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Isolation, purification, characterization and antigenic evaluation of GPI-anchored membrane proteins from *Leishmania (Viannia) braziliensis*

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Abstract

GPI-anchored proteins from the plasma membrane of *Leishmania (Viannia) braziliensis* promastigotes were isolated, characterized and their migration pattern compared with those from other *Leishmania* species. In all cases the SDS-PAGE migration patterns were obtained under reducing and non-reducing conditions, using DL-dithiothreitol (DTT) as a reducer agent. Our results reveal that under reducing conditions the SDS-PAGE migration pattern is modified as a consequence of the disruption of disulphur-bonds and protein transformation. This is demonstrated when in non-reducing conditions the *L. (V.) braziliensis*-GPI-anchored proteins pattern showed a group of bands over the 100 kDa, and two more bands of 52 kDa and 50 kDa in four different isolates, whereas under reducing conditions the major GPI-anchored protein fractions were detected as bands of 63 kDa, 50 kDa and an increase of peptides between 34 kDa and 22 kDa. Similar modifications were detected in the SDS-PAGE migration patterns of GPI-anchored protein fractions from *L. (Leishmania) donovani, L. (L.) mexicana* and *L. (L.) amazonensis* run under the same reducing conditions. Antigenic evaluation carried out by Western blot revealed the presence of two very specific *L. (V.) braziliensis*-GPI-anchored protein bands of 50 kDa and 28 kDa. These bands were specifically recognized by anti-*L. (V.) braziliensis*-GPI-anchored protein serum from experimentally immunized animals. These two peptides were not detected when GPI-anchored protein fractions from *L. (L.) donovani, L. (L.) mexicana* and *L. (L.) amazonensis*, were challenged with the same anti-serum. The present results lead us to suggest the use of these two peptides as biochemical markers to identify and differentiate leishmaniasis caused by *L. (V.) braziliensis*. The lack of immunogenicity observed here with the peptide gp63, a very common protein detected in *Leishmania* species, is considered.
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Keywords: GPI; Membrane proteins; *L. (V.) braziliensis*; Biochemical marker

1. Introduction

The process of diagnosing leishmaniasis lacks, at present, of a unique diagnostic method to surely declare an individual infected by any of the *Leishmania* sp. Among the most popular techniques to diagnose infected people the leishmanin–skin test (LST), the parasitological and the serological methods appear to be the more used ones (Liew and O'Donnell, 1993). Regarding to serological tests for leishmaniasis most of them are based on crude antigens (Ag) from promastigotes culture forms to perform IFAT, ELISA, IP, DA and Western blot, among others, which are still far from reliable (Isaza et al., 1997; Añez et al., 2007). Looking for solving the problem of reliability of the diagnostic methods several glyco-conjugates have recently been identified and characterized from the surface of *Leishmania* promastigotes. Among these, lipo-phospho-glycan, glycosyl-inositol-phospholipids and other surface proteins like those recognized as gp63, gp46/m2 and PSA-2 have been tested (Handman and Goding, 1985; Oriandi and Turco, 1987; McConville and Bacic, 1988). The described surface molecules appear to be similarly anchored to the membrane of *Leishmania* by glycosyl-phosphatidyl-inositol (GPI), although in a different structural way (Ferguson, 1997, 1999; Schneider et al., 1990; Low, 1989). This kind of anchoring to the surface has been

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described in many microorganisms, specially *Trypanosomatidae* (Ferguson, 1997, 1999; Añez-Rojas et al., 2006). Among glycoproteins, gp63 has been recognized as one of the more abundant proteins detected in the surface of *Leishmania* promastigotes. This protein has been characterized as a metal-protease, incriminated in the resistance to complement mediated lyses and to the adhesion to macrophages (Brittingham et al., 1995; Russell and Wilhelm, 1986). On the other hand, gp63 has also been considered as an immune-specific Ag to diagnose leishmaniasis (Heath et al., 1987).

The present paper deals with the isolation, purification and biochemical characterization of GPI-anchored membrane proteins from promastigote culture forms of *Leishmania (Viannia) braziliensis*. Its protein pattern was also compared to those detected in *Leishmania (Leishmania) mexicana, L. (L.) amazonensis* and *L. (L.) donovani* in an attempt to discriminate among them. In addition, assays on the antigenic evaluation of *L. (V.) braziliensis*-GPI-anchored membrane proteins were performed in order to select proteins acting as potential candidates to be used as biochemical markers in specific serological diagnosis.

2. Materials and methods

2.1. Parasites

Promastigote forms kept in NNN culture medium from four isolates of *L. (V.) braziliensis* were selected to carry out the present work. Prior to the experiments, identification of the parasites was corroborated by a *L. (Viannia)*-specific PCR assay as previously indicated (Guevara et al., 1992). The *L. (V.) braziliensis* isolates were identified as MHOM/Ve/75/LMR-75; MHOM/Ve/90/ZC-90; MHOM/Ve/80/CEM-80; MHOM/Ve/87/AS-87; all of them isolated and maintained at the laboratory for parasitological research, Faculty of Science, Universidad de Los Andes, Mérida, Venezuela. Similarly, international reference isolates of *L. (L.) mexicana* (MHOM/Br/00/JdO-00 and MORY/Pa/71/OC-71), *L. (L.) donovani* (MHOM/SD/00/Kartoum-00) and *L. (L.) amazonensis* (IFLA/Br/67/PH8), were included in this study for comparison purposes. In all cases parasites were kept at 25 °C until they grew up to obtain masses of flagellates (13×10^8/ml). Once the culture reached the exponential phase, the parasites were collected and spun at $3000 \times g$ at 4 °C for 15 min and then washed three times in PBS, pH 7.4 to collect a pellet, for each isolate.

2.2. Isolation and purification of L. (V.) braziliensis-GPI-anchored membrane proteins

GPI-anchored membrane proteins from the four chosen isolates of *L. (V.) braziliensis* were obtained using the partition Triton X-114 method described by Ko and Thompson (1995) and recently modified by Añez-Rojas et al. (2006). This methodology allowed us to obtain three different fractions separated according to their solubility in the detergent used. These included the hydrophilic proteins (HP), the integral hydrophobic proteins (hp) and the GPI-anchored proteins (for details see Ko and Thompson (1995) and Añez-Rojas et al. (2006)). Similar procedure was carried out to obtain GPI-anchored proteins from the isolates of other reference species used in this work.

2.3. Protein measurement

Concentration of protein fractions (HP, hp and GPI) was estimated according to Lowry et al. (1951) using bovine serum albumin as standard.

2.4. Gel electrophoresis

L. (V.) braziliensis-GPI-anchored proteins gel electrophoresis was carried out on 12% sodium dodecyl sulphate polyacrylamide (SDS-PAGE) on a 0.75 mm thick minigel in a Mini-Protean system (Biorad®) and stained with Coomassie blue R-250 as described by Laemmli (1970). Gels were run at constant voltage (100 V). GPI-anchored protein fractions were precipitated with 3 volume of cold acetone and re-suspended in sampling buffer (0.2% SDS, 10% glycerol, 168 mM Tris–HCl, pH 6.8). Samples were heated at 100 °C for 5 min in the presence or absence of 100 mM DL-dithiothreitol (DTT), in order to obtain samples under reducing and non-reducing conditions, respectively. The molecular mass markers were from Biorad®.

2.5. Rabbit immunization with L. (V.) braziliensis-GPI-anchored membrane proteins and detection of polyclonal antibodies

Immunizations were carried out in rabbits with extracts of *L. (V.) braziliensis*-GPI-anchored membrane proteins following a schedule of three challenges, 15 days each. The first immunization was done by subcutaneously inoculating 80 μg of protein, followed by a second and third challenge with 40 μg and 60 μg of GPI-anchored membrane proteins, respectively. Prior to inoculation, proteins were re-suspended in PBS and mixed 1:1 with Freund's adjuvant (v/v) to a 1 ml final volume. The rabbit serum anti-*L. (V.) braziliensis*-GPI-anchored proteins was collected 15 days after the last immunization. The anti-serum was then titered using three different serological methods including IFAT (Camargo, 1966), TAD and ELISA (Añez et al., 2003).

2.6. Biotinylation of L. (V.) braziliensis promastigote surface proteins

L. (V.) braziliensis promastigotes at exponential phase from NNN culture medium were collected and used for biotinylation of surface proteins. They were washed three times with cold PBS pH 7.2 and re-suspended at a concentration of 1×10^8 promastigotes/ml. They were later added with 0.5 mg/ml sulpho-NHS-LC-biotin, incubated 20 min on ice with soft agitation and then washed three times in the same solution as recommended by Altin and Pagler (1995).

2.7. Western blot

After purification of biotinylated parasites from different isolates, a SDS-PAGE was carried out with the separated *L.*

(V.) braziliensis-GPI-anchored protein fractions, which were electro-transferred to PVDF membrane (Immobilon P) and located by Western blot using ExtrAvidin® peroxidase conjugate and developed with 3,3′-diaminobenzidine and H_2O_2. On the other hand, the non-biotinylated GPI-anchored membrane protein fractions of *L. (V.) braziliensis*, were blocked 1 h with buffer 10 mM Tris–HCl, 150 mM NaCl, pH 8 supplemented with 2% bovine serum albumin (BSA), and then washed three times for 15 min with tris-buffered-saline (TBS). They were incubated 1 h with anti-GPI rabbit serum 1:100 in the same buffer and then washed three times for 15 min each. In this case the electro-transferred membranes (Immobilon P) were incubated 30 min with IgG-peroxidase anti-rabbit conjugate, washed and developed with 4-chloro-1-naphthol and H_2O_2.

3. Results

3.1. Isolation, purification and characterization of *L. (V.) braziliensis*-GPI-anchored proteins

Several repeated assays revealed a well stabilized and reproducible GPI-anchored protein patterns with the isolates of *L. (V.) braziliensis* used during the study. This indicates the reliability of the separation methodology and its reproducibility to obtain specific GPI-anchored protein fractions. The estimation of the solubilized membrane proteins of the *L. (V.) braziliensis* isolates was about 1% from the total cellular proteins in all cases. The presence of *L. (V.) braziliensis*-GPI-anchored membrane protein fractions was evidenced in biotinylated parasites under reducing (50 kDa and 28 kDa) and non-reducing (>100 kDa, 50 kDa and 28 kDa) conditions and are shown in Fig. 1. The results also revealed that the migration pattern observed in SDS-PAGE for the *L. (V.) braziliensis*-GPI-anchored membrane proteins were modified in the presence of DTT as a reducer agent which

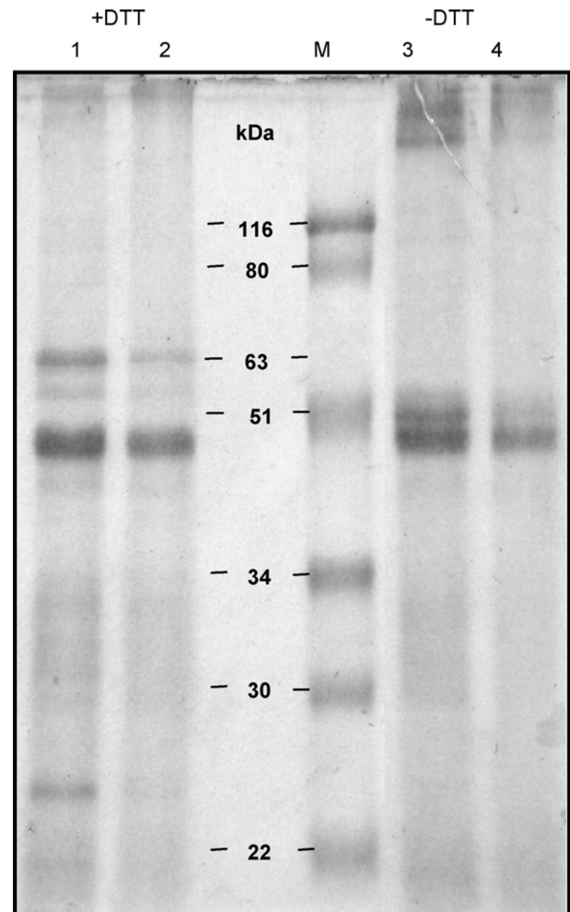

Fig. 2. *L. (V.) braziliensis*-GPI-anchored membrane protein migration patterns in SDS-PAGE under reducing (+DTT) and non-reducing (−DTT) conditions and stained with Coomassie blue. Lanes 1–2: +DTT (1) MHOM/Ve/75/LMR-75 −50 μg and (2) MHOM/Ve/90/ZC-90 −50 μg; Lanes 3–4: −DTT same isolates order and fraction amounts; M: molecular mass marker.

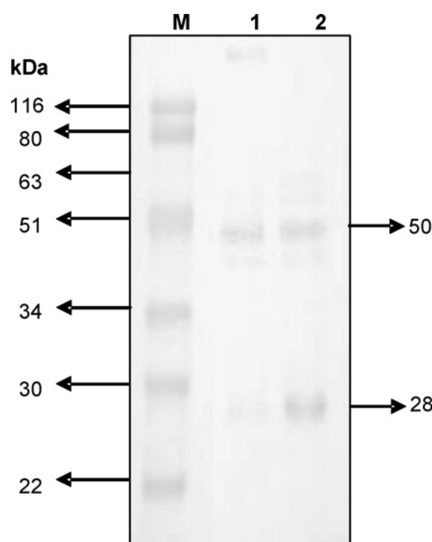

Fig. 1. Detection of GPI-anchored membrane proteins in biotinylated promastigotes of *Leishmania (Viannia) braziliensis* (MHOM/Ve/75/LMR-75) under reducing (+DTT) and non-reducing (−DTT) conditions. Western blot developed with ExtrAvidin®-peroxidase conjugate. M: molecular mass marker; Lane 1: 15 μg −DTT; Lane 2: 15 μg +DTT.

showed a different migration pattern than that observed in the protein fractions treated under non-reducing (−DTT) conditions (Fig. 2). This appears to be evident when, in the absence of DTT as reducer agent, the GPI-anchored proteins pattern showed a strong group of bands over the 100 kDa together with two more bands of 50 kDa and 52 kDa in the *L. (V.) braziliensis* isolates. On the contrary, in the presence of DTT the major GPI-anchored proteins were detected as bands of 63 kDa, 50 kDa and an increase of peptides between 34 kDa and 22 kDa in Coomassie blue stained gels. Details on the observed differences are shown in Fig. 2. Replication of the experiments revealed that the observed bands over 100 kDa under non-reducing conditions appears to be a high molecular mass complex, which is disjointed and split up by the effect of DTT as a reducer agent. This is demonstrated by the disappearance of the high molecular mass complex and the appearance of a higher intensity in peptides under the 34 kDa as observed in *L. (V.) braziliensis* isolates when DTT was used (Fig. 2). In addition, in biotinylated GPI-anchored protein fractions of *L. (V.) braziliensis* an increase in intensity of the band of 28 kDa molecular mass and the absence of the high molecular mass complex under reducing conditions (+DTT) appear to corroborate these observations

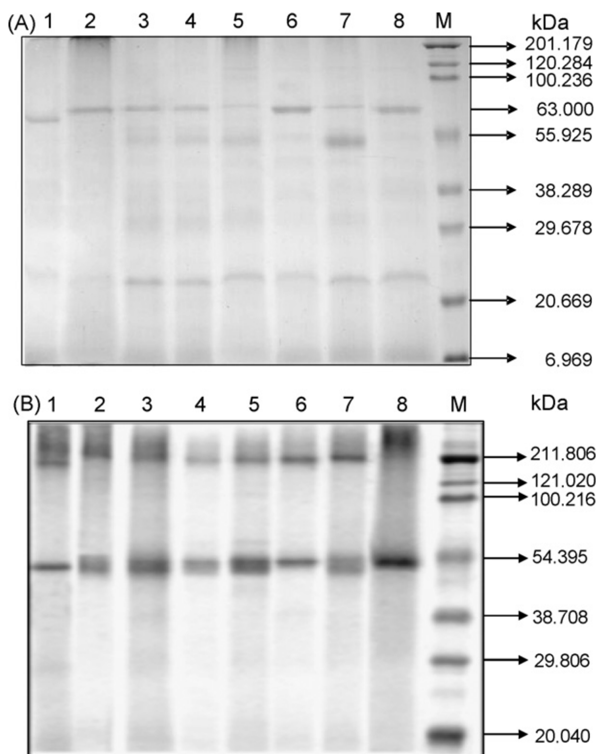

Fig. 3. (A) GPI-anchored membrane protein fractions from different *Leishmania* species detected in SDS-PAGE under reducing conditions (+DTT) and stained with Coomassie blue. Each lane with 30 μg protein with 100 mM DTT. Lane 1: *Leishmania (Leishmania) donovani* (MHOM/SD/00/Kartoum; Lane 2: *L. (L.) amazonensis* (IFLA/Br/67/PH8); Lane 3: *L. (V.) braziliensis* (MHOM/Ve/75/LMR-75); Lane 4: *L. (V.) braziliensis* (MHOM/Ve/90/ZC-90); Lane 5: *L. (V.) braziliensis* (MHOM/Ve/80/CEM-80); Lane 6: *L. (L.) mexicana* (MHOM/Br/JDO); Lane 7: *L. (V.) braziliensis* (MHOM/Ve/87/AS-87); Lane 8: *L. (L.) mexicana* (MORY/Pa/71/OC-71); Lane M: molecular mass marker. (B) As in (A) but under non-reducing conditions (−DTT).

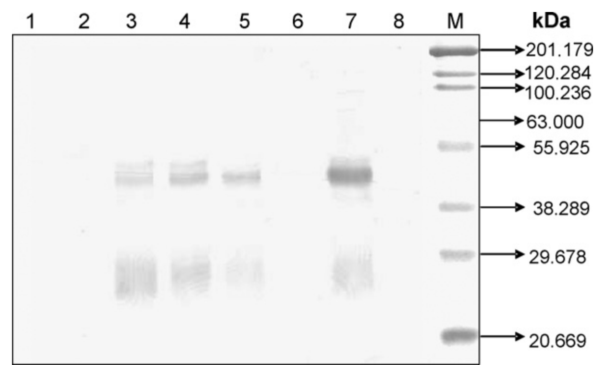

Fig. 4. Immunogenicity of GPI-anchored membrane proteins from different *Leishmania* species. Western blot revealed with anti-*L. (V.) braziliensis*-GPI-anchored protein sera. Each lane with 15 μg protein fraction in presence of 100 mM DTT. Lane 1: *L. (L.) donovani*; Lane 2: *L. (L.) amazonensis*; Lanes 3–5 and 7: *L. (V.) braziliensis*; Lanes 6 and 8: *L. (L.) mexicana*. Isolates identification as in Fig. 3.

(Fig. 1). Similar results were observed when different species of *Leishmania* were compared. In Fig. 3 different patterns detected in the GPI-anchored membrane proteins migration in various species of *Leishmania* in the presence (Fig. 3A) or absence (Fig. 3B) of DTT, are shown. These patterns may be used as a tool to discriminate among species of this parasite.

3.2. Identification of immunogenic L. (V.) braziliensis-GPI-anchored membrane proteins

Prior to these experiments the titters of antibodies anti-*L. (V.) braziliensis*-GPI-anchored proteins in immunized rabbit sera were measured using three different serological methods. Titters of 1:2048, 1:4096 and 1:800 were respectively detected when IFAT, DAT and ELISA were used, which indicates a good response to the respective antigens.

Western blots revealed the response to rabbit immune-sera anti-*L. (V.) braziliensis*-GPI-anchored protein fractions generated when challenged with the GPI-anchored membrane protein of different *L. (V.) braziliensis* isolates. In this case, from the major GPI-anchored membrane proteins detected under reducing conditions (see Fig. 3), only two peptides of 50 kDa and 28 kDa revealing immunologic activities were identified (Figs. 1 and 4). Similar results were obtained when sera from patients infected with *L. (V.) braziliensis* were used (results no shown). In no cases in the present work the commonly reported *Leishmania* peptide of 63 kDa, was detected in Western blot performed under reducing conditions (+DTT). This fact may suggest the lack of immunogenic activity of this protein when obtained in the presence of reducer agents as DTT. No bands at all were detected when GPI-anchored membrane protein fractions of isolates of *L. (L.) donovani, L. (L.) mexicana* and *L. (L.) amazonensis* were exposed to the *L. (V.) braziliensis*-GPI-anchored protein rabbit immune-sera, indicating a high specificity in the response. Details on the results of the Western blot carried out using GPI-anchored membrane protein fractions from eight isolates of four different species of *Leishmania* in the presence of anti-*L. braziliensis*-GPI-anchored protein rabbit sera are shown in Fig. 4.

4. Discussion

The *Leishmania*-GPI-anchored membrane proteins analyzed in the present work were identified by its SDS-PAGE migration pattern, which showed 2–3 major peptides and molecular mass similar to those previously obtained by other researchers in surface proteins by radio-iodionation and ulterior immune-precipitation (Lemesre et al., 1985; Heath et al., 1987; Hernandez et al., 1989; Araújo Soares et al., 2003). In our system, confirmation of the presence of GPI-anchored proteins was carried out by using biotinylated *Leishmania*-culture forms to obtain biotin marked GPI-anchored proteins. Thus, the obtained results surely indicated the presence of GPI-anchored protein fractions and their location in the plasma membrane of *Leishmania* promastigotes used during the experiments. The present results also allowed us to demonstrate that the presence of DTT, as a reducer agent, was able to modify the migration pattern detected in SDS-PAGE for the *L. (V.) braziliensis*-GPI-anchored membrane proteins.

It is possible to explain the above indicated differences considering that, by the effect of the reducer agent (DDT), the GPI-anchored membrane proteins of different *Leishmania*

species may modify their molecular mass as a consequence of the disruption of the disulphur-bond, transforming the protein structure and allowing the displacement through the gel, in which bands at different levels are detected, which are not seen under non-reducing conditions.

Regarding the detection by Western blot of immunogenic activity of *L. (V.) braziliensis*-GPI-anchored membrane proteins obtained under reducing conditions, our results revealed the presence of two very specific protein fractions detected at 50 kDa and 28 kDa, which were specifically recognized by the anti-*L. (V.) braziliensis*-GPI-anchored protein serum obtained from an experimental model. Similar results were observed using sera of patients suffering leishmaniasis from endemic areas in Venezuela (results will be shown elsewhere). Neither other GPI-anchored protein fractions from four different *L. (V.) braziliensis* isolates, nor protein fractions from *L. (L.) donovani, L. (L.) mexicana* and *L. (L.) amazonensis*, were able to react against the specific antibodies from *L. (V.) braziliensis*-GPI-anchored membrane protein. Undoubtedly, the proven specific antigenicity and sensitivity of the GPI fractions obtained under reducing conditions lead us to consider the polypeptides of 50 kDa and 28 kDa present at the *L. (V.) braziliensis*-GPI-anchored membrane protein fraction, as biochemical markers to identify and differentiate tegumentary leishmaniasis caused by *L. (V.) braziliensis*.

Contrary to previous reports regarding the activity of the gp63 protein in *Leishmania*, in our system this protein was not recognized by the anti-*L. (V.) braziliensis*-GPI-anchored membrane proteins. This fact lead us to suppose a modification of the epitopes present under non-reducing conditions, which may explain the high specificity of the immune-serum to recognize specifically the two peptides of 28 kDa and 50 kDa, whose epitopes were not modified. This fact also appears to indicate that the conformational structure of the GPI-anchored membrane proteins may vary according to the reducing condition where the parasite's proteins are maintained. This also may indicate that the antigenic capability of the major GPI-anchored membrane proteins in *Leishmania* should depend on the disulphur-bonds, which could bring important consequence on the host immune response. This change in the antigenicity appears to be very similar to that observed when it is schismed the GPI-anchor of the protein as previously reported (Butikofer et al., 2001; McGwire et al., 2002). In conclusion, the effect caused by DTT as reducer agent on *Leishmania*-GPI-anchored membrane proteins are expressed by the modification of their molecular mass, and by the observed changes in the obtained patterns when immune-detection methods are used.

Finally, to explain the lack of immunogenicity observed with the gp63, a very common protein detected in all the species of *Leishmania* under non-reducing conditions, it is very likely that the presence of cystein and proline may confirm that gp63 has a tertiary structure maintained by disulphur-bonds. This seems to be the case since all the gp63 sequences in different *Leishmania* species have a high similarity in their aminoacid sequences (BLAST), with a high level of conservation at the 20 cystein and 11 proline positions. Similar results have been reported comparing gp63 sequences in *Crithidia fasciculata, Trypanosoma brucei* and *Leishmania guyanensis* (El-Sayed and Donelson, 1997). This common structure may explain some results including: (i) reduction of the *Leishmania*-gp63 migration in PAGE under reducing conditions, (ii) cross reaction between different gp63 when used as specific Ag for detecting leishmaniasis and (iii) the lack of reports on gp63 immune-detection under reducing conditions.

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