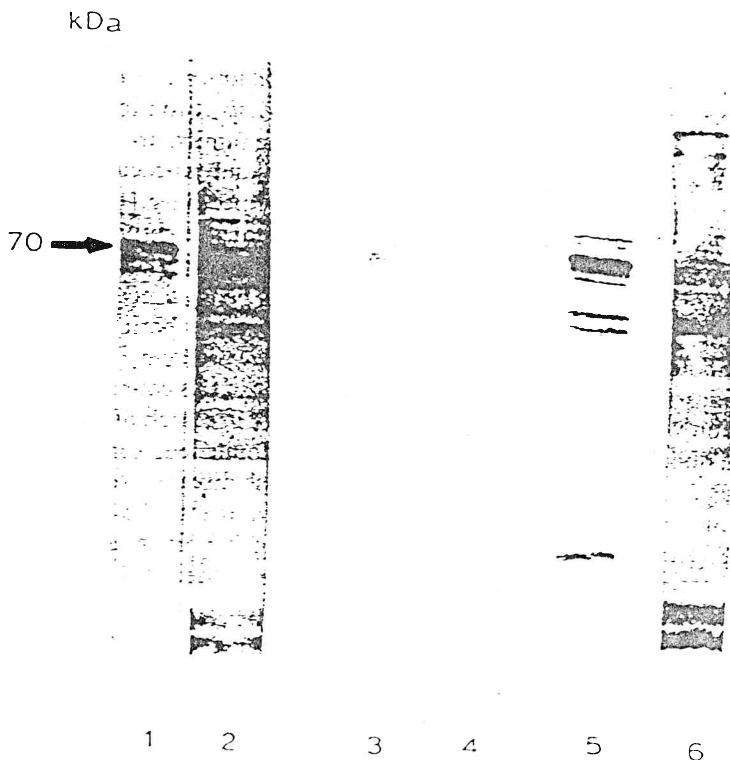


Fig. 1. Western blot analysis of mixed infected serum binding to *T. cruzi* and *L. b. braziliensis* proteins. Homogenates of both parasites were electrophoresed on 7 – 15% linear polyacrylamide gradient gels and blotted. Each slot contained approximately 30 μg of total protein. Lanes 1, 3, and 4, *T. cruzi* trypomastigote extracts probed with a 1:500, 1:1000, and 1:3000 dilution of the reference serum PM (serum c in Fig. 4), respectively; lane 2, *L. b. braziliensis* promastigote extract probed with a 1:500 dilution of the reference serum PM. Lane 5, *T. cruzi* trypomastigotes probed with a 1:500 dilution of the serum FR (serum e in Fig. 4); lane 6, *L. b. braziliensis* promastigote extract probed with a 1:500 dilution of the reference serum FR.

Levin and others [4]. The screening and the antibody detection were performed as previously reported [4, 5]. Serum reactivity to the recombinant phage was analyzed by phage dot array immunoassay [4, 5]. One microliter (10[illegible] pfu) of different phage were arrayed on lawns of *Escherichia coli* Y1090. The phage were grown, blotted and reacted with different sera. Wild-type λgt11 was included as a negative control, whereas phage JL7, JL8, and JL5 were included as positive controls of *T. cruzi* infection [8].

### 3.3. *Purification of recombinant β-galactosidase-HSP70 fusion protein*

*E. coli* Y1089 bacteria were lysogenized with the phage HSP70-RA1. The lysogenic bacteria were cultured and induced as described [4]. The β-galactosidase fusion protein was purified and used to affinity-purify anti-HSP70 monospecific antibodies from sera of individuals with the double infection [4, 5].

### 3.4. *Monoclonal and monospecific antibodies*

Affinity-purified monospecific anti-HSP70 antibodies immunoselected as previously described [4], and the monoclonal antibody D4F18 [9], were used to confirm the identity of cloned peptides. The latter was raised against a recombinant encoding the C-terminal region of the *Plasmodium falciparum* HSP70 [9], and it was found to react with the same peptide sequence that is recognized by the monoclonal antibody G10C9 described by Mattei et al. [10], namely the HSP70 *P. falciparum* peptide ANGILNVTAVEKSTGK. It is noteworthy that it recognized the 70-kDa heat-shock proteins from several protozoan species as well as the corresponding mammalian protein [9, 10].

### 3.5. *Western blots*

*L. b. braziliensis* promastigotes and *T. cruzi* trypomastigotes were cultured and lysed in the presence of a mixture of various antiproteolytic agents

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1   CCGCTGACGCTTGGCATCGAGACGGCGGGTGGCGTCATGACGTCGCTGATCAAGCGCAAC
     P  L  T  L  G  I  E  T  A  G  G  V  M  T  S  L  I  K  R  N
61  ACGACGATTCCGACCAAGAAAAGCCAGATCTTCTCGACGTACCCGGACAACCAGCCGGGC
     T  T  I  P  T  K  K  S  Q  I  F  S  T  Y  P  D  N  Q  P  G
121 GTGCACATCCAGGTCTTTGAGGGGGAGCGTGCGATGACGAAGGACTGCCACCTGCTCGGC
     V  H  I  Q  V  F  E  G  E  R  A  M  T  K  D  C  H  L  L  G
181 ACATTCGACCTGTCCGGCATCCCGCCGGCGCCGCGCGGTGTGCCCCAGATTGAGGTGACC
     T  F  D  L  S  G  I  P  P  A  P  R  G  V  P  Q  I  E  V  T
241 TTTGACCTCGACGCCAACGGCATCCTGAACGTGTCCGCGGAGGAGAAGGGCACCGGCAAG
     F  D  L  D  A  N  G  I  L  N  V  S  A  E  E  K  G  T  G  K
301 CGCAACCAGATTGTCATCACGAACGACAAGGGCCGCCTGAGCAAGGCGGACATTGAGCGC
      R  N  Q  I  V  I  T  N  D  K  G  R  L  S  K  A  D  I  E  R
361 ATGGTGTCCGAGGCTGCCAAGTACGAGGCGCAGGACAAGGAACAGCGCGACCGCATTGAC
     M  V  S  E  A  A  K  Y  E  A  Q  D  K  E  Q  R  D  R  I  D
421 GCAAAGAACGGTCTTGAGAACTACGCATTTTCGATGAAGAACACCGTAAACGAGCCGAAC
     A  K  N  G  L  E  N  Y  A  F  S  M  K  N  T  V  N  E  P  N
481 GTCGCTGGCAAGATTGAGGAGGCCGACAAGAACACGATTACGAGTGCCGTGGAGGAGGCA
     V  A  G  K  I  E  E  A  D  K  N  T  I  T  S  A  V  E  E  A
541 CTGCAATGGCTGAACAACAACCAGGAGGCCAGCAAGGAGGAGTACGAGCACCGCCAGAAG
     L  Q  W  L  N  N  N  Q  E  A  S  K  E  E  Y  E  H  R  Q  K
601 GAGCTGGAGAACCTGTGCACGCCCATCATGACGAAGATGTACCAGGGCATGGGCGCGGGC
     E  L  E  N  L  C  T  P  I  M  T  K  M  Y  Q  G  M  G  A  G
661 GGCGGTATGCCCGGAGGTATGCCTGGTGGAATGCCCGGAGGTATGCCTGGTGGAATGCCC
     G  G  M  P  G  G  M  P  G  G  M  P  G  G  M  P  G  G  M  P
721 GGGGGCATGCCTGGCGGCGCGAACCCGTCGTCTTCGTCAGGACCGAAGGTGGAGGAAGTG
     G  G  M  P  G  G  A  N  P  S  S  S  S  G  P  K  V  E  E  V
781 GACTGAGAGCGCATCCCCGAAGATGTTCTCATGGCGGCGTCTGCTCGCGAACCAATAGCC
     D  *
841 CGTTGGTTTTCTCCCTTGTAGAGCGTAGAGGTCTGCGACAAACCCAGCTGCCATCACTAT

901 TTTTATTATTGGTTTTTTCCCCTCTCCATTATTATTATTATTATTATTGTTTTTCTTCGC

961 TCTCCATTATTATTATTGTTATTATTATTATTACGGTTGTTAAAAAAAAAAAAAAA
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Fig. 2. Nucleotide and deduced amino acid sequence of the HSP70-RA1 cDNA fragment, EMBL accession number X58715.

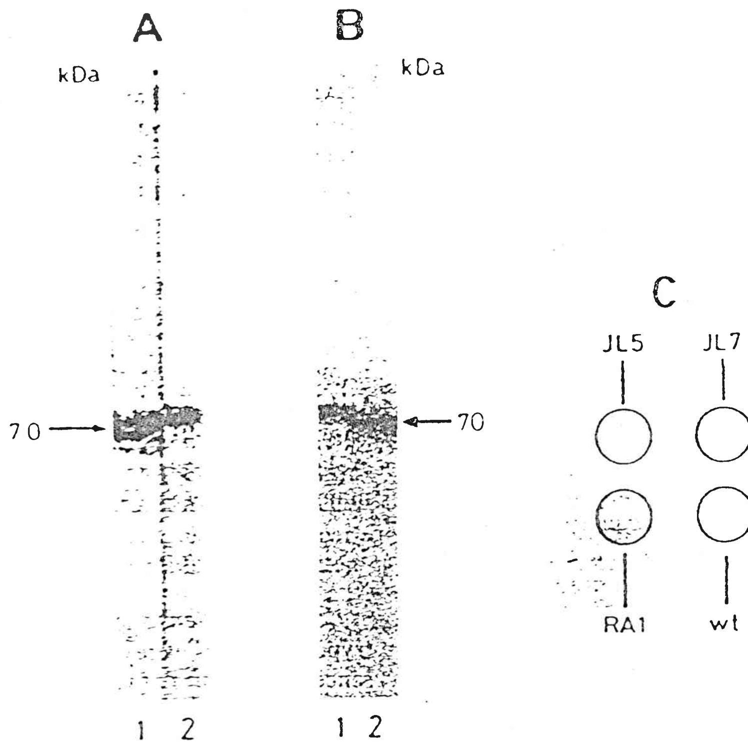


Fig. 3. Identification of *T. cruzi* and *L. b. braziliensis* 70-kDa heat-shock proteins. (A) Western blots of *T. cruzi* trypomastigote lysate (lane 1), and *L. b. braziliensis* promastigote lysate (lane 2) probed with anti-HSP70-RA1 immunoselected antibodies. (B) Western blots of *L. b. braziliensis* promastigote lysate (lane 1), and *T. cruzi* trypomastigote lysate (lane 2), probed with the monoclonal antibody D4F18. (C) Recombinant clones JL7, JL5, and HSP70-RA1, and λgt11 wild type (wt) probed with the monoclonal antibody D4F18.

as previously informed [4]. Polypeptides were analyzed by denaturing polyacrylamide gel electrophoresis and blotted [4].

### 3.6. *Characterization of cDNA clones*

Base sequencing was carried out according to protocols reported elsewhere [4, 5]. the *T. cruzi* 70-kDa heat-shock protein cDNA sequence, HSP-70-RA1, has the EMBL accession number X58715.

## 4. Results

The antigen recognition profile of the sera from Bolivian individuals with the mixed infection was assessed by phage dot array immunoassay and Western blotting. As expected, all sera displayed a varied degree of immunoreactivity to the *T. cruzi* recombinants JL7, JL8 and JL5, known to react with a great majority of chagasic sera (Fig. 4, sera a, b, c, d, and e) [8]. Furthermore, on Western blots of *L. b. braziliensis* promastigote extracts the sera recognized several bands ranging from low (15 and 20 kDa), to high (>150 kDa) molecular weight, showing an intense reaction with a group of proteins of about 70 kDa (Fig. 1, lanes 2 and 6). On Western blots of *T. cruzi* trypomastigote extracts the sera reacted with several proteins. The most intense reaction targeted a band of about 70 kDa (Fig. 1, lanes 1, 3, 4, and 5).

To identify this *T. cruzi* protein, 50 000 recombinants from a λgt11 bloodstream trypomastigote cDNA library were screened using a 1:3000 dilution of the reference serum PM (Fig. 1, lane 4). At this serum dilution no reactivity to the JL7, JL8, and JL5 *T. cruzi* recombinants was observed. The screening revealed that only two recombinant phage RA1, and RA2 out of 50 000 were reactive.

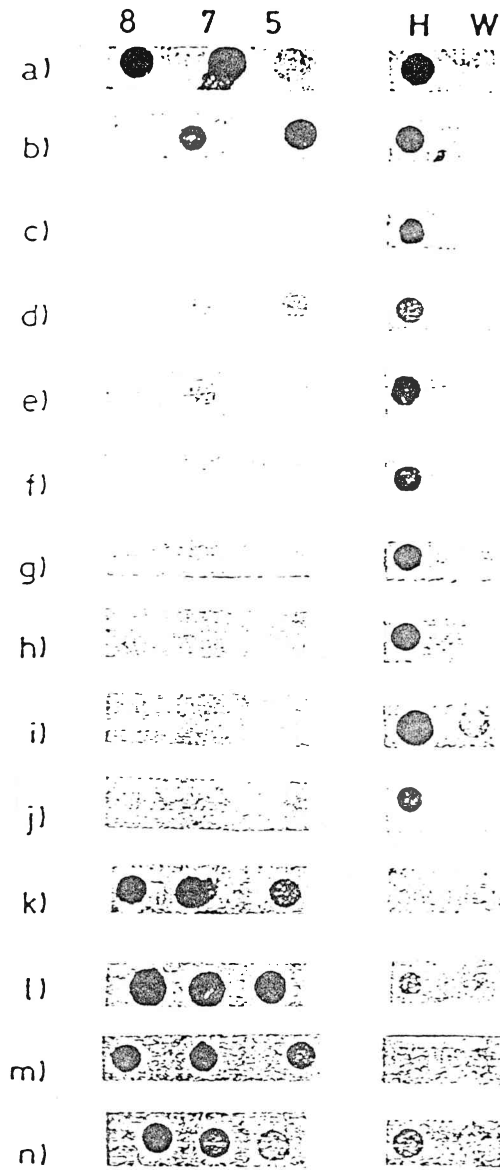


Fig. 4. Arrays of recombinant clones probed with different sera. Serum samples a – e were sera from patients with the mixed infection, dilutions of these sera were 1:500, except reference serum c (serum PM in Fig. 1), and serum e (serum FR in Fig. 1) that were diluted 1:200. Serum samples f – j were sera from patients with espundia, dilutions of the serum samples f, g, and h were 1:500, whereas the ones for serum samples i and j were 1:200. Serum samples k – n were from chronic Chagas patients, dilutions of these sera were 1:500. Lane 8 shows recombinant JL8; lane 7, recombinant JL7; lane 5, recombinant JL5; lane H, recombinant HSP70-RA1; and lane W, λgt11 wild type.

### 4.1. *Characterization of the cDNA insertions*

Insert cDNAs from these two clones were recloned into the vector M13mp18 and sequenced. The RA1 recombinant had a 1016 bp cDNA insert, its nucleotide and deduced amino acid sequence is shown in Fig. 2. The RA2 cDNA was slightly shorter than the RA1 insert (939 bp), and its sequence was identical to the one shown in Fig. 2, extending from base 77 to the poly(A)$^+$ tail. The amino acid

sequence derived from RA1 cDNA showed that the last aspartic codon was followed by a TGA stop codon and by another stop codon, TAG, 72 bases downstream. The insert has a run of 15 adenine residues at its 3′ terminus, indicating that the cloned peptide corresponds to the carboxy-terminal portion of a parasite protein. Sequence comparison indicated that it encodes the C-terminal portion of the *T. cruzi* 70-kDa heat-shock protein (HSP70), being more than 90% homologous to the HSP70 amino acid sequences of *T. cruzi* Maracay and Peru strains [11].

### 4.2. *Anti-RA1 monospecific antibodies and the monoclonal D4F18 react with the* L. b. braziliensis *HSP70 protein*

The antibody select technique was used to verify that the RA1 recombinant carried sequences encoding a *T. cruzi* polypeptide. In Western blots of total proteins from trypomastigotes, the immunoselected anti-RA1 antibodies reacted with a band of about 70 kDa (Fig. 3A, lane 1). The same antibodies recognized an *L. b. braziliensis* polypeptide of about 70 kDa (Fig. 3A, lane 2).

To further corroborate the identity of peptides targeted by the immunoselected antibodies, Western blots of *T. cruzi* and *L. b. braziliensis* antigens were probed with the monoclonal antibody D4F18. It reacted with proteins of about 70 kDa from both parasites (Fig. 3B, lanes 1 and 2), and with the RA1 recombinant (Fig. 3C). These results ratify the cross-reactive nature of the 70-kDa heat-shock proteins from these related protozoan parasites.

### 4.3. *Anti-RA1 reactivity of sera from individuals with different infections*

The reactivity of different sera with the HSP70-RA1 recombinant antigen and other cloned *T. cruzi* antigens was evaluated using a phage dot array immunoassay. The sera from individuals chronically infected with *T. cruzi* reacted strongly with the recombinants JL7, JL8, and JL5, but weakly with the HSP70-RA1 antigen (Fig. 4, sera k, l, m, and n, and results described in [12]). No anti-HSP70-RA1 reactivity was detected in acute chagasic sera (not shown). The serum samples from individuals infected by *L. b. braziliensis*, patients with espundia, presented high anti-HSP70-RA1 activities, but no reactivity to the other *T. cruzi* antigens (Fig. 4, sera f, g, h, i, and j). The sera from patients with the mixed infection showed positive reactions to all or some of the *T. cruzi* recombinant antigens that are currently used as Chagas diagnostic reagents (recombinants JL7, JL8, and JL5), and presented a very strong reactivity to HSP70-RA1. This high level of anti-HSP70 reactivity, and the positive reactions with the *T. cruzi* diagnostic antigens defined a serologic pattern characteristic of the *T. cruzi/L. b. braziliensis* mixed infection (Fig. 4, sera a, b, c, d, and e).

## 5. Discussion

A cloning scheme was designed to identify the 70-kDa polypeptide from *T. cruzi* and *L. b. braziliensis*, targeted by the sera from patients with a *T. cruzi/L. b. braziliensis* mixed infection. To do so, a *T. cruzi* trypomastigote cDNA expression library was constructed and screened with a serum from an individual with the mixed infection (reference serum PM, Fig. 1, lanes 1, 2, 3, and 4; serum c in Fig. 4). This serum was used as immunoprobe because, at high dilutions, it reacted with only one *T. cruzi* polypeptide (Fig. 1, lane 4). Two phage were isolated; their nucleotide, and the corresponding amino acid sequences confirmed the results in Fig. 1, since the isolated phage, RA1 and RA2, encoded portions of the same antigen, namely *T. cruzi* HSP70 (Fig. 2).

The cross-reactive nature of the HSP70 antigens from flagellates has been firmly established [13]. Based on the following results, this report confirmed the cross-reactivity between the *T. cruzi* and the *L. b. braziliensis* HSP70: (a) anti-RA1 immunoselected antibodies reacted with both *L. b. braziliensis* and *T. cruzi* 70-kDa HSPs; (b) both 70-kDa antigens reacted with the D4F18 monoclonal antibody; and (c) sera from individuals infected only by *L. b. braziliensis* reacted with the *T. cruzi* HSP70-RA1 recombinant.

Accordingly, the analysis of the results in Fig. 4 suggested that the profile of anti-HSP70 reactivities displayed by the sera from patients with mixed parasitosis was contributed mainly by the *L. b. braziliensis* infection, and to a lesser extent by the chronic *T. cruzi* infection. This hypothesis

seemed valid because chronic chagasic individuals showed low or moderate levels of anti-HSP70 reactivity [12], whereas the sera from patients with espundia reacted intensely with the HSP70 recombinant (Fig. 4, sera f, g, h, i, and j).

We conclude that the serologic pattern of the mixed infected sera, sharing humoral features of both parasitosis, such as the positive reactions to the *T. cruzi* recombinants JL7, JL8, and JL5 (diagnostic of *T. cruzi* infection), and the intense anti-HSP70 response (characteristic of mucocutaneous leishmaniasis), may be employed to complete the clinical characterization of the patients with this double infection.

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