MB-1 – CHARACTERIZATION OF THE LARGEST SUBUNIT OF RNA POLYMERASE II OF *TRYPANOSOMA CRUZI*

Ejchel, T.F. , Ramirez, M.I. & Schenkman, S.
Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, R. Botucatu 862, 8º andar, São Paulo, SP, Brasil.

In higher eukaryotes transcription of protein coding genes is mediated by RNA polymerase II (polII). The largest subunit of this enzyme contains a carboxy-terminal domain that is formed by repeated serine-rich heptapeptides. Phosphorylation of this domain plays an important role in the transition between transcription initiation and elongation, the coupling of transcription with splicing, and termination. In contrast to most eukaryotes, in Kinetoplastidae a typical carboxy-terminal domain is absent. This could explain the polycistronic transcription by pol II-like enzyme in these organisms. Here, we have started to characterize the largest pol II subunit in *Trypanosoma cruzi*, since there is a decrease in transcription when the parasite differentiates into non-proliferative and infective forms. Southern blots of genomic DNA digested with various enzymes hybridized with a probe corresponding to the amino-terminal region of *Trypanosoma brucei* pol II. Similar bands hybridized with a 180 bp PCR fragment based on the *T. cruzi* EST (TENF0479), suggesting that this EST corresponds to pol II in *T. cruzi*. This fragment contains 56% similarity to the carboxy-terminal domain of *T. brucei* pol II and encodes for a peptide that lacks the typical heptapetides. The probes generated in this study will allow us to screen libraries to clone the entire pol II gene of *T. cruzi*.

Supported by FAPESP, CNPq

MB-2 – RNA STRUCTURE AND POST-TRANSCRIPTIONAL REGULATION OF α AND β-TUBULIN GENES OF *TRYPANOSOMA CRUZI*

Bartholomeu, D.C.; Silva, R.A.; Batista, M.G.; Galvão, L.M.C.; Donelson, J.E. & Teixeira, S.M.R.
Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil and Department of Biochemistry, University of Iowa, USA

Tubulins are cytoskeletal proteins present as components of pellicular, mitotic and flagellar microtubules in kinetoplastid protozoans. In several kinetoplastid parasite, including *Trypanosoma cruzi*, the differentiation process that occurs during their life cycle, involves a cytoskeleton reorganization that causes changes in the cell shape. In order to study the role of tubulin during differentiation, we proposed to determine the structure of the genes encoding alfa and beta tubulin subunits and to analyze their expression during the life cycle of different strains of *T. cruzi*.

Probes specific for α and β tubulin were obtained through PCR amplification using primers derived from a genomic sequence deposited in GenBank which contains part of the α/β tubulin gene cluster. They were used in Northern blot and nuclear run on analysis and for the screening of cDNA libraries. Cultures of three strains of *T. cruzi* (Tulahuén, Y and CL-Brener) were prepared and total RNA was purified from amastigotes, trypomastigotes and epimastigotes. For all three strains, Northern blot analyses showed that α and β tubulin mRNAs are more abundant in epimastigote when compared to amastigote and trypomastigote forms. As also shown by others, we have detected only one transcript of 2,0 kb corresponding to a-tubulin, while two transcripts with approximately 1,9 kb and 2,3 kb were detected using probes specific for β-tubulin. Accordingly, we have isolated, from a Tulahuém cDNA library, one class of a tubulin cDNA and two classes of cDNA encoding β-tubulin with main differences in their 3'UTRs. In at least one cDNA of each class we were able to map the SL addition site and poly-adenylation sites, thus determining the exact sequences of 5' UTR, 3' UTR and intergenic regions. In the Y strain, we have observed significant differences in RNA levels when the larger, 2.3 kb β-tubulin message is compared to the smaller β- tubulin mRNA during the three stages of parasite life cycle. Since nuclear run on assays indicated that α and β tubulin genes are transcribed to an equal extent in epimastigote and amastigote forms, we conclude that these genes are under a post-transcriptional control. Plasmid containing the luciferase reporter gene and sequences corresponding to the 3'UTR plus intergenic regions of α and β-tubulin transcripts have been constructed and will be tested in transient transfection assays to identify the sequences responsible for the post-transcriptional regulation of tubulin gene expression.

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MB-3 – GENE TRANSFECTION OF *TRYPANOSOMA CRUZI* WITH A MINOR CYSTEINE-PROTEINASE ISOFORM (CRUZIPAIN 2), BUT NOT WITH CRUZAIN, POTENTIATES TRYPOMASTIGOTE INFECTIVITY IN MAMMALIAN CELLS 'IN VITRO'

Lima, A.P.C.A., Schimtz, V.P., Aparício, I.M., and Scharfstein, J.
Instituto de Biofísica Carlos Chagas Filho, C.C.S., UFRJ, Rio de Janeiro, Brasil.

Cysteine proteases are important for *T. cruzi* survival since their inhibition blocks parasite development in infected cells (Meirelles *et. al*., 1992 Mol. Biochem. Parasitol. 52:175-184; Harth *et. al*., 1993 , Mol. Biochem. Parasitol. 58:17-24). Encoded by a large number (~130) of closely related polymorphic genes, these proteinases possess a papain-like central domain linked to an unusual C-terminal extension which is not essential for proteolytic activity. The major cysteine-proteinase (cruzipain or cruzain) is a cathepsin L-like lysosomal enzyme whose X-ray structure (catalytic domain) was recently solved (McGrath *et al.*, 1995 J. Mol. Biol. 245:251-259). The present study was motivated by earlier description of cDNA sequences (Lima *et. al*., 1994, Mol. Biochem. Parasitol. 67:333-338) which predicted the existence of isoforms containing non-conserved amino-acid substitutions in positions that could possibly affect their substrate specificity. These predictions were confirmed when one of these isoforms, namely cruzipain 2, was expressed in *S. cerevisae.* As reported elsewhere in this meeting (Reis, F. C. G., *et al.* 1999; Lima, A. P. C. A., *et al.*, submitted), cruzipain 2 exhibits marked differences from the cruzain archetype. The realization that this isoform is expressed preferencially in trypomastigotes and amastigotes (yet being detectable in epimastigotes) has encouraged the undertaking of phenotypic analysis of *T. cruzi* DM28c transfected with I) one full copy of the Dm28c cruzipain 2 gene, II) one copy of a full-length gene encoding a Dm28c cruzain-like isoform (93% aa. identity) or III) the cruzipain 2 gene lacking the C-terminal extension, cloned in the pTEX plasmid. The parasites were selected for growth in LIT in the presence of 200 or 800 ug/ml of G418 and, after 6 weeks, epimastigotes were differentiated into trypomastigotes upon incubation of stacionary phase cultures in Grace's insect cell medium for 5 days. Tissue culture trypomastigotes were then maintained by weekly passages in Vero cells and used in invasion assays. Our results showed a marked increase in mammalian cell invasion but these effects were only observed with *T. cruzi* lines that were transfected with a full length gene copy of cruzipain 2. Furthermore, we observed that the amastigote forms of these parasites, but not the ones transfected with the cruzain-like gene or with the cruzipain 2 gene lacking the C-terminal extension, grew faster than Dm28c wild type in the infected cell. Taken together, these results indicate that the the activity of cruzipain 2 isoform(s) may be involved in host cell parasite invasion and intracellular growth.

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MB-4 – CHARACTERIZATION OF A NEW MEMBER OF DNAJ-LIKE PROTEIN FAMILY IN *TRYPANOSOMA CRUZI*

Salmon, D., Carreira, M. A. C. and Goldenberg, S.
Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Av. Brasil 4365, Rio de Janeiro, R-J, 21045-900, Brasil.

In eukaryotes, DnaJ-like proteins belong to the highly conserved heat shock protein 40 (hsp40) family and are involved in regulation of the molecular chaperones hsp70 (DnaK-like protein) mediating the biogenesis of proteins. The basic mechanism of action of the hsp70s is the ability to bind and release polypeptides which are in non-native conformations in a ATP-dependent manner (protein folding); the sub-cellular compartmentalization of different hsp70 members and their specific interaction with different DnaJ-like proteins have allowed to these chaperones to be involved in a variety of protein biogenesis pathways including assembly and disassembly of protein complex, proteolysis, translocation of proteins into organelles and translation initiation. In *T. cruzi*, it has been described that the DnaJ-like family consists of 5 members (tcj1-4 and TcDJ1) sharing a highly conserved N-terminal sequence known as J domain which allows the association with hsp70 proteins. Recently, we have cloned and sequenced a gene encoding a novel *T. cruzi* DnaJ-like protein which has been isolated serendipitously in a *T. cruzi* genomic DNA library. This sequence contains an open reading frame of 1014bp encoding a 338 amino acid protein. In contrast to the other members of *T. cruzi* DnaJ-like family which are encoded by single copy genes, the novel DnaJ-like protein is encoded by multicopy genes which are not organized in tandem, as in the case of other *T. cruzi* hsp genes, but spread into the genome of the parasite. Moreover, the gene encoding the novel DNA-J like is present and highly conserved in five species of the subgenus Trypanozoon suggesting that this putative chaperonin might play a major role in the biogenesis processes of trypanosomatids (Bringaud et al., 1998). Northern blot analysis and semi-quantitative RTPCR revealed that this new trypanosome DnaJ-like gene is transcribed as a 1,4kb mRNA and that the level of expression of this steady state RNA increases during the *in vitro* metacyclogenesis. Sequence analysis indicates that the novel DnaJ-like encoding protein is related to a cytosolic DnaJ-like protein of *S.cerevisiae*, SIS1 (50% homology), involved in translation initiation. Related to this observation, the hydrophilic character of this DnaJ-like protein and the absence of both a detectable N-terminal putative peptide signal and a C-terminal CAAX motif (substrate for prenyl modification of some DnaJ-like proteins) suggest that this protein is probably cytosolic. Moreover, both yeast SIS1 protein and the new *T. cruzi* DnaJ-like protein share common a J domain followed by a glycin-rich region and they lack the CRR repeat region involved in the substrate binding of some DnaJ-like proteins. This unusual structural feature suggests that the SIS1 protein and this new DnaJ-like protein might have a related function. Herein, using a specific rabbit antibody raised against the his-tagged DnaJ-like protein without its J domain, we show by western blot analysis that this protein is present as a polypeptide of 36,5 KDa corresponding to the MW expected by analysis of the primary polypeptide sequence, hence suggesting that this protein is unmodified (not prenylated nor glycosylated). Moreover, its translation is developmentally regulated and follows the same expression pattern of mRNA expression during the metacyclogenesis. We are currently investigating by confocal microscopy the localization of the novel DnaJ-like T. cruzi protein in vivo. Preliminary results confirm a cytosolic localization.

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MB-5 – *TRYPANOSOMA CRUZI* GENOME INITIATIVE: THE MOLECULAR KARYOTYPE OF THE CHROMOSOMES OF *T. CRUZI* (CLONE CL BRENER) AND THE ASSIGNMENT OF CHROMOSOME MARKERS

Patricio E. Porcile[1], Marcia R. Santos[1], Mirian S. Carmo[1], Renata Torres de Sousa[1], Adeilton Brandão[2], Turan Ürméney[3], Edson Rondinelli[3], Hernan Lorenzi[4], Mariano J. Levin[4], W M. Degrave[2] and José Franco da Silveira[1].
1. Departamento de Micro, Imuno e Parasitologia, Escola Paulista de Medicina, UNIFESP, R. Botucatu 862, CEP-04023-032, SP, Brasil (email: porcilepat.dmip@epm.br); 2. Departamento de Bioquímica e Biologia Molecular, IOC, FIOCRUZ, RJ, Brasil; Instituto de Biofísica Carlos Chagas Filho, UFRJ, RJ, Brasil; 4. Ingebi, Buenos Aires, Argentina.

As part of the *Trypanosoma cruzi* Genome Initiative, we have hybridized chromoblots carrying *T. cruzi* chromosomal bands with a panel of cloned sequences (6 repetitive sequences, 42 genes encoding structural proteins and RNAs, 129 ESTs, 9 STSs).

The karyotype of the of *Trypanosoma cruzi* CL Brener clone was studied by pulsed gel electrophoresis (PFGE) in conditions that allowed 20 chromosomal bands to be detected (Cano et al. 1995, *Mol Biochem Parasitol 71*: 273). The hybridization patterns observed could be divided into four classes: i) markers hybridizing to a single chromosomal band; ii) markers hybridizing mapping two or more bands; iii) markers hybridizing to all bands; and iv) markers hybridizing to the slot only.

Thirty two ESTs hybridized exclusively to a single band, and about 40% of them were mapped on the megabase bands XX (3.5 Mb) and XIX (3.35 Mb). About 50% of the ESTs used in this work were mapped on two or more chromosomal bands. Several ESTs hybridized to two bands which may correspond to size-polymorphic homologous chromosomes. About 10% of the ESTs hybridized with all bands. Complete nucleotide sequencing of these ESTs showed the presence of repetitive sequences, like SIRE (Vazquez et al. 1994, *Mol Biochem Parasitol* 64: 327), at the 5' or 3' untranslated regions. It is noteworthy that 60% of the ESTs and genes encoding proteins or structural RNAs were mapped on the megabase chromosomal bands. The association of the majority of the ESTs and protein or RNA encoding genes studied in this work with the megabase chromosomes suggested that these chromosomes carry essential information of *Trypanosoma cruzi*. Finally, several ESTs hybridized only to the DNA trapped in the well that may correspond to kinetoplast maxicircle DNA. It is konwn that maxicircle kDNA does not migrate into a PFG.

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MB-6 – GENETIC DIVERSITY IN *TRYPANOSOMA CRUZI* ISOLATES FROM HUMANS, VECTORS AND RESERVOIR HOSTS IN THE DISTRICT OF BARCELOS, AMAZONAS STATE: EPIDEMIOLOGICAL IMPLICATIONS

Pacheco RS[1]; Araujo, IB[2]; Pires, MQ[1]; Silva, AR[1]; Carrara, NC[2]; Santos, LC[2]; Junqueira, ACV[2] & Coura, JR[2]
1-Dept. Bioquímica e Biologia Molecular, 2- Dept. Medicina Tropical, Instituto Oswaldo Cruz - FIOCRUZ

In rural areas of the district of Barcelos , northern part of the State of Amazonas, Brazil, cases of Chagas disease are sporadic and the human infection can be ascribed to either accidental contact with infected sylvatic vectors or to human migrations. However, it was recently reported (Coura et al.. 1994 *Rev. Soc Bras. Med. Trop. 27*: 251-253; Coura et al. 1996 *Trans.R.Soc.Trop. Med.Hyg. 90*: 278-279) the "attacks" by *Rhodnius brethesi* on "piaçava" (*Leopoldinia piassaba*) gatherers in a rural locality on a tributary river of the Rio Negro, district of Barcelos. In the present study, the genetic diversity, of 22 *Trypanosoma* isolates from *R. brethesi* (16), *Didelphis marsupialis* (2) and humans (4), proceeding from Barcelos and different "piaçava" areas in that district, was demonstrated by multilocus enzyme electrophoresis (MLEE) using a system of 11 enzymatic loci, restriction fragment length polymorphisms of kDNA (RFLP) and molecular hybridization analyses. Aiming to contribute to the epidemiology and to the understanding of the dynamics of the disease' transmission in such areas the pheno and genotypic typing of the circulating strains was carried out adding further information to previous studies (Coura et al. 1996 *loc. cit.*; Fernandes et al. 1998 *Am.J.Trop.Med.Hyg. 58*: 807-811). The heterogeneity of the *T. cruzi* isolates was remarkable at both nuclear and mitochondrial DNA levels. MLEE and phenetic analyses have allowed the discrimination of the isolates into two distinct phenetic groups separated by a coeficient of similarity lesser than 0.25. Among the circulating *T. cruzi* populations 6 distinct isoenzymatic patterns were distinguished. Although 62.5% of the isolates from *R. brethesi* had been clustered within the group of sylvatic zymodemes (Z1), three phenotypic patterns , one identical to the Z1 prototype and two Z1 variants could be included in the same cluster. The corresponding Z1 variant zymodeme of *T. cruzi* was found infecting opossuns and triatomines in a same area. In addition, such Z1 variant zymodeme seems to be a wide spreading genotype as it was found in vectors and reservoir hosts in Barcelos and in "piaçava" areas. *T. cruzi* populations recovered from human together with three isolates from *R. brethesi* were clustered in a second group. In this group, zymodemes detected in humans and vectors were distinct suggesting, at least by this parameter, that such *T. cruzi* population found in *R. brethesi* was not responsible for human infections. Likewise, no association was found neither with Z1 nor with Z2 zymodemes. One out of the four human isolates studied was typed as an isoenzymatic variant of the San Agostin reference strain of *T. rangeli* and clustered in a separated group. Results were confirmed by hybridization with specific probes. This finding corroborates previous data on PCR amplification of mini-exon gene (Coura et al 1996 *loc. cit*). Genotypic heterogeneity was detected in human isolates by RFLP analyses. The patterns obtained have shown more polymorphisms in *T cruzi* than in *T. rangeli* populations. Interestingly, parasites retrieved from a vector displayed the same RFLP pattern when compared to the human *T. rangeli* isolate. Subsequent hybridizations with a subgenus *Schizotrypanun* specific oligonucleotide (Pacheco et al. 1996 *Parasite 3*: 207-209) also revealed no sequence homology. The data reported in this study certified the high heterogeneity of *T. cruzi* populations that circulate in sylvatic environments. Further investigation will be necessary to correlate genetic diversity with clinical and biological peculiarities.

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MB-7 – MOLECULAR ANALYSIS OF THE GENETIC VARIABILITY OF BRAZILIAN *TRYPANOSOMA CRUZI* ISOLATES

Santos, S.S. , Cupolillo, E*, Junqueira, A. C. V. , Coura, J. R., Fernandes, O./**
Laboratório de Doenças Parasitárias, Departamento de Medicina Tropical, Instituto Oswaldo Cruz, Fiocruz., Av. Brasil, 4365, Rio de Janeiro; Departamento de Imunologia, IOC, Fiocruz *; Departamento de Patologia, UERJ.**

The basis for the clinical and epidemiological variability of Chagas disease is unclear. Genetic differences among parasite strains are likely to determine the putative course of the disease in a given individual. We have been analyzing the ribosomal DNA internal transcribed spacer of *Trypanosoma cruzi* isolates from Amazonas, Piauí and Minas Gerais, in order to correlate the genetic variability of this locus with the geographical distribution of the parasite and the clinical presentation of the disease. Ribosomal RNA genes (rDNA) are highly conserved and have proven to be useful in phylogenetic analysis (Fernandes et al., 1994 *Mol. Biochem. Parasitol. 66*: 26221-271). Trypanosomatid rDNA exhibits an unusual organization where the coding regions for the three large and five small ribosomal RNA molecules are separated by internal transcribed spacers (ITS) that show extensive variability. ITS are relatively small and flanked by highly conserved segments to which PCR primers can be designed.

In this study, the ITS corresponding to non-coding regions between the small and the large sub-units of the rRNA gene were co-amplified by PCR with the 5.8S rDNA. The PCR products were further digested with restriction endonucleases and analyzed by acrylamide gel electrophopresis (RLFP). Quantitative similarities of the RFLP profiles were used to cluster the stocks. Although genetic polymorphism could be evidenced intra-cluster, a phenetic analysis of the results demonstrated a clear dimorphism of the isolates that corresponded to the geographical distribution of the stocks. The PCR products were cloned into the pUC18 vector to determine the DNA sequence in order to generate area-specific molecular probes and to perform phylogenetic analysis. The dimorphism found in this locus presents a strong correlation with the previously described major phylogenetic lineages of the parasite.

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MB-8 – BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF *TRYPANOSOMA CRUZI* ISOLATES FROM *TRIATOMA VITTICEPS* (STAL, 1859) (HEMIPTERA: TRIATOMINAE) NATURALLY INFECTED IN SANTA MARIA MADALENA AND CONCEIÇÃO DE MACABU (RIO DE JANEIRO, BRAZIL)

Gonçalves, T.C.M.[1], Nogueira, R.S.[2], Nehme-Russel, N.S.[3], Chaves, I.C.[3], Sousa, M.A.[1]
Departamentos de Entomologia[1], Protozoologia[2] e Bioquímica e Biologia Molecular[3], Instituto Oswaldo Cruz, FIOCRUZ, Avenida Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brasil.

Recently, 466 specimens of *Triatoma vitticeps* were collected in Santa Maria Madalena municipal district (465) and in a neighbouring district, Conceição de Macabu (only one specimen). The intestinal contents of those insects were examined under the optical microscope evidencing that nearly 60% of them were infected with flagellates (Gonçalves *et al.* 1998, *Mem. Inst. Oswaldo Cruz* 93: 711-717). Sixty-eight isolates were obtained in axenic cultures and 27 were identified as *T. cruzi* by the typical morphology of the metacyclics from culture and blood stages from experimentally infected mice, as well as by sensibility to complement lysis of their epimastigotes (Gonçalves *et al.* 1996, *Mem. Inst. Oswaldo Cruz* 91 Suppl.: 219). The aim of this study was to evaluate the parasite diversity in six sites (5 belonging to the municipality of Santa Maria Madalena and 1 to Conceição de Macabu). *T. cruzi* isolates are being characterized by biometric parameters, and also by molecular approaches (k-DNA minicircle fingerprints and molecular karyotype using pulsed-field gel electrophoresis). Morphometric analysis of epimastigotes and trypomastigotes from axenic cultures are being performed using one isolate from each site. Great genomic diversity among the isolates was evidenced by kinetoplast or nuclear DNA analysis, with the exception of two strains from distinct sites and belonged to the same schizodeme. These results evidence a complex epidemiologic situation in the regions under study, suggesting a great diversity of infection sources to *T. vitticeps* and emphasizing the sylvatic habit of this species.

MB-9 – *TRYPANOSOMA CRUZI*: TWO OR THREE GROUPS? ONE OR MORE SPECIES?

Carlos Robello [1],[2], Otto Pritsch[1], Alfonso Cayota[1], Francisco Gamarro [2], Santiago Castanys [2] and Fernando Alvarez-Valin [3]
[1] Depto. de Bioquímica, Fac. de Medicina, Montevideo, Uruguay
[2]Instituto de Parasitología y Biomedicina "Lopez-Neyra". Granada, España
[3]Sección Biomatemáticas, Fac. de Ciencias, Montevideo, Uruguay

For the purpose of investigating the evolutionary relationships among strains from the human parasite *Trypanosoma cruzi*, we have determined the nucleotide sequence (in 16 *T. cruzi* stocks) of a DNA fragment having approximately 1030 nucleotides in length. Phylogenetic analysis shows that *T. cruzi* contains at least three major groups of strains, a result that contradicts previous phylogenetic inferences based on data from polymorphism. On the other hand, we have performed a comparative analysis of the relative extend of nucleotide divergence in *T. cruzi* in relation to the divergence between Leishmania species using the gene encoding pteridine reductase. The results presented in this work unambiguously show that the most distant *T. cruzi* strains are at least as divergent as the complex of Leishmania species that contains *L. major* and that including *L. mexicana.*

MB-10 – KARYOTYPE VARIABILITY IN *TRYPANOSOMA CRUZI*: ANALYSIS OF ESTS GENERATED IN THE *T. CRUZI* GENOME PROJECT

Vargas, N., Pedroso, A., Silva, M.N., Schumacher, R.I. and Zingales, B.
Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Caixa Postal 26.077, CEP 05599-970 São paulo, SP, Brasil

A variety of biochemical and molecular approaches have confirmed the great genetic diversity in *T. cruzi* isolates A convergence among investigators regarding the clustering of *T. cruzi* strains into two principal groups exists. These groups have been designated TC1 (corresponding to: Zymodeme 1, Miles et al., 1977; Group 1, Tibayrenc, 1995; Lineage 2, Souto et al., 1996; Type III, Andrade et al., 1974; Ribodeme II/III, Clark and Pung, 1994) and TC2 (equivalent to: Zymodeme 2; Group 2; Lineage 1; Type II; Ribodeme I, same refs.). The *Trypanosoma cruzi* Genome project was launched in 1994 by a Consortium that includes 15 laboratories in the North Hemisphere and in Latin America. CL Brener clone (TC2) was chosen as the reference organism, and its biological and molecular parameters as well as its karyotype have been characterized (Zingales et al., 1997; Cano et al., 1995). Presently almost 10,000 ESTs (Expressed Sequence Tags) are available for CL Brener. In order to further evaluate the karyotype variability in *T. cruzi* we investigated the distribution of approximately 30 ESTs and other cloned gene sequences in the chromosomal bands of the following strains and clones: CL Brener; Esmeraldo cl3; Y (TC2); NRcl3; SO3cl5; SC43cl1 (Group 1/2, Souto et al., 1996); Dm28c; G; Sylvio-X10cl1; YuYu (TC1). Total DNA content was determined by flow cytometry on SYTOX-Green and Propidium Iodide (PI)-stained individual clones. The values obtained are in the range of 172 to 200 fg DNA per cell. The chromosomal bands were separated by PFGE (Cano et al., 1995) and stained with ethidium bromide. The general conclusion was that TC2 and Group 1/2 karyotype is composed of bands of higher molecular size (0.45 to 3.5 Mbp) in relation to TC1 (0.55 to 2.8 Mbp). This observation was confirmed following hybridization with a *T. brucei* telomeric probe. Hybridization with ESTs and some cloned sequences gave three general patterns: (a) Recognition of a single chromosomal band of the same molecular size in all strains; (b) Recognition of chromosomal bands of the same size in strains of TC2 and Group 1/2 and with bands of lower molecular size in TC1; (c) Hybridization with two chromosomal bands (homologous chromosomes) in TC2 and Group 1/2 and, in most cases, with one chromosomal band of lower molecular size in TC1. Another interesting observation is that strains of Group 1/2 have the same hybridization pattern with all probes used so far. Analysis of three ESTs that were mapped in the same chromosomal band in CL Brener indicates that two of theses ESTs are located on the same band (of different sizes) in all isolates. Most probably these ESTs are genetically linked. On the other hand the third EST is localized in different chromosomal bands in some isolates. Data for each probe will be presented and discussed.

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MB-11 – CHROMATIN ORGANIZATION IN DIFFERENT *TRYPANOSOMA CRUZI* STAGES

Porto, R. M., Elias, M. C. Q. B.,. & Schenkman, S.
Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina-Universidade Federal de São Paulo, R. Botucatu 862, 04023-062, São Paulo, SP, Brasil

The organization of the chromatin plays an important role in the regulation of transcription and replication in eukaryotic cells. We have previously found that in *T. cruzi*, the nuclear structure varies between different stages of this parasite and reflects different transcription states. Transcription is diminished in infective and non-proliferative forms. These forms have nuclei rich in heterochromatin, while the nuclei of highly proliferative and non-infective forms has a large nucleolus and peripheral heterochromatin. In this report we have studied whether the nuclear organization are related to the chromatin structure. Nuclei of different parasite stages were treated with different concentrations of *Microccocal* nuclease (MNase) and the DNA fractionated by agarose gel electrophoresis. In all stages we found a typical nucleosome ladder pattern. However, we found that the chromatin of epimastigotes are more resistant to MNase than metacyclic and culture-derived trypomastigote. As the chromatin of epimastigotes and trypomastigotes are equally sensitive to digestion with DNase I, the different Mnase susceptibility might be due to the access of the enzyme to DNA located between each nucleosome. Therefore, we analyzed the histones in the different stages by Triton-Acid-Urea PAGE, which separates proteins according to their hydrophobic properties. Epimastigotes show a different pattern of histone H1 like proteins when compared with trypomastigote forms. These data suggest that differences in the MNase susceptibility reflects a different chromatin organization in the actively transcribing parasites.

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MB-12 – A *TRYPANOSOMA CRUZI* CDNA ENCODING A NEW AMASTIGOTE REPETITIVE ANTIGEN WITH HOMOLOGY TO RIBOSOMAL PROTEIN AND WITH A HISTONE-LIKE DOMAIN.

daRocha WD, Macêdo CDS, Vieira SB, Corradi RV, Bartolomeu, D.C., Donelson J.E., Teixeira SMR.
Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil and Department of Biochemistry, University of Iowa, USA

A cDNA expression library of amastigote of *T. cruzi* was screened with sera derived from two groups of patients: asymptomatic patients (AS), and individuals presenting cardiac manifestations typical of chronic Chagas disease, chagasic cardiopathy (CC). Among all positive clones isolated so far, approximately 62% of them were found to encode parasite antigens containing various tandemly repeated sequences, the majority of them has been previously characterized. Partial sequence analysis of all positive clones isolated so far showed that five cDNAs encode a new repetitive antigen with 61% similarity with the eukaryotic ribosomal protein L7a. Analysis of the complete sequence of one of these cDNAs reveals that it encodes a protein, named TcAg-29, containing a Lys-Pro-Ala-rich repeat (KPAA) which has not yet been described in *T. cruzi*. Sequence alignment with another, almost identical cDNA clone containing the SL sequence indicated that the large, 319 amino acid ORF encodes the complete protein predicted by the nucleotide sequence. The repetitive sequence, which is present at amino terminus of TcAg-29 is also found in histone H1 from *Chironomus dorsalis*. At the C-terminus, TcAg-29 presents significant homology to a regulatory protein (algP) from *Pseudomonas aeruginosa*. In addition, the amino acid sequence of *Tc*Ag-29 has three putative nuclear localization signals (NLS) and many phosphorylation sites for PKC and Casein kinase II. Based on these sequence comparisons, it has been hypothesized that TcAg-29 might have a regulatory function, involving a DNA-binding activity in the nuclei. Southern blot analysis revealed that TcAg-29 is encoded by a single gene in the genome of at least two strains of *T. cruzi*. Northern blot analysis showed that *Tc*Ag-29 is trancribed into a 1.3 kb mRNA that is present at the same level in all three forms of the parasite. We have also began the characterization of the antigenic properties of this molecule, using the recombinant protein expressed in *E. coli*. Western blot analysis showed that the recombinant antigen is present in *E. coli* extracts with an apparent molecular weight of 40 kDa and strongly reacts with sera from 50 % of patients with Chagas' disease. This reactivity is however, independent of the clinical manifestation of the disease. Since the recombinant TcAg-29 also reacts with sera derived from normal individuals, we suspected it might contain epitopes that are target for natural antibodies. To better characterized its reactivity and to prepare antibodies against the recombinant protein we have cloned distinct fragments encoding only the N-terminal or the C-terminal part of the molecule to be expressed as a fusion with His tags.

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MB-13 – MOLECULAR CHARACTERIZATION OF *TRYPANOSOMA CRUZI* AMASTIGOTE CDNA CLONES

Verbisck, N.V. [1], Araya, J. [2], Santos, M.R.M.[1], Engman, D.[3], Mortara, R. A. [1], Franco da Silveira, J. [1]
[1]Departamento de Micro, Imuno e Parasitologia, UNIFESP, Rua Botucatu, 862, São Paulo 04023-062, SP, Brasil (email: newtonvisk.dmip@epm.br); [2]Dept. Tecnologia Médica, Universidad de Antofagasta, Antofagasta, Chile; [3]Departments of Pathology and Microbiology-Immunology, Northwestern University Medical School, Chicago, Illinois, USA

T. cruzi amastigote expresses specific surface membrane proteins that might be targets of humoral and cell-mediated immune responses, and might be involved in the interaction of the parasite with mammalian cells. We have previously screened a *T. cruzi* (Sylvio X-10/4 strain) amastigote cDNA library with Tt34c1 probe, that encodes a trypomastigote stage-specific 85-kDa surface glycoprotein (gp85) previously described (Takle & Cross, *Mol Biochem Parasitol*, 48:185, 1991), and 4 recombinant clones (Sx23, Sx12, Sx38 and Sx42) were isolated and characterized. Northern blot analysis showed that transcripts homologous to clone Sx23 are expressed in amastigotes and trypomastigotes whereas sequences homologous to clones Sx12, Sx38 and Sx42 are expressed only in the amastigote stage.

Clone Sx23 encodes a polypeptide that shows a high degree of homology (77% of identity) with the carboxy-terminal domain of the gp85, gp90 and Tc-85 surface glycoproteins of the *Trypanosoma cruzi* gp85/sialidase superfamily. Clone Sx23 also displays >90% of identity at nucleotide level with gp85 sequences found at the subtelomeric regions (Chiurillo et al., *Mol Biochem Parasitol* 100:173, 1999). These results suggest that clone Sx23 encodes a member of the *T. cruzi* gp85/sialidase superfamily.

The sequences of cDNA clones Sx12, Sx38 and Sx42 show >90% of identity at nucleotide level with the 3' region of the cDNA Tt34c1, and with many *T. cruzi* telomere-associated sequences (Chiurillo et al., *Mol Biochem Parasitol* 100:173, 1999). The peptides translated from the open reading frames found in the clones Sx12, Sx38 and Sx42 do not share any homology with the members of *T. cruzi* gp85/sialidase superfamily. Sequences found in clones Sx12, Sx38 and Sx42 are present in 2.0-3.0 kb-transcripts of amastigotes. This result indicates that the 3' region of the gp85 gene (Tt34c1) is transcribed in the amastigote stage, and it may encode peptides that not belong to the gp85 family.

Southern blot hybridizations showed that sequences homologous to the clones Sx12, Sx23, Sx38 and Sx42 are present in multiple copies, and distributed in several chromosomes. cDNAs Sx42 and Sx23 can be found in a same region of the genome linked to the other members of *T. cruzi* gp85/sialidase superfamily.

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MB-14 – ISOLATION AND CHARACTERIZATION OF *TRYPANOSOSMA CRUZI* CDNA CLONES ENCODING AMASTIGOTE ANTIGENS RECOGNIZED BY SERA FROM CHAGAS' DISEASE PATIENTS

daRocha WD, Macêdo CDS, Vieira SB, Corradi RV, Cunha-Neto E*, Donelson JE**, Teixeira SMR.
Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brasil. * Instituto do Coração, Universidade de São Paulo, São Paulo, SP, Brasil ** Department of Biochemistry, University of Iowa, USA

Characterization of *Trypanosoma cruzi* antigens using recombinant DNA techniques has been limited to the screening of antigens expressed in epimastigote and trypomastigote stages of the parasite life cycle. Although an increasing list of experimental evidences is suggesting that *T. cruzi*-infected host cells are target by MHC class I-restricted CD8+ T-cells, very few studies focusing on antigens expressed in the amastigote stage have been reported so far. As a long term goal for the present study, we propose to evaluate a large number of recombinant antigens derived from *T. cruzi* amastigotes, according to the humoral and cellular immune responses they elicit in chronically infected chagasic patients. Sera derived from two groups of patients were used in our studies: asymptomatic patients (AS), that represent the majority of persons chronically infected with *T. cruzi*, and individuals presenting cardiac manifestations typical of chronic Chagas disease or chagasic cardiopathy (CC). From an expression amastigote cDNA library, we have isolated 72 positive clones, with the vast majority of them reacting equally with antibodies from both pools. cDNA inserts in various positive clones have been characterized and partial nucleotide sequences of 39 cDNAs have been determined so far. Forty eight percent of the clones were found to encode ribosomal proteins, 17,9% heat shock proteins, 15,3% encoding flagellar proteins and five cDNA clones (12,8%) showed no homology with previously known sequences. Two recombinant antigen (pA29, a novel repetitive *T. cruzi* antigen and a clone with sequence homologous to the flagellar protein, PAR-2) have been further characterized by Western blotting using serum from each one of the 12 patients from both groups. One of them, PAR-2 was produced as recombinant His-fusion protein in bacteria and submitted to affinity chromatography using Ni-NTA colums. The recombinant PAR-2 antigen reacts with sera from 7 patients of the AS group and 8 patients from CC group but not with healthy individuals. In addition, our preliminary Western blot results with recombinant PAR-2 antigen indicated that, in the majority of reactions with sera from CC patients, it can be observed a stronger response when compared with sera from AS patients.

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MB-15 – CLONING OF PUTATIVE Δ^{12}(ω6)-OLEATE DESATURASE FROM *TRYPANOSOMA CRUZI*

Rosa A. Maldonado[1]and Alan H. Fairlamb[2].
[1]Universidade de São Paulo, ICB II, Depto. de Parasitologia, 05508-900 SP-São Paulo, Brazil; [2]Dept. of Biochemistry, MSI/WTB, University of Dundee, Dundee DD1 5EH, UK.

Although total lipids represent approximately 20% of the dry weight of *T. cruzi*, very little is known of the lipid metabolism and its enzymes in this parasite. A previous study showed that epimastigotes forms can synthesize oleic (C18:1) and linoleic acid (C18:2) from stearic (C18:0) and oleic acid, respectively (Aeberhard, E *et al.*, 1981, Lipids, 16: 623-625). This result suggests the presence of $\Delta^9$and Δ^{12} desaturases in the parasite. Mammals are able to synthesize oleic acid from stearic acid, but are unable to synthesize the essential fatty acid linoleic acid, since they lack the Δ^{12} desaturase.

During attempts to clone the C14-sterol demethylase gene using PCR with degenerated primers, we obtained a PCR product with a sequence showing high homology to the Δ^{12} (ω6)-oleate desaturase from plants (Liu, Q *et al.*, 1999, Plant Physiol. 120: 340-340; Chen, B.Y. & Janes, H.W., 1995, Plant Physiol. 109: 1498-1498). The genomic clone of the Δ^{12} (ω6)-oleate desaturase from *T. cruzi* was obtained from a size selection library. Northern blot analysis indicated that the gene is transcribed in epimastigote and trypomastigote forms. This gene is present in more than one copy in *T. cruzi.* Pulsed field analyses at high stringency conditions showed that OD_{tc} is located in the chromosome of 610 kb in the strain X10/7 and Dm28c, while in the strains Cl and Y it was found in two chromosomes of 765 and 1.0 kb, respectively. At low stringency, pulsed field analyses showed the presence of this gene in *Crithidia deane*, *Herpetomonas megaseliae, and Endotrypanum* (LU88). The full characterization and expression of this gene is in progress.

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MB-16 – CONSTRUCTION OF AN EXPRESSION VECTOR FOR TRANSFECTION IN *TRYPANOSOMA CRUZI*

Fragoso, S.P.[1], Carreira, M.A.C.[1], Buck, G.[2], Goldenberg, S.[1]
(1) Laboratório de Regulação da Expressão Gênica, Departamento de Bioquímica e Biologia Molecular – Instituto Oswaldo Cruz – RJ and (2) Dept. Microbiology and Immunology, Virginia Commonwealth University, Richmond, Virginia, E.U.A.

The comprehension of the mechanisms of gene regulation operating in *T.cruzi* and the role that a specific gene plays in the biology of the parasite has increased by the recent advances in techniques for introducing exogenous DNA (transfection). The experiments in this field are based either on the measuring of the level of expression of reporter gene, flanked by putative regulatory sequences from the parasite, or on the knock out of a normal gene from the genome of the parasite, by replacing it with a gene encoding resistance to an antibiotic.

Another important tool in the study of the molecular aspects of the biology of *T.cruzi* is the construction of expression vectors. The construction of those vectors has taken advantage of many intergenic regions from trypanosomatids that may be used as a putative promoter. However the expression of genes from these vectors is transient because the plasmids lack a selection marker. Recently the ribosomal promoter from different strains of *T.cruzi* has been characterized and it has been used to drive the expression of reporter genes transiently.

We have constructed a *T.cruzi* expression vector (pTcEX) where the ribosomal promoter controls the expression of the gene of interest. The advantage of that plasmid, however, is that it has a selection marker (*neo* gene) that was cloned under the independent control of an upstream region of the TcTOP2 gene, which was found in our laboratory to be able to direct a high level of transcription of *neo* gene. In pTcEX the ribosomal promoter is followed by a 163 bp region obtained from the 5' untranslated region (5'UTR) of TcDJ1 gene (a member of the heat-shock DnaJ gene family) in order to provide the 3' splice site for mini-exon addition in the 5' end of the transcript from the cloned gene. The signal for poly-A tail addition in the transcript is provided by a 700 bp from the 3' untranslated region (3'UTR) of TcPEPCK gene (a gene constitutively expressed in *T.cruzi*). A BamHI cloning site is positioned between the 5'UTR-TcDJ1 and 3'UTR-TcPEPCK.

The vector pTcEX is being currently used to overexpress different stage-regulated genes in order either to determine their localization in the parasite using antibodies against myc tag or analyze their role in the triggering of the metacyclogenesis process. Besides that the plasmid is also being used to overexpress topoisomerase II enzyme fused with 6xHis tag sequence in order to permit its purification by Ni-NTA column, thus overcoming the problem of lacking of solubility or activity when the protein is expressed in bacteria or yeast.

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MB-17 – THE USE OF GREEN FLUORESCENT PROTEIN TO STUDY TRANSFECTION AND SELECTION OF EXOGENOUS PROTEINS IN *TRYPANOSOMA CRUZI*

Ramirez , M.I., Yamauchi, L.M., De Freitas , L.H., and Schenkman, S.
Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo - Escola Paulista de Medicina. R Botucatu 862. 8 andar, CEP 04023-062, São Paulo-SP, Brasil

We have previously constructed a modified pTEX vector containing green fluorescent (GFP) gene in fusion with the amino-terminal domain of histone (H2) gene under control of the 18S ribosomal RNA promoter (p33) to establish improved conditions to transfect and select transformants of *Trypanosoma cruzi*. This vector allows a rapid detection of GFP transiently expressed after electroporation, and a continuous monitoring of the growth of stable transfected parasites. Here we found that all class I strains express transiently GFP in the first few hours after electroporation. Strains CL, Tulahuen and F are particularly more susceptible to transient GFP expression. Expression is detected 6 hours after electroporation, peaks between 12 and 36 hours, depending on the strain, and disappears after 60 hours. Addition of geneticin just after transfection delays the transient expression for about 24 h, but also in this case fluorescent cells are not visible after 72 h. Addition of geneticin 12 h after electroporation, between 400 and 800 mg/ml, results in the recovery of fluorescence in most of the surviving parasites after 3-4 weeks. Lower, or higher concentration of antibiotic results in slow recovery. Addition of 10% of total human blood diminished the recovery time probably due to the presence of growing factors. When we used the same plasmid without the ribosomal promoter we could not see transient expression, and stable fluorescent parasites were recovered after longer periods of time in the presence of geneticin These results suggest that the low transfection efficiency in *T. cruzi* is due to the elimination of most of transfected plasmids in the first hours and that the transient expression is detected due to a high transcription level obtained with the ribosomal promoter.

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MB-18 – *TRYPANOSOMA CRUZI:* MOLECULAR CLONING OF A GENE CODING FOR A PUTATIVE VACUOLAR PROTEIN

Carlos Robello[a,b], Bruno Dallagiovanna[a], Alfonso Cayota [b], Otto Pritsch [b], Santiago Castanys[c], Francisco Gamarro[c] and Ricardo Ehrlich[a,].
[a] Departamento de Bioquímica, Facultad de Ciencias, Universidad de la República, Montevideo-Uruguay. [b] Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, Montevideo-Uruguay. [c] Departamento de Bioquímica y Farmacología Molecular, Instituto de Parasitología y Biomedicina “López Neyra”, CSIC, Granada-Spain.

We have characterized a putative vacuolar gene of *Trypanosoma cruzi* named Tc38. This gene codes for a 337 amino acid protein with a predicted molecular mass of 38 kDa and a significant homology to the plant storage vacuolar protein γ-3-hordein involved in the transport and targeting of prolamins to the vacuole of developing barley endosperm. Western Blot analysis using a polyclonal antiserum against recombinant *Tc38* revealed that the protein is differentially expressed in the different life stages of the parasite, showing a higher expression in the epimastigote stage. Immunofluorescence studies suggest that the protein is located in vacuolar structures in epimastigotes. Our results suggest that *Tc38* could be related to protein storage in *T. cruzi.*

MB-19 – SELECTION OF SPECIFIC TRANSCRIPTS BELONGING TO THE TWO MAIN PHYLOGENETIC LINEAGES OF *TRYPANOSOMA CRUZI*

Toma, H.K.; Yamada-Ogatta, S.F.*.; Fernandes, O.#Goldenberg, S.*. & Krieger, M.A.*
Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ. *Departamento de Bioquímica e Biologia Molecular, #Departamento de Medicina Tropical, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, RJ.

T. cruzi strains can be grouped in two distinct phylogenetic groups based on the heterogeneity of the intergenic region of their mini-exon genes. In order to identify differential transcripts between these two phylogenetic lineages, we are using the recently described method of Representation of Differential Expression or RDE (Krieger & Goldenberg, 1998 – Parasitol. Today 14: 163) for the amplification and cloning of lineage-specific genes.

This technique consists in the subtractive hybridization of cDNA populations of tester molecules with an excess of driver molecules. The non-hybridized tester molecules are then selectively amplified by PCR. In parallel we have used the genomic DNA from strain Y (representing the lineage 1) and F (representing the lineage 2) in subtractive amplification analysis. After DNA digestion with the restriction endonuclease BamHI, the populations were hybridized one against each other, with a molar excess of the driver population and selectively amplified by PCR. These subtractive amplifications were done for three cycles with increasing amounts of driver molecules. The molecules obtained by this technique were cloned in plasmid Bluescript and hybridized to test their specificity. Five clones specific to the Y strain and one to the F strain have been isolated so far. We are presently sequencing these clones and testing their specificity in different isolates of *T.cruzi*.

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MB-20 – CHARACTERIZATION OF TWO GENES SPECIFICALLY EXPRESSED DURING *T. CRUZI* METACYCLOGENESIS

Ávila AR; Krieger MA; Ogatta,SFY, Monteiro VS, Fragoso, SP. Dallagiovanna, B. and Goldenberg S
Departamento de Bioquímica e Biologia Molecular, Avenida Brasil 4365, Rio de Janeiro - RJ - Brasil, 21045-900.

We have developed a method, named Representation of Differential Expression (RDE), that is based on the PCR amplification of DNA sequences unique to a given cell population (tester), after hybridization to an excess of DNA from a related cell population (driver) to remove sequences common to tester and driver populations.

During the *in vitro* metacyclogenesis of *T.cruzi*, differentiating epimastigotes adhere to the culture flasks and are released to the culture medium upon transformation into metacyclic trypomastigotes. In order to characterize genes expressed at different times of the metacyclogenesis process, we have used cDNAs obtained from polysomal mRNAs of 24h-adhered epimastigotes and exponentially growing epimastigotes as tester and driver populations, respectively, in a RDE procedure.

A gene (24h-Ad) transcribing a 630 nucleotides mRNA that is not present in the polysomal fraction of epimastigotes, was fully sequenced and characterized in terms of its genomic organization. Search on the GeneBank showed that this gene did not display any homology to available gene sequences. Southern blot analysis and pulsed field gel electrophoresis indicated that it exists as low copy number gene in *T. cruzi* Dm28c. There are at least three copies of the 24h-Ad gene organized in tandem repeats of 2,8 kbp in the genome of *T. cruzi*. Interestingly, we have observed that the 2,8 kbp repeats contain at least a unknown gene in addition to the 0,6 kbp 24h-Ad gene. This gene transcribes a 1,3 Kb mRNA and its expression follows the expression pattern of the 24h-Ad gene. In order to gain further insight into the biological function of the 24h-ad gene, we are using two alternative procedures to determine the cellular localization of the encoded polypeptide. One of them consists in raising an antiserum against the gene product cloned in an expression vector and using the antiserum against the protein in cellular localization analysis by immunofluorescence. The other approach is to generate *T.cruzi* transfectants expressing a recombinant protein composed by 24h-ad and myc-tagged protein sequences, using a monoclonal antibody against the myc-tagged protein to localize the recombinant protein in the parasite.

Support: PRONEX, PADCT, CNPq, PAPES-FIOCRUZ

MB-21 – MOLECULAR CHARACTERIZATION OF A *T. CRUZI* MULTIGENE FAMILY DIFFERENTIALLY EXPRESSED DURING METACYCLOGENESIS

Dallagiovanna, B. Plazanet-Menut, C. Ogatta SFY*, Krieger MA, Ávila AR & Goldenberg S
Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Av. Brasil 4365, 21045-900, Rio de Janeiro, RJ, Brasil * *Departamento de Microbiologia, Universidade Estadual de Londrina, Centro de Ciências Biológicas, Londrina, PR, Brasil

T. cruzi metacyclogenesis can be mimicked in vitro using chemically defined medium (Contreras et al. 1985, MPB 16:315-327). Under these conditions differentiating epimastigotes adhere to the culture flasks and are released to the culture medium upon transformation into metacyclic trypomastigotes. The development of Representational Differential Expression (RDE) has lead to the cloning of *T. cruzi* stage-specific genes (Krieger and Goldenberg 1998, Parasitol Today 14: 163-166). The method consists in a selective amplification of genes specifically expressed by a given population (tester) following subtraction with a reference population (driver).

Using the RDE method with a tester DNA population consisting of PCR-amplified cDNA synthesized from mRNA extracted from adhered differentiating epimastigotes we selected one clone (clone88) whose expression is linked to the metacyclogenesis process. Genomic analysis showed that multiple copies of clone 88 are present in *T. cruzi* being most of them localized in a single high molecular weight chromosome. Genomic and cDNA libraries of *T cruzi* Dm28c clone were screened using clone 88 as a probe. Several genomic and cDNA clones were isolated and sequenced. All of the cDNA clones were fully processed displaying the mini exon and poly A sequences, pseudogenes were also found as part of this gene family. The nucleotide identities between the different members of the family sequenced ranged from 75 to 98 %. A short open reading frame was found in the different copies encoding for a cystein-rich peptide of an average of 60 amino acids.

Expression of the gene family during metacyclogenesis was studied at the mRNA level. While total RNA Northern blot showed that these genes are equally transcribed throughout the differentiation to trypomastigotes, analysis of the polysomal RNA showed that the mRNAs are lowly represented in epimastigotes being mobilized to the polysomal fraction during metacyclogenesis with a peak of accumulation at 24 hours.

Supported by PRONEX, PADCT, CNPq, CAPES and PAPES-FIOCRUZ

MB-22 – CHARACTERIZATION OF TWO GENES EXPRESSED BY METACYCLIC TRYPOMASTIGOTES OF *TRYPANOSOMA CRUZI*

Yamada-Ogatta SF*, Krieger MA, Toma, HK, Ávila AR, Monteiro VS., Linss, J., Fragoso, SP & Goldenberg S
Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Av. Brasil 4365, 21045-900, Rio de Janeiro, RJ, Brasil * Departamento de Microbiologia, Universidade Estadual de Londrina, Centro de Ciências Biológicas, Londrina, PR, Brasil

Previous work in our laboratory resulted in the development of the RDE method for the amplification and cloning of *T. cruzi* stage specific genes (Krieger & Goldenberg, 1998 – Parasitol. Today 14: 163). This method was applied for studying genes expressed specifically by metacyclic trypomastigotes obtained *in vitro*, using cDNAs obtained from polysomal RNAs extracted from trypomastigotes and epimastigotes of *T. cruzi* Dm28c as tester and driver populations, respectively.

After three cycles of subtractive amplification, the molecules obtained were cloned in plasmid Bluescript. As a result, we have obtained two clones, clone #10 and clone #30, with respective sizes of 0.8 kbp and 1,0 kbp. Northern blot analysis confirmed that these clones are expressed mainly by trypomastigotes. Both were fully sequenced and characterized in terms of their genomic organization. Southern blot analysis indicate these genes exist as low copy number in *T. cruzi* Dm28c. We are presently raising antisera against recombinant proteins derived from these genes in order to determine their precise localization within the parasite.

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MB-23 – CARACTERIZATION OF THE HSP10 GENE IN *TRYPANOSOMA CRUZI*

Fernandes M, Rondinelli E, Ürményi TP
Programa de Biologia Molecular, Instituto de Biofísica Carlos Chagas Filho CCS, Bloco G, Ilha do Fundão 21949-900 Rio de Janeiro, Brasil

The HSP10/HSP60 chaperone machine, a highly conserved protein apparatus, composed of HSP60 and HSP10 proteins, is responsible for helping celular proteins to reach their stable tridimensional conformation. The chaperone machine also reduces protein denaturation in stressing conditions and prevents the formation of protein aggregates. The presence of this chaperone machine is restricted to eubacteria, mitochondria and chloroplasts. It is believed that HSP60 and HSP10 form a barrel-shaped structure which encloses the postulated folding intermediates of a polypeptide substrate.

The *Trypanosoma cruzi* HSP60 gene has been previously isolated and caracterized in our laboratory (Giambiagi-de Marval *et al*, 1993 - MBP 59 : 25 - 31). In order to characterize the other component of the *T.cruzi* chaperone machine, HSP10, we used a cDNA clone obtained from the EST sequencing effort of the *T.cruzi* genome project (Verdun et al, 1998 - Infect. Immun. 66 (11) : 5393 - 5398). Preliminary northern blot experiments show an mRNA of about 0.5 Kb, the level of wich is increased by heat shock at 37°C and 40°C. We are currently performing Southern blot and genomic library screening experiments.

MB-24 – CHARACTERIZATION OF AN ARF FAMILY GENE IN *TRYPANOSOMA CRUZI*

Freire, A.S., Paixão, J.C., Lopes,U.G.
Instituto de Biofísica Carlos Chagas Filho - UFRJ

Arf is a Ras-related small monomeric GTPase, and is ubiquitary found across eukaryotic species being quite conserved in sequence. It was first described as a cofactor of cholera toxin, and has been best characterized as the molecule that controls traffic of vesicles at initiation of the process of budding vesicles. Recently Arf was characterized as an activator of phospholipase D.

Little is known about the traffic of vesicles and the role of GTPases in *Trypanosoma cruzi*. In order to investigate the role of monomeric GTPases in the biology of *T. cruzi* we aimed to clone Ras-related genes through the development of a specific RT-PCR based method (Paixão et al. 1996), which generated several products related to monomeric GTPases. In this work we use one of these products as a probe in the screening of a *T. cruzi* CL Brenner cDNA library.

We have sequenced the full coding region of na Arf gene in *T. cruzi*. We have found the conserved motifs for ligation and hidrolysis of GTP for Arf family as well as the merystoilation glicene2 residue in the N terminus. Southern blot analysis showed a profile compatible with one copy per haploid genome. Moreover, Northern blot assay detected a 1.0 kb transcript was found in epimstigote stage.

We aim to gain insight about Arf function through immunolocalization and transfection assays.

This work was sponsored by CAPES, PRONEX and FAPERJ.

MB-25 – CHRACTERIZATION OF THREE RAB GENES OF *T. CRUZI*

Araripe, J.R.[1]; Cunha e Silva, N.L.[2]; Ürmenyi, T.P.[1]; de Souza, W.[2] & Rondinelli, E.[1]
Lab. de Metabolismo Macromolecular Firmino Torres de Castro[1], Lab. de Ultraestrutura Celular Hertha Meyer[2], Instituto de Biofísica Carlos Chagas Filho, UFRJ, Rio de Janeiro, RJ.

Rab proteins are involved in controlling vesicle transport in the exo and endocytic pathway. We have previously demonstrated that *Trypanosoma cruzi* has a *RAB*7 homologue gene that is single copy in the genome of this parasite. The product of this gene, *TcRAB*7 protein, localizes in a structure at the anterior region of the epimastigote form of CL Brener clone, DM28 and Y strains, near the flagelar pocket, basal bodies and kinetoplast.

To further investigate if *TcRAB*7 was associated with the kinetoplast, we co-stained the cells with propidium iodide, a nucleic acid binding dye. Our results demonstrated that the protein localizes very close, but not juxtaposed, to the kinetoplast. Metacyclic trypomastigotes presented weaker staining, when compared with epimastigotes, but it was still present as a single structure at the anterior region, very far from the kinetoplast and basal bodies. Several structures were stained in amastigotes all over the cytoplasm.

In order to study the function and the importance of other rab genes in *T. cruzi*, we obtained 3 clones homologues to *RAB* sequences. All of them were isolated by Dra. Lena Aslund (Uppsala University, Sweden) and Dr. Wim Degrave (FIOCRUZ,Brazil) from our cDNA libraries of epimastigotes constructed for the genome project of *T. cruzi*, two clones from the normalized and one from the non-normalized libraries. One of the clones, homologue to mouse *RAB*23 sequence, was used as a probe to hybridize total RNA and to restriction enzyme digested DNA of *T. cruzi*. The pattern of hybridization suggests that, like *TcRAB*7 gene, the putative *RAB*23 gene is present either as a single copy or as a discrete represented gene. This gene is transcribed as a single 1.2 kb mRNA. We are now performing the characterization of the other genes homologous to *RAB*5 of *T. brucei* and *RAB*C of *D. discoideum*.

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MB-26 – MOLECULAR CHARACTERIZATION OF TCRHO1: A RHO FAMILY GTPASE IN *TRYPANOSOMA CRUZI*

Nepomuceno-Silva, J.L., Mendonça, S.M., de Melo, L.D.B., de Sá, F.G., Redner, P., Benedito, A.B. & Lopes, U.G.
Laboratório de Parasitologia Molecular, Instituto de Biofísica Carlos Chagas Filho, CCS, Bloco G, Sala G1-57, Cidade Universitaria, UFRJ, Rio de Janeiro, RJ, Brasil

In order to characterize genes of the Ras superfamily GTPases in *Trypanosoma cruzi*, we have previously cloned a RT-PCR fragment which sharing around 40% of homology with members of the Rho family of small GTPases. In other organisms studied so far, this gene family is involved in signal transduction pathways leading to rearrangements of the actin cytoskeleton which triggers modifications of cellular morphology. We have used this fragment as a homologous probe in the characterization of a Rho family GTPase gene in *T. cruzi*, named Tcrho1. Genomic Southern blot suggested that Tcrho1 is a single copy gene on Dm28c *T. cruzi* genome. Screening of a Dm28c lEMBL3 genomic library produced a genomic clone containing the whole Tcrho1 *locus*. Sequencing of subclones revealed that Tcrho1 has an open reading frame of 831 bp, predicting a protein of 277 aa and around 31 kDa which shares around 60% homology with several Rho family GTPases. The predicted peptide sequence spans five conserved functional domains related to GTP binding and hydrolysis in its sequence. It has also an essential domain related to post-translational lipid modification engraved on its carboxi-termini. Phylogenetic analysis suggest that Tcrho1 places at an intermediary position between Rho and Rac/Cdc42 subgroups of Rho family GTPases. Mapping of 5'region by RT-PCR unveiled at least five different transcripts derived of alternate trans-splicing. Northern blot analysis of epimastigote forms revealed an mRNA around 1200nt in length. The cellular function of Tcrho1 is still unknow. However functional studies are in progress in order to characterize the role of Tcrho1 in the parasite physiology.

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MB-27 – IDENTIFICATION OF A RIBOSOMAL PROTEIN L27 OF *TRYPANOSOMA CRUZI*

Perone, D.**; Santos, M.A.*; Castro, L.I.R.; Cicarelli, R.M.B.
Departamento de Ciências Biológicas, FCF, UNESP, Araraquara, São Paulo.

Since the small nuclear ribonucleoproteins (snRNPs) are also involved in the *trans-splice* processing of pre-mRNA of *Trypanosoma cruzi*, the initial goal of our study was to isolate *T. cruzi* snRNPs to identify the differences between the protozoan and the human snRNPs; as well as the possibility of involvement of anti-*T. cruzi* snRNPs antibodies in the autoimmunity of Chagas' disease. To clone snRNPs we screened a *Trypanosoma cruzi* epimastigote cDNA library with a purified polyclonal antibody raised against Sm binding site of yeast snRNPs. This technique enable us to obtain a clone with an open reading frame that codes for a protein of 133 amino acids. GeneBank comparative analysis reveled high homology between the *T. cruzi* polypeptide and ribosomal proteins L27 from: *H. sapiens*, *S. cerevisiae* e *C. elegans*. To express the recombinant *T. cruzi* ribosomal protein L27 in *E. coli*, we subcloned the fragment into the expression vector pET32(a). After induction of the recombinant bacteria with 5 mM IPTG, we were able to isolate the recombinant antigen by rapid affinity purification using Ni^{2+} columns. Subsequent immunoblot studies demonstrated that the purified recombinant ribosomal protein L27 of *T. cruzi* is recognized by anti-Sm sera. Similar result was described previously where the authors showed that anti-Sm autoimmune antibodies from SLE patients were able to cross-react with a mouse ribosomal protein (Yoshihisa et al., J. of Immunol, 143:1915-1920). These results suggest that we cloned a gene that probably encodes the ribosomal protein L27 of *Trypanosoma cruzi*. The significance of sharing common epitopes with yeast Sm binding site is not clear yet, however it brings up the possibility of *T. cruzi* ribosomal protein L27 to be involved in the autoimmunity of Chagas' disease.

Financial Support: **CAPES, *FAPESP

MB-28 – IDENTIFICATION AND CHARACTERIZATION OF A TELOMERE-BINDING PROTEIN PRESENT IN ALL STAGES OF *TRYPANOSOMA CRUZI*

Melo-Godoy, P.D. and Schenkman, S.
Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo, R. Botucatu 862, 8° andar, São Paulo, S.P., Brasil.

Telomere-binding proteins are involved in several processes, such as, interaction with telomerase, telomere-mediated chromosome stabilization, and control of the gene expression. In this work, we have investigated the presence of these proteins in *T. cruzi* nuclear extracts. An oligonucleotide containing *Trypanosoma cruzi* telomere sequence was used in gel retardation analysis to detected DNA-protein interactions. We found the formation of specific complexes with nuclear extracts of *T. cruzi* epimastigote, trypomastigote, intracellular trypomastigote and amastigote forms. The complex detected in the gel retardation assay is resistant up to 800 mM NaCl and could be detected in several parasite strains. The binding activity (Tctel) was retained on immobilized single-strand, but not double-strand telomere sequences, and could be eluted with the 500 mM NaCl. The binding activity was purified by gel filtration as a protein of 50 kDa. Ultraviolet crosslinking experiments in the gel retardation complex also reveals that Tctel has 50 kDa. Although a similar binding activity was detected in nuclear extracts of mammalian cells, the Tctel originates in the parasite, since it was detected in epimastigote forms that growth in absence of mammalian cells. The characterization of this protein may help to understand the mechanisms that control telomere maintenance in rapidly dividing cells.

Supported by FAPESP

MB-29 – DIFFERENTIAL GENE EXPRESSION OF A MACROPHAGE CLONAL LINE TRANSFECTED WITH MINICIRCLE KDNA OF *TRYPANOSOMA CRUZI*

Simões-Barbosa, A., Argañaraz, E.R., Barros, A.M., Nitz, N., Correa, R. & Teixeira, A.R.L.
Laboratório Multidisciplinar de Pesquisa em Doença de Chagas, Faculdade de Ciências da Saúde, Universidade de Brasília. CP 04536. CEP 70919-970, Brasília, DF, Brasil.

Several authors have shown a lack of correlation between the presence of the parasite and the development of tissue lesions in chronic Chagas' disease (Teixeira *et al*, 1990, *Am. J. Trop. Med. Hyg.*, 43: 146-148; Bestetti *et al*, 1997, *Inter. J. Cardiol.*, 58: 199-209). We have previously shown the transfection of *T. cruzi* minicircle kDNA in the host cell by natural infection (Teixeira *et al*, 1991, *Rev. Soc. Bras. Med. Trop.*,.24: 55-58; Teixeira *et al*, 1994, *Mut. Res.*, 305: 197-209). Moreover, we identified autoimmune antibodies in a panel of Chagas patients sera reactive against epitopes in the membrane of the kDNA transfected cells, which strongly suggested alteration of gene expression (Simões-Barbosa *et al*, 1999, *Mem. Inst. Oswaldo Cruz*, 94(1), accepted for publishing).

Here, we confirmed the integration of a *T. cruzi* kDNA minicircle sequence in a macrophage clonal line by PCR and Southern blot hybridization, with primers and probe for the conserved region of kDNA, respectively. Then, we obtained total RNA from control uninfected and from transfected macrophage clonal line, which was subjected to parallel differential display of mRNA with various primers combination. This suitable technique allowed us to detect a gene overexpressed in the transfected macrophage clonal line only, as shown by northern blot hybridization. We are performing 5´RACE (5´ Rapid Amplification of CDNA Ends) to obtain its full-length transcript of 9,5kb. This finding suggests that a *T. cruzi* kDNA minicircle sequence transfected in a macrophage clonal line may generate phenotypic alteration of gene expression in the host, thus creating perspectives for sheding light on the variability of clinical manifestations of Chagas' disease.

Financial support: CAPES

MB-30 – *T. CRUZI* UNIVERSAL MINICIRCLE SEQUENCE-BINDING PROTEIN (UMS-BP) MRNA ACCUMULATES UPON ACRIFLAVINE TREATMENT

Amorim RO, Rondinelli E, Silva R
Programa de Biologia Molecular, Instituto de Biofísica Carlos Chagas Filho CCS, Bloco G, Ilha do Fundão 21949-900 Rio de Janeiro Brasil

The kinetoplast DNA (kDNA) is found in the single mitochondrion of the members of the Trypanosomatidae family. It consists of catenated DNA minicircles and maxicircles. They are interlocked topologically to form a disc-shaped DNA network. The replication of kDNA is described as a process in which individual minicircles are detached from the disc-shaped network and replicated. Then, the minicircles are reattached to the periphery of the disc. The kinetoplast DNA minicircles initiate their replication at a conserved 12-nucleotide sequence, the Universal Minicircle Sequence (UMS, 5'-ggggttggtgta-3'). The nucleotide sequences in minicircles of most species of trypanosomatids are heterogeneous. However, in all species studied so far, the universal minicircle sequence (UMS) is conserved.

To study the replication of *T. cruzi* kDNA we have treated epimastigote cells with two intercalating drugs: ethidium bromide (1; 5 and 10 µg/ml) and acriflavine (10; 25 and 50 µg/ml) in order to block cell division. We have observed a decrease in the number of cells in treated cultures. After 5 days of continuous drug treatment we have observed an increase in the number of metacyclic trypomastigotes. The UMS-BP-coding mRNA, involved with minicircle replication, accumulates after 24 hours in the presence of acriflavine (5µg/ml) but did not accumulate in the presence of ethidium bromide (25µg/ml) compared with control cells. Preliminary results indicate that the accumulation of mRNA could be a general effect since the mRNA coding for ATPase is also accumulated in the presence of acriflavine.

MB-31 – ETHIDIUM BROMIDE SELECTIVELY MUTATES K-DNA IN CLONES OF *TRYPANOSOMA CRUZI*

Contreras, V.T., De Lima, A.R., Navarro, M.C. & Arteaga, R.Y.
Centro de Biología Molecular de Parásitos (Centro BioMolP). Facultad de Ciencias de la Salud. Universidad de Carabobo. Valencia. Venezuela.

The k-DNA of parasitic trypanosomatids consists of minicircles and maxicircles. k-DNA plays an important role in the life cycle of these organisms, and the genetic stability of minicircle sequences are used routinely as markers in intraespecific variation studies of *T. cruzi*. Several authors have observed no alteration in k-DNA of *T. cruzi* populations maintained in long-term culture. In fact, lines produced by successive cloning provide genetically homogeneous populations. However, dyskinetoplastic forms can be induced in trypanosomatids by ethidium bromide (EB) treatment, and such treatment appears to specifically affect k-DNA without interfering with nuclear DNA synthesis. Here, we demonstrate that treatment with EB of a cloned *T. cruzi* line yields stable changes in k-DNA restriction patterns. Epimastigote LIT cultures at the end of the log phase (2 x 107 p/ml) were incubated with 5 ug EB/ml for 18 hours at 27 ºC, and metacyclogenized in TAU3AAG medium for 72 hours at 27 ºC. Differentiated parasites were treated with fresh guinea pig serum and separated on DEAE cellulose. The putative dyskinetoplastic (dk) metacyclics obtained were cultivated in LIT medium for several months. Controls of the parental strain (EPm), and the parental clone line (EPm6) were similarly processed, but without the EB treatment. The parasite morphology was studied by Giemsa stain. After five months in culture, epimastigote forms were cloned, or subcloned in LIT medium after serial dilutions (1 parasite/well) in 96 well plates, and incubated for 28 days at 27 ºC. Samples of k-DNA derived from positive wells were compared by schizodeme analyses (restriction enzyme polymorphism).

Obtained results were as follows: a) Treatment with EB produced a total of 33% of dk metacyclics in EPm6 parasites; b) The metacyclic and epimastigotes derived from the cultured, EB treated dk metacyclics were morphologically indistinguishable from the control parasites; c) Three clones were obtained from the parental strain EPm, 12 subclones from the parental clonal line EPm6, and 4 dk subclones from parasites treated with EB; d) Two k-DNA restriction patterns were detected in clones derived from untreated parental strain EPm, while all the k-DNA patterns of the subclones derived from EPm6 were identical to each other and to the parental EPm6 clonal line. In contrast, subclones derived from dk metacyclic trypomastigotes showed 3 k-DNA patterns which differed significantly from the parental EPm6 k-DNA schizodeme. These results demonstrate that the parental EPm strain is genetically heterogeneous, and that the long-term maintenance in culture of the cloned EPm6 line did not change its k-DNA pattern. Furthermore, our data indicates that treatment with EB can produce stable changes in the k-DNA of *T. cruzi* without modifying the biological properties of the parasites.

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MB-32 – A NEW APPROACH TO THE INVESTIGATION OF PROCESSES REGULATING NETWORK TOPOLOGY IN *T. CRUZI*

Lys Guilbride and Sergio Schenkman
Escola Paulista de Medicina, UNIFESP, São Paulo, Brasil

Earlier work showed that the kDNA network in *T. cruzi* intracellular amastigote parasites is large and replicative, whereas in trypomastigote forms, the kDNA network is non-replicative, small and topologically distinct. Purified trypomastigote parasites were induced to transform morphologically into amastigote-like forms by axenic culture at 37° C. Networks isolated at 48 hours from amastigote-like parasites have the shape and size similar to networks found in intracellular amastigote parasites, indicating topological network transformation occurs and accompanies morphological transformation. However, these networks are apparently non-replicative, as assayed by the fluorescein incorporation technique. This suggests that during morphological parasite transformation at 37°C, the process regulating network topology is active, while the process involved in network replication remains inactive or lags behind. It is therefore conceivable that change in network topology can occur independently, and is not necessarily linked to the process of network replication. This provides a convenient system for further investigation into processes regulating network topology.

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MB-33 – EFFECT OF PHOSPHOLIPASE C IN THE INTERACTION HUMAN PLACENTAL TROPHOBLAST -*TRYPANOSOMA CRUZI*

Sartori M.J.[1], Frank FM. [2], Lin S.[1], Mezzano L.[1], Malchiodi E. L.[2], Fabro S. P. de[1]
1-IIa. Cátedra de Histología, Embriología y Genética e Instituto de Biología Celular. Fac. Ciencias Médicas. Universidad Nacional de Córdoba. Ciudad Universitaria (5016). Córdoba. Argentina. E-mail: msartori@cmefcm.uncor.edu
2-Catedra de Inmunologia, Facultad de Farmacia y Bioquimica, Universidad de Buenos Aires.

Previous works have demonstrated that Placental Alkaline Phosphatase (PLAP) activity decreases in plasma of chagasic pregnant women in the third trimestre. *In vitro,T. cruzi* induces changes on the protein pattern of human syncytiotrophoblast and alkaline phosphatase activity is modified by trypomastigotes in cultured human placental villi. The process of invasion of the *T. cruzi* into the host cell has been studied not only in phagocytic cells but also in non professional phagocytic ones (Meirelles 1987, Prioli 1991). It is known that membrane enzymes participate in cellular invasion by other microorganisms (Walker 1983), but it is still not clear if they are related to the invasion of the *T.cruzi*. The PLAP is an IgG receptor in the placental trophoblast (McNabb 1976) and it is anchored to the membrane by a glycosyl-phosphatidylinositol molecule, which could be solubilized by Phospholipase C (PL-C) (Van Hoof 1994). In this work we compared *T. cruzi* invasion into normal human placental villi and into PL- C (Sigma) pretreated-villi. Central villi of placental cotyledones were pre-treated for four hours with PL- C (1,5 mg/ml in a final volumen of 12,5 µl/ml) , washed with PBS and co-cultured with $1x10^6$ bloodstream trypomastigotes (Tulahuen strain) in a final volume of 500 µl of M-199 cultured media. After four hours, the placental tissues were washed with PBS and processed for PCR (Moser 1989) , zymmogrames and Western Blot, and histochemical studies. In zymmogrames, heterocygotic-PLAP placentas present two bands (85 KDa and 100 KDa), from which only one (85 KDa) remains after the treatment with the PL- C. In Western-Blot only a 85 KDa band is visualized, in PL- C- treated placenta and control. PLAP was also detected histochemically and immunohistologically using a monoclonal anti PLAP. In both cases, the enzyme is well labeled in control placentas and PL- C -pretreated and infected placentas. Instead, it is less labeled in not-pretreated and infected placentas. Using hematoxilina/eosine stained, amastigotes are easily observed in the placentas without PL- C treatment, but in PL- C pretreated placentas amastigotes could not be seen, in spite of a carefull search. The absence of parasites was confirmed by PCR because the expected 188 bp band could not be seen in placentas pretreated with PL- C previously to the *T. cruzi* co-culture, while it could be seen in control placentas without pretreatment and co-culture with trypomastigotes. We conclude that the pretreatment with PL- C before infecting with *T.cruzi* difficults the invasion of the parasite into the placental villi, as demonstrated by optical microscopy and PCR. Besides, PLAP expression is modified only when *T.cruzi* invasion takes place, otherwise it remains like the control.

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MB-34 – SPECIFIC BINDING OF POLY[DT-DG] AND POLY[DC-DA] SEQUENCES BY PROTEIN NUCLEAR EXTRACTS FROM *TRYPANOSOMA CRUZI* EPIMASTIGOTES..0

Duhagon, M.A., Dallagiovanna, B., Ehrlich, R. and Garat, B.
Sección Bioquímica, Facultad de Ciencias, Universidad de la República, 11400 Montevideo, Uruguay

Mechanisms of gene expression regulation in *Trypanosoma cruzi* are not yet well understood. Recently special attention has been focused on protein-DNA interaction involving DNA or RNA targets. Dinucleotide repeats are frequently found in intergenic regions, though no precise function has been ascribed to them. Here, we analyze the frequency of poly $[dT\text{-}dG]_n$ repeats in *T.cruzi* genome and address the issue whether these repeats constitute a signal for specific protein binding.

We found that the frequency of these repeats is higher in non-coding sequences than in coding ones. When present, and particularly when n>8, it is common to find more than one repeat in the same region. Electrophoretic mobility shift assays using *T. cruzi* epimastigote nuclear extracts demonstrated the existence of at least one sequence specific single strand binding activity for each strand (poly $[dT\text{-}dG]_{15}$ and poly $[dC\text{-}dA]_{15}$). Competitors used in this work, allowed us to discard bindings due to RNA or single strand DNA non sequence specific binding proteins. Sequence specificity was studied by competition with two random oligodeoxynucleotides and a poly[dT] oligodeoxynucleotide as a polypyrimidine tract. Though *E. coli* single strand binding protein was also able to band shift these probes, no sequence specificity was observed. Strand polarity was observed. Preliminary characterization enable the estimation of functional dissociation rates with half life of about 1 minute and 10 minutes for specific complexes with poly $[dT\text{-}dG]_{15}$ and poly $[dC\text{-}dA]_{15}$ probes respectively. The corresponding functional equilibrium constants were about 10^{-9} M^{-1} and 10^{-10} M^{-1}.

Putative roles for these proteins are briefly discussed in this communication. Purification of the corresponding proteins is in process so as to allow an accurate determination of the physicochemical parameters of the complexes. The elucidation of the amino acidic sequence of these proteins could give us information about their putative biological function.

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MB-35 – IMMUNOPRECIPITATION OF SMALL NUCLEAR RNAS OF *TRYPANOSOMA CRUZI* BY ANTI-TMG ANTISERUM

Castro, L.I.R.; Perone, D.*; Martins, E.C.**; Bosetto, M.C**.; Cicarelli, R.M.B.

Many small nuclear U RNAs of eucaryotic cells are found in vivo complexed to proteins forming the small nuclear ribonucleoproteins (snRNPs). The snRNPs are involved in the process of *splicing* of pre-mRNA and each one is named according to the snRNA it contains. The U1, U2 and U5 snRNPs hold only one snRNA while U4/U6 snRNPs comprise both U4 and U6 snRNAs. The U snRNAs are very abundant and vary their size from 57 to 216 nucleotides. Except for U6 snRNAs, all the others carry on their 5' end a trimethylguanosine (2,2,7-trimethylguanosine). Although well described to mammal cells, snRNAs have not been characterized yet in the parasite *Trypanosoma cruzi*. In this study, our goal was to isolate the U snRNAs by immunoprecipitation of *Trypanosoma cruzi* and HeLa cells nuclear extracts with anti-trimethylguanosine antisera. The RNAs obtained after digestion of the pellet with proteinase K, were separated on polyacrilamide/urea gel and visualized by silver staining. As expected for HeLa cells, we obtained bands correspondent to U1, U2, U4, U5 and U6 RNAs while for *T. cruzi* we were able to visualize only bands with sizes correspondent to U2, U4 and U5 RNAs. These results suggest that at least U2, U4 and U5 are present in the nucleus of this protozoan cell and could be a constitutive part of *T. cruzi* spliceosome.

Financial Support: *CAPES, **CNPq

MB-36 – THE ROLE OF SATELLITE DNA SEQUENCES OF *TRYPANOSOMA CRUZI*

Elias,M.C.Q.B. and Schenkman, S.
Disciplina de Biologia Celular, Escola Paulista de Medicina - UNIFESP.

The life cycle of *Trypanosoma cruzi* includes replicative (epimastigotes and amastigotes) and infective (trypomastigote) forms. We have found that both polymerase I and II transcription activity decrease 10 fold in infective forms as assessed by transcription of isolated nuclei, or lysolecithin permeable parasites. In all stages the transcriptional activity is proportional to the copy number of most of the genes, in agreement with the idea that in this parasite the genes are constitutively transcribed and the expression is controlled at the post-transcriptional level. Exception are genes described as satellite DNA which are 195 bp repeats and correspond to about 10% of the entire parasite genome. These sequences are transcribed at much less extent, indicating that they are under transcriptional control, or to silencing mechanism. To better understand satellite DNA role, we have studied their localization by *in situ* hybridization and by pulse field electrophoresis of chromosomal size DNA. We found that satellite DNA sequences form 10-12 patches at the nuclear periphery of proliferative forms in a similar way of the dense plates of heterochromatin observed by electron microscopy, which has been shown to be involved with segregation of chromosomes during mitosis in trypanosomes. Pulse-field electrophoresis analysis show that satellite DNA sequences are present in a subset of large chromosomes and form long patches of repeats (> 30 kB). Based on these observations, we suggest that *T. cruzi* satellite DNA may form structures involved in chromosome segregation during mitosis, and might be related to the kinetophore or centromeres and for this reason are poorly transcribed.

Supported by FAPESP

MB-37 – CHAGAS DISEASE REACTIVATION IN AIDS PATIENTS: TREATMENT CONTROL USING PCR AND GENETIC CHARACTERIZATION OF SUB-POPULATIONS OF *T. CRUZI* IN BLOOD AND CEREBRAL FLUID

Lages-Silva, E[1,3], Silva-Vergara, ML[2], Matos, A[1], Macedo, A[4], Chiari, E[3] & Ramirez, LE[1]
[1]Disciplina de Parasitologia, Departamento de Ciências Biológicas, FMTM, Uberaba, MG; [2]Disciplina de Doenças Infecciosas e Parasitárias, Departamento de Clínica Médica, FMTM, Uberaba, MG; [3]Departamento de Parasitologia; [4]Departamento de Bioquímica e Imunologia, ICB/UFMG, Belo Horizonte, MG

The opportunistic character of *Trypanosoma cruzi* has been reported mainly in cases of co-infection with the human immunodeficiency virus. In such cases, chagasic infection reactivates, invades the central nervous system (CNS) and has high lethality. The factors involved in this process are unknown but appear to depend on host immune response and characteristics common to certain parasite populations. This study follows one case of Chagas disease reactivation in an AIDS patient. *T. cruzi* genetic characteristics as well as blood and cerebral fluid parasitemia before and during benznidazol treatment were analyzed. The patient is a 63-year-old male with the following profile: 1,000 leukocytes/mm^3, CD4+ 67/mm^3 and CD8+ 313/mm^3. He was hospitalized in HE-FMTM with meningoencephalitis that did not respond to the initial treatment for toxoplasmosis. Later, *T. cruzi* was detected in blood and cerebral fluid and the patient was treated with benznidazol (7mg/Kg) for 60 days. Blood and cerebral fluid samples were collected before treatment (T0), and at 7 (T1) and 22 (T2) days post-treatment. Direct, microhematocrit, LIT medium culture, PCR and LSSP-PCR examinations were performed on the samples. Direct and microhematocrit evaluations indicated higher parasitemia in the cerebral fluid than the blood at T0, dropping sharply in T1 and being negative in T2. Cultures of cerebral fluid and blood were positive in T0 and T1 but were negative in T2. *T. cruzi* DNA was intensely amplified by PCR in dilution higher than 1:200 for cerebral fluid and blood in both T0 and T1. In T2, we verified that blood continued to be strongly positive in PCR analyses while DNA amplification in cerebral fluid was weak and only occurred in undiluted samples (negative in 1:10, 1:100 and 1:200 dilutions), which suggests the disappearance of circulating parasites in this fluid. Using the LSSP-PCR technique, we genetically characterized sub-populations of parasites present in the cerebral fluid and blood and observed genetic signatures with profiles that indicated a single population. PCR is a helpful tool for early detection of the parasite in cerebral fluid and for evaluating treatment efficacy in patients with reactivated infections of the CNS. It is necessary to define genetic patterns of *T. cruzi* populations related to CNS involvement in order to determine preventive measures for immunosuppressed patients.

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MB-38 – PCR-BASED DIAGNOSIS FOR CHAGAS' DISEASE IN TREATED OR NON-TREATED PATIENTS LIVING IN ENDEMIC AREA

Teixeira, M.G.M., Oelemann, W.M.R., Caseca, A., Borges-Pereira, J. & Peralta, J.M.
Departamento de Imunologia, Instituto de Microbiologia, UFRJ-CCS, Cidade Universitaria, Ilha do Fundao, 21941-090 Rio de Janeiro and Departamento de Medicina Tropical, IOC-FIOCRUZ, Avenida Brasil 4365, 21045-900 Rio de Janeiro.

In recent years, various investigators reported the use of the polymerase chain reaction (PCR) in the diagnosis of chronic chagasic infection, and a variety of *T.cruzi*-specific amplification target sequences have been described. Most of the published studies use kDNA minicircle sequences as target molecules, due to their abundance in the parasite genome. Each parasite contains a netwok of approximately 20.000 concatenated minicircles, each of which possesses four copies of target sequences.

When compared with xenodiagnosis or hemoculture the PCR technique has shown a consistend increase insensitivity. However, low sensitivity of PCR in comparing serologic tests has also been reported. Because of these diaparities, the true and potencial usefulness of PCR for chronic Chagas disease diagnosis further investigation.

In this study we analysed 87 specimens from individual living in endemic area for Chagas disease, Virgem da Lapa, MG. Some patients were submitted to a specific chemotherapy.

Total DNA was isolated by phenol/chroform extraction followed by ethanol precipitation. The primers 121/122 were designed to amplify *T.cruzi* kenotoplast DNA minicircle sequence (kDNA). The amplified products were analysed by agarose gel electrophoresis, ethidium bromide staining and by na enzyme-linked immunosorbent assay (ELISA).

In substitution of the hot start method, were utilized the Platinum Taq DNA polymerase (GIBCO). Platinum Taq DNA polymerase is derived from recombinant Taq DNA polymerase by binding of a thermolabile inhibitor containing monoclonal antibodies to Taq DNA polymerase to provide automatic hot start.

The PCR technique was compared with some serological methods, indirect immunofluorescence (IFI), ELISA, western blotting (WB) and INNOLIA.

The results of serological methods showed 12 negative samples and 75 positive samples. All the negative samples serologically, were negative in PCR. The PCR technique detected 50 of them 75 positive samples sorogically only.

MB-39 – EVALUATION OF BLOOD AND TISSUES PARASITISM BY MEANS OF BLOOD CULTURE, PEROXIDASE ANTIPEROXIDASE AND POLYMERASE CHAIN REACTION IN HAMSTERS INFECTED AND REINFECTED WITH THE SAME STRAIN OF *TRYPANOSOMA CRUZI*.

Cabrine, M.S., Lages-Silva, E., Matos, A., Chapadeiro, E., Ramirez, L.E.
Disciplina de Parasitologia, Departamento de Ciências Biológicas da Faculdade de Medicina do Triângulo Mineiro-FMTM, Uberaba, MG.

The role of *Trypanosoma cruzi* in the pathogenesis of the lesions found in Chagas'infection chronical phase is questionable, especially due to the difficulty in finding the parasite either in the blood or in the tissues. With the development of more sensitive methods able for detecting either antigens or fragments of the *T. cruzi* genome, the importance of the parasite as a stimulator of histopathological processes is now being recovered. In this work, we have determined tissue and blood parasitism in hamsters infected and reinfected with VIC strain of *T. cruzi*, by using the techniques of blood culture, Peroxidase antiperoxidase (PAP) and Polymerase Chain Reaction (PCR). Six groups of 20 hamsters each were used, one of them as the control group. The other ones were infected and reinfected with one to five inocula of 2,000 blood trypomastigotes of VIC strain, at intervals of 45 days between them. After ten months the animals were bleeded, sacrificed and autopsied. The blood was used to carry out blood culture and PCR (performed with the S35 and S36 starters which amplify a fragment of 330 bp of *T. cruzi* minicircle). PAP was performed with the tissues of: heart, salivary gland, pre-stomach, stomach, small and large intestines, cecum, kidney, liver, spleen, testis, epididymis, seminal vesicle, bladder and skeletal muscle. PCR was performed with the tissues of heart, esophageal-gastric, pyloric and ileocecal junctions and testis. With tissue PCR only the animals which received one and five inocula were studied. The presence of *T. cruzi* was detected in the blood through blood culture in 16.7%, 37.5%, 11.1%, 42.9% and 25%, and through PCR in 100%, 100%, 50%, 60% and 75% for the animals with one, two, three, four and five inocula, respectively. The positivity of PAP was distributed among the groups of animals autopsied, in the following way: only in the heart in the animals with one inoculum; in the heart, stomach and bladder (12.5%); pre-stomach, stomach (25%) e testis (37.5%) in the group with two inocula; in the testis (50%) and epididymis (20%) in the animals with three inocula; in the pre-stomach, epididymis and skeletal muscle (14.3%) and testis (28.6%) in the group with four inocula; and in the heart, salivary gland, cecum, kidney, liver, and skeletal muscle (25%), epididymis (75%) and testis (100%) in the group with five inocula. The evaluation of tissue parasitism by means of PCR detected *T. cruzi* DNA in 100% of hearts and junctions in the groups evaluated. The positivity in the testis was 100 and 75%, respectively, for the groups with one and five inocula.

These results emphasize the presence of *T. cruzi* in blood and tissues in the Chagas' disease chronical phase in hamster. In spite of PAP easily detecting the parasite in the tissues, the PCR has shown a greater sensitivity and homogeinity in detecting kDNA sequences of *T. cruzi* either in blood (50-100%) or tissues (75-100%). Blood culture was very oscillating varying from 11.1 to 50% in the different groups of animals. The reinfection determinates an increasing of tissue parasitism especially in the testis, thus suggesting that this may be an organ reservoir of parasitism in hamster chronical infection.

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MB-40 – POLYMERASE CHAIN REACTION AS A LABORATORIAL TOOL TO EVALUATE PARASITOLOGICAL CURE IN CHRONIC CHAGASIC PATIENTS SUBMITTED TO SPECIFIC THERAPY

Fernandes, O.[1,2], Macedo, V.[3], Cardoso, A.[1], Marques, P.[1], Silveira, C.[3], Morel, C.[1] & Britto, C.[1]
[1]Fundação Oswaldo Cruz, RJ, Brasil - Instituto Oswaldo Cruz, Departamentos de Medicina Tropical & Bioquímica e Biologia Molecular; [2]Universidade do Estado do Rio de Janeiro, RJ, Brasil; [3]Universidade de Brasília, DF, Brasil, Departamento de Medicina Tropical

The evaluation of etiologic treatment for chronic Chagas disease faces with the absence of any clear-cut criterion of cure. The low degree of parasitemia and the persistence of positive immunologic reactions represent some of the difficulties involved in addressing therapeutic efficacy. In order to define whether the polymerase chain reaction (PCR) could be used as a laboratorial method for evaluating cure in Chagas disease after specific treatment, we investigated 80 xenopositive chronic chagasic patients from two distinct Brazilian endemic areas: Mambai (State of Goias) and São Felipe (State of Bahia). Kinetoplast specific sequences from *Trypanosoma cruzi* were used as target for the PCR amplification method, to detect the presence of parasite mitochondrial DNA in total blood. The basic objective of this study was to compare the PCR results with xenodiagnosis performed 20 years after the specific chemotherapy with benznidazol (5mg/kg/day - 8 weeks). A 330 bp fragment originated from kinetoplast DNA was detected in only 27/80 patients (34% of positivity), while the xenodiagnosis showed positive results in 13/80 (16%). Therefore, the performance of one single PCR after treatment revealed parasite clearance in 66% of the individuals, while the negativation of xenodiagnosis was observed in 84%. All the anti-*T. cruzi* serological assays performed concomitant with the PCR and xenodiagnosis, after completion of chemotherapy, were consistently positive or doubtful. It has always been difficult to monitor the cure phenomenon by serology, since a humoral response against the parasite antigens remains for a very long period. We conclude that the PCR has a clear advantage over conventional techniques for direct detection of *T. cruzi* in chagasic patients that underwent chemoterapy, showing a higher sensitivity when compared with the xenodiagnosis results. A more careful evaluation should be carried out by analyzing these patients in a follow-up survey, to show that the treatment was really effective.

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MB-41 – DEVELOPMENT OF A SHUTTLE VECTOR FOR OVEREXPRESSION STUDIES IN *LEISHMANIA*

Franco,A.R.; Floeter-Winter,L.M.; Uliana, S.R.B.
Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Caixa Postal 66208, 05389-970 São Paulo, SP

Based on the rRNA promoter region of *Leishmania amazonensis* (Uliana et al, Mol. Biochem. Parasitol. 76:245-255,1996) a plasmid was constructed (pARHyg) containing, downstream from the promoter, a synthetic splicing acceptor site, the hygromycin phosphotransferase gene (*hyg*) and a synthetic polyadenilation site. Promastigotes of *L. amazonensis* and *L. major* transfected with this plasmid grew in the selective drug only for a few passages. The introduction of the *L. major meta1* coding sequence in the construct generated stable transfectants, suggesting that the presence of *Leishmania* genomic sequences could have a role in the stability of the episome. A high level of *meta 1* protein was detected in those cells (Uliana et al., Exp. Parasitol., in press).

A new construct was then obtained by cloning 2 kb of the 3' *meta 1* flanking sequence downstream to the *hyg* gene (pARHyg3'). Transfectants obtained with this construct were stable in both promastigotes *in vitro* and lesion amastigotes. The RNA analysis of pARHyg3' transfectants showed a discrete band of 1.3 kb when the Northern blot was probed with the *hyg* coding region, in contrast to the diffuse pattern observed for pARHyg transfectants, hybridized to the same probe. The precise processing site is likely to be provided by the *meta 1* 3' region. To verify the importance of accuracy in the RNA processing event for the stability of the episome and to map the possible sequence responsible for this regulation, new constructs will be generated containing smaller fragments of the *meta 1* flanking region.

Supported by FAPESP and CNPq.

MB-42 – CYSTEINE PROTEINASES OF *LEISHMANIA*: MECHANISMS OF TARGETING TO THE LYSOSOME

Linda Khalili Boukai, Diamar da Costa-Pinto, Lucas Siqueira Trindade, Adriana C. Lima, Maurílio J. Soares,* Diane McMahon-Pratt and Yara M. Traub-Cseko.
Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro, R.J., Brazil and *Yale Medical School, New Haven, CT, USA.

Protein trafficking in trypanosomatids has been found to be similar to that of higher eukaryotes in many instances, but differences exist for all subcellular locations investigated so far. The unique mechanisms of targeting exploited by trypanosomatid parasites could serve as chemotherapy targets.

In mammals, the trafficking mechanisms of lysosomal enzymes is well studied and involves mannose 6-phosphate receptors. Alternative mechanisms exist, and vacuolar sorting of carboxypeptidase Y in yeast was shown to be related to aminoacids present in the propeptide region.

We have investigated the mechanisms of targeting of the cysteine proteinase Lpcys2 of *Leishmania pifanoi* to the lysosome. We studied the role of N-glycosylation in this process by creating constructs containing Lpcys2 mutated in the two putative N-glycosylation sites located either in the mature or C-terminal extension regions of the proteinase. A double mutant was also generated. These were transfected into *L. major* and visualized with polyclonal antibody that shows no cross reaction with endogenous proteinases. Lysosomal labeling was observed at the same levels of the control in all cases. A slightly lower count of gold particles in the mutants in the C-terminal extension asparagine was observed.

We also studied the putative role for signal peptides in targeting by fusing either the pre-pro region or the C-terminal extension of Lpcys2 to GFP. While parasites transfected with the latter showed a bright cytoplasmic fluorescence, the pre-pro construct transfectants showed localized fluorescence compatible with a lysosomal localization, indicating the presence of a signal in this region. The cellular localization of GFP was confirmed by colocalization experiments using an antibody against a lysosome specific cathepsin B of *L. major*.

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MB-43 – CLONING AND EXPRESSION OF A GENE ENCODING A CYSTEINE PROTEINASE OF *L. (L.) AMAZONENSIS* AMASTIGOTES

Lasakosvitsch, F., Oliveira, T.R., Beyrodt, C.G.P., Araya, J., Silveira, J.F. & Barbiéri, C.L.
Disciplina de Parasitologia, Escola Paulista de Medicina - Universidade Federal de São Paulo, Caixa Postal 20.342, São Paulo, 04023-062, SP.

An antigen of apparent molecular mass of 30 kDa (p30) was identified in *L. (L.) amazonensis* amastigotes and shown to be implicated in lymphoproliferative responses in BALB/c mice mediated by $CD4^+$ Th1. Characterization of p30 was carried out by use of a monoclonal antibody directed to the antigen and purification of p30 by immunoaffinity chromatography revealed that it presents cysteine proteinase activity. Immunoelectron microscopy studies were also performed and showed the predominance of p30 in megasomes of *L. (L.) amazonensis* amastigotes. Active immunization of BALB/c mice with p30 was able to induce a significant degree of protection against homologous infection (Beyrodt et al., 1997, Infect. Immun. 65, 2.052). The aim of the present work was to clone and sequence the gene encoding p30 as well as to express the recombinant protein in a bacterial expression system.

Two distinct cysteine proteinase genes of *Leishmania (L.) pifanoi*, Lpcys 1 and Lpcys 2, were characterized (Traub-Czeko et al., 1993, Mol. Biochem. Parasitol. 57: 101-116) and corresponding genes in *Leishmania (L.) mexicana* were also described (Mottram et al., 1992, Mol. Biochem. Parasitol. 6: 1925-1932). The gene p30 of *Leishmania (L.) amazonensis* was isolated by PCR amplification using specific primers derived from *Leishmania (L..) pifanoi* Lcys1. A 1.06-kb fragment was amplified from the genomic DNA of *L.(L.) amazonensis* amastigotes, and cloned in the vectors pUC18 and pGEX.

Nucleotide sequence analysis of the amplified fragment showed a high degree of homology to the sequences of previously cloned cysteine proteinase genes of *L (L.) pifanoi* and *L. (L.) mexicana* (96% of identity), and *L. (L.) major* and *L. (L.)* chagasi (86%). The peptide translated from the open reading frame found in the 1.06-kb fragment shares 98% of identity with the cysteine proteinases of *L (L.) pifanoi* and *L. (L.) mexicana*. A comparative analysis of the gene p30 with different *Leishmania* cysteine proteinase genes revealed changes that can be explained by point mutations. The genomic organization of gene p30 was analysed by Southern blotting. The 1.06-kb fragment was hybrized to the *Leishmania (L.) amazonensis* genomic DNA, and a single band was detected with several restriction enzymes suggesting that gene p30 is present in a few copies in the genome. Analysis of the p30 recombinant protein is currently in progress and the protection conferred by the native and recombinant p30 will be compared in BALB/c model.

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MB-44 – EXPRESSION AND CRYSTALLIZATION OF *LEISHMANIA TARENTOLAE* ADENINE PHOSPHORIBOSYLTRANSFERASE GENE

Silva, M.[1,2], Oliva, G. [1], Caruso, C.S.[1,2], Beltramini, L.[1] & Thiemann, O.H.[1]
[1] Laboratório de Cristalografia de Proteínas e Biologia Estrutural, Departamento de Física e Informática, Instituto de Física, Universidade de São Paulo, Brasil. [2] Instituto de Química, Universidade de São Paulo, São Carlos, SP, Brasil.

Phosphoribosyltransferases (PRTases) are inportant enzymes involved in the recycling of purine nucelotides. The mammalian cells can synthesize the purinenucleotides de novo and are only dependent on the salvage pathway during phases of rapid cell growth. Parasitic protozoan of the order *Kinetoplastida* rely exclusively on the purine salvage pathway. Kinetoplastid protozoa from the genus, *Leishmania*, possess three enzymes involved in the salvage pathway, adenine PRTase (APRT), hypoxanthine-guanine PRTase (HGPRT) and xanthine PRTase (XPRT). APRT is responsible for catalyzing the conversion of adenine and α-D-5-phosphoribosyl-1-phosphosphate (PRPP) into adenosine-5-monophosphate 9AMP) and pyrophosphate (PPi) by the nomeric inversion of the ribofuranose ring. This difference in purine nucleotide metabolism between the mammalian host and protozoan parasites has stimulated considerable interest in the salvage pathway as a target for chemotherapy. Our purpose is to explore this metabolic difference and develop new chemotherapy alternatives for the treatment of Leishmaniasis. Utilizing the recombinant form of *L. tarentolae* APRT, the enzyme will be crystallized and its atomic structure solved.

In our study, the APRT gene has been cloned into the expression vector pET29a(+). An expression protocol was developed and the enzyme obtained in then cytosolic fraction of *Escherichia coli*. The APRT protein was purified to homogeneity in one-step affinity chromatography over an AMP-Agarose column and concentrated up to 12mg/ml. Crystallization screens have been performed in hanging-drop experiments. Proteins crystals have been obtained at 4ºC, with 2-propanol as crystallization agent. X-ray diffraction data are being collected to elicit the complete structure determination. In conclusion, we observed that the *L. tarentolae* APRT could be concentrated maintaining its stability for extended periods of time. the concentrated protein could be crystallized and the crystals obtained are of sufficient quality for diffraction studies.

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MB-45 – CLONING AND CHARACTERIZATION OF *LEISHMANIA TARENTOLAE* HYPOXANTHINE-GUANINE PHOSPHORIBOSYLTRANSFERASE GENE

Thiemann, O. H. [1], Alfonzo, J. D. [2] , Simpson, L. [2] & Oliva, G. [1]
[1] Departamento de Física e Informática, Instituto de Física, Universidade de São Paulo, Caixa Postal 369, São Carlos, 13560–970, SP, Brasil. [2] Howard Hughes Medical Institute, UCLA, Los Angeles, CA, USA.

Parasitic protozoa of the order *Kinetoplastida* are unable to synthesize purines de novo and rely on the salvage pathway for the recycling of ribonucleotides. Phosphoribosyltransferases (PRTases), adenine PRTase (APRT) (EC 2.4.2.7), hypoxanthine-guanine PRTase (HGPRT) (EC 2.4.2.8) and xanthine PRTase (XPRT) (EC 2.4.2.22, are enzymes involved in the recycling of purine nucleotides. The mammalian host however can synthesize the purinenucleotides de novo as well as recycle purines during phases of rapid cell growth. HGPRT is responsible for catalyzing the conversion of hypoxanthine or guanine and α-D-5-phosphoribosyl-1-pyrophosphate (PRPP) into inosine-5-monophosphate (IMP) or guanosine-5-monophosphate (GMP) and pyrophosphate (PPi) respectively. Our purpose is to explore this difference in purine nucleotide metabolism between the mammalian host and protozoan parasites for the development of alternatives for the treatment of Leishmaniosis.

For this purpose the *hgprt* gene was cloned from a *Leishmania tarentolae* genomic library and the sequence determined. The *L. tarentolae hgprt* gene contains a 633 nucleotides open reading frame that encodes a 210 amino acids protein. The predicted amino acid sequence has high identity to other HGPRTases. The *hgprt* gene from *L. tarentolae* was cloned into a suitable expression vector and expressed in *Escherichia coli* cells. A purification protocol was established, and the purified protein was found to retain enzymatic activity. The steady-state kinetic parameters are being determined for the recombinant enzyme. Initial crystallization trials for the screening of conditions are being performed on the recombinant HGPRT.

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MB-46 – MOLECULAR CLONING OF STEROL 24-METHYLTRANSFERASE FROM *LEISHMANIA MAJOR*

Jiménez-Jiménez, C., Fárez-Vidal, E., Gállego, C., Ruiz-Pérez, L.M. and González-Pacanowska, D.
Instituto de Parasitología y Biomedicina “López-Neyra”. Consejo Superior de Investigaciones Científicas. Granada, Spain.

One of the major differences between cholesterol and ergosterol is the presence of the C24 alkyl side chain in the case of ergosterol. Inhibitors of this step should selectively target sterol biosynthesis in *Leishmania*. Sterol 24-methyltransferase methenylates the steroid nucleus at position 24 and known inhibitors of the enzyme are 24 and 25-azasterols which have been shown to inhibit the growth of both *Leishmania* (Haughan et al, 1995, Biochem. J. 308, 31-38) and *Trypanosoma cruzi* (Urbina et al, 1996, Chemotherapy 42, 294-307). Degenerate oligonucleotides were designed complementary to conserved sequences of yeast and plant methyl transferases. In PCR reactions using *Leishmania major* genomic DNA as template we have obtained an amplified DNA band of approximately 340 bp that, once sequenced, was identified to be highly homologous to 24-methyltransferase. The PCR amplified DNA was used as a probe to screen a *Leishmania major* cDNA library (ZAP Express ™). Several positive clones have been analyzed and sequenced. Four phagemids were rescued from the library and one of them presented a 2.4 kb insert which contained the coding sequence for 24-methyltransferase. Although the spliced leader was not evidenced at the 5’ end, the cDNA contains an open reading frame of 1059 bp, a similar length to genes coding other methyl transferases and the codon usage was in good agreement with that described for *Leishmania* genes. A polypyrimidine tract is present 27 bp upstream the methionine considered as the initiation codon. The deduced amino acid sequence gives a protein of 353 residues which is similar to the 24-methyltransferase from *Ricinus communis* (overall identity was 48.1% and similarity 57.1%) and *Saccharomyces cerevisiae* (identity 43% and similarity 52.1%). Results concerning the chromosomal localisation and genomic organisation of the gene will be presented.

MB-47 – CLONING AND CHARACTERIZATION OF *LEISHMANIA* (*L.*) *DONOVANI* INOSINE URIDINE NUCLEOSIDE HYDROLASE

Santana, D.M.*,**, Sturm, N.R.**, Palatnik de Sousa, C.B.* and Campbell, D.A.**
* Instituto de Microbiologia "Prof. Paulo de Góes" , CCS, Cidade Universitária, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, RJ, Brasil, CEP 21941-590, CP 68040, immgcpa@microbio.ufrj.br. [1]
**Department of Microbiology, Immunology & Molecular Genetics, UCLA School of Medicine, University of California, Los Angeles, CA, USA.

The Fucose-Mannose Ligand (FML) is an antigenic extract from the promastigote form of *L.*(*L.*) *donovani* that can be used to diagnose human visceral leishmaniasis. It also elicits a strong and specific protective response in animal models. The major immunoprotective component of FML is a 36-kDa glycoprotein fraction, designated GP36, that is recognized by sera from hyperimmune rabbits and human Kala-azar patients and by murine monoclonal antibodies. The GP36 fraction can be isolated from FML by SDS-PAGE and used as an antigen that protects mice from experimental infection. Because GP36 is a strong candidate for an effective vaccine against visceral leishmaniasis we chose to test the antigenicity and protective capacity of recombinant DNA-derived protein. To this end, we have cloned the gene encoding an antigenic component of the GP36 fraction.

To clone the gene, we first obtained peptide sequence from gel-isolated GP36. A 19-amino-acid peptide sequence was obtained from a tryptic peptide (p106). Degenerate oligonucleotides based on the p106 sequence were used to screen a cosmid library of *L. donovani*. DNA sequence analysis of a 1.5-kb *Cla*I-*Sac*II fragment from a cosmid revealed an open reading frame that contained the complete p106 peptide. The predicted protein was 78.4% similar to the Inosine Uridine Nucleoside Hydrolase (IUNH) of *Crithidia fasciculata*. The genomic organization of the *L.*(*L.*) *donovani IUNH* was evaluated by Southern blot analyses, which showed it to be a single copy gene. Northern blot analysis showed that the *L. donovani IUNH* yields two messenger RNAs: a major band of 2.1 kb and a minor band of higher molecular weight.

Recombinant *L. donovani* IUNH (rIUNH) was expressed in *E. coli* from the pMAL-c2 plasmid. rIUNH was cleaved from the maltose-binding fusion protein by Factor X and purified by Q-Shepharose ion exchange chromatography. Western blot analysis showed that the rabbit anti-GP36 polyclonal serum recognized the rIUNH of *L. donovani*. The rIUNH will be used as an antigen in diagnostic assays and for experimental vaccination in animal model against visceral leishmaniasis. We are also using *Leishmania* as a eukaryotic system to overexpress the rIUNH from an episome. This approach will be used to determine its subcellular localization and clarify its function. In *Crithidia fasciculata* IUNH plays an important role in the acquisition of preformed purines from host sources. Since IUNH activity has not been identified in mammals, this protein is a potential target for selective drug design in addition to diagnosis and vaccination studies.

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MB-48 – MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF IMPDH GENE IN *LEISHMANIA AMAZONENSIS*: PUTATIVE ROLE IN APOPTOSIS

Pitaluga, A. N.[1,2], Traub-Cseko, Y. M.[1], Moreira, M. E. C.[2]
[1]Lab. de Biologia Molecular de tripanossomatídeos, DBBM/IOC, FIOCRUZ, RJ, BRASIL. [2] Programa de Medicina Experimental, Pesquisa Básica, INCa, RJ, BRASIL. E-mail: pitaluga@gene.dbbm.fiocruz.br

Some Trypanosomatids are digenetic parasites and, as a hallmark in their life cycle, they infect two genetically distinct organisms, sand flies of *Phlebotominea* family (as vectors) and mammals as definitive hosts. A crucial moment of the natural history of leishmaniasis is the inoculation of these parasites in mammals, when they suffer a heat shock. This abrupt temperature shift kills most of the parasites and 20 percent of them die by apoptosis. This cell death is a genetically regulated event that could be triggered by many pathways, like dNTPs pool imbalance. *Trypanosomatidae* display a total require for purines, as they lack the intracellular machinery to synthesize them by the "de novo" pathway. As a result the enzymes devoted to metabolism of purines are extremely important to the parasite. One of these enzymes is Inosine-5'-Monophophate Dehydrogenase (IMPDH), which converts Inosine Monophosphate (IMP) to Xanthosine Monophosphate (XMP). It is important to mention that an inhibitor of IMPDH, named Tiazofurin, induces apoptosis by depletion of GTP in K562 cells. Could heat shock reduce the expression of IMPDH leading to dNTPs pool imbalance and apoptosis in *L. amazonensis*? The first step to answer this question is the characterization of IMPDH gene in this parasite. An RT-PCR was made and showed an 353bp fragment of 94,3% identity with *L. donovani* IMPDH, differentially expressed in heat-shocked and non heat-shocked conditions. An screening of a genomic library of *L. amazonensis* was made against this probe (IMPDH-P^{32}). The clones identified by autoradiography were digested and hibrydizated against the same probe to confirm the presence of IMPDH fragments. The positive fragments were subcloned (Sure Clone Kit – Pharmacia) and are being sequenced at this very moment. After total gene characterization, biochemical and differential display assays will be made to investigate the role of IMPDH in *L. amazonensis* apoptosis. These could also lead to the development of tripanocidal drugs based on IMPDH substrate analogs.

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MB-49 – TRANSFECTION OF *LEISHMANIA (V.) GUYANENSIS* WITH THE *LBPGPA*, A GENE INVOLVED IN ANTIMONY RESISTANCE

Anacleto, C., Campos, J. S., Cisalpino, P. S., Ferreira, A. V. B.*, Abdo, M. C. B., Fernandes, A. P. S. M., Moreira, E. S. A.
Depto. Microbiologia, *Depto. Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG.

One of the mechanisms for drug resistance in *Leishmania* involves an increase in active efflux by overexpression of a membrane p-glycoprotein. We have previously isolated a glucantime-resistant strain of *Leishmania (V.) guyanensis* which showed an extrachromosomal amplification. This amplicon contains a gene, *lbgpgpA* which shows homology to the *L. tarentolae pgpA* gene (*ltpgpA*). In an attempt to investigate the involvement of the *lbpgpA* gene with the oxianion-resistance phenotype, we have performed trasfection experiments with fragments isolated from the amplicon that contained the gene. A 5.1 Kb *ApaI/EcoRI* fragment containing the *lbgpgpA* ORF and a 6.7 Kb *EcoRI/EcoRI* fragment containing this ORF plus 1,6Kb of the upstream region were cloned into the vector pSNAR and transfected by electroporation. Susceptibility tests of transfectants to both glucantime and antimony tartrate indicated that they showed low level resistance. PFGE and RFLP of transfectant clones showed that the 5.1 Kb fragment was stable in the vector after trasnfection and was replicated as an episome. On the other hand, the clone containing 1.6 kb 5' region of the *lbgpgpA* ORF was integrated in a 550 Kb chromosome. Additional data suggested that part of an inverted repeat is contained in the 1.6 kb upstream region and seem to be involved in gene rearrangement in this parasite. The transfection experiments and cross-resistance studies indicated that under the conditions tested the *lbgpgpA* gene is involved in low level resistance to antimonials, such as glucantime and antimony tartrate. The number of copies of the introduced fragments found in the transfectants is not compatible with the resistance level suggesting that as in *L. tarentolae* resistance to oxianions in *L. (V.) guyanensis* is multifactorial.

Financial Support: CAPES, CNPq.

MB-50 – EXPRESSION AND PURIFICATION *LEISHMANIA AMAZONENSIS* ARGINASE IN *E. COLI*

da Silva E.R., Pioker F.C. & Floeter-Winter L.M.
Departamento de Parasitologia - Instituto de Ciências Biomédicas - USP - C.P. 66208 São Paulo SP

The open reading frame of *Leishmania amazonensis* arginase coding gene was amplified by PCR, using genomic DNA as template and oligonucleotides encompassing an *Eco*RI site besides the 5'and 3'end coding regions. Two strategies were used to clone the PCR product into the expression vector pRSET-B (Invitrogen). Strategy A: direct cloning of amplified product into the *Eco*RI site to produce a fusion peptide containing a tag of six histidines. Strategy B: in the *Nde*I site an *Eco*RI site was generated by the ligation of *Eco*RI linker (Biolabs). The new site for *Eco*RI was then used to clone the PCR product in such manner that the expressed product did not have the his tag. The two constructions, A and B, were used to transform *E. coli* (BL-21 DE3pLysS) cells. After induction with IPTG, the transformants, obtained from both constructions A and B, expressed the recombinant enzyme. Extract of these cells was prepared by freeze-thaw (N_2 liquid/42^{o}C) in MOPS 100 mM. A fraction of each extract was incubated in MOPS 100 mM containing $MnCl_2$ 50 mM at 37^{o}C for 4 hours to activate the recombinant arginase. Both extracts, A and B showed arginase activity.

The initial purification of arginase produced by transformant A was performed by affinity chromatography in Ni^{2+} resin. After the fractionation, the electrophoresis analysis showed the presence of a major band with the expected size and some bacterial protein contaminants. It was obtained about 5 mg of arginase to each L of induced *E. coli* culture (A_{600}=1,2).

Purification of protein produced by transformant B was initiated by affinity chromatography in Arginine Sepharose 4-B resin (Pharmacia). The recombinant arginase only was able to bind to the resin if the enzyme was activated with Mn^{2+}. On the other hand the activated enzyme, probably, converted the arginine to ornithine and urea destroying the column. The system A expressed a fusion peptide tag of histidine linked to the recombinant arginase and this can make it difficult to concentrated the enzyme to perform the crystallization process. Generally, it is necessary to remove the his tag to concentrated enzyme. So, on one side, the strategy A has the advantage of being producing a recombinant protein without the his tag, but on the other side it is more difficult to purified. To purify the recombinant enzyme express in the strategy B another affinity resin or chromatographic methods should be used.

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MB-51 – DETERMINATION OF THE *TRANS-SPLICING* SITE OF *LEISHMANIA AMAZONENSIS* ARGINASE CODING MRNA

Castilho T.M., da Silva E.R. & Floeter-Winter L.M.
Departamento de Parasitologia - Instituto de Ciências Biomédicas - USP C. P. 66208 São Paulo SP

Arginase is one of the enzymes involved in the urea cycle that is active in promastigotes of *Leishmania* (Camargo, 1979). The complete nucleotide sequence of the open reading frame of *L. amazonensis* arginase was described to be 990 bp. The mRNA transcribed from the arginase gene, however, was identified as a 4.4 kb long product (da Silva & cols, 1998). The physiological role of the approximately 3.5 kb untranscribed RNA is not known, but they may be involved in regulation of gene expression. Little is known about regulation of gene expression in *Leishmania*. The trypanosomatids, however, presents a special mechanism of mRNA maturation known as *trans*-splicing. A small RNA leader, or mini-exon, is inserted into the 5' end of the mRNA before its exportation to cytoplasm. The recognition site of the *trans*-splicing is described to be a polypyrimidine tract followed by an AG. On the other hand, the 3' end of non-transcribed mRNA may, in some examples, be responsible for the expression of stage-specific products. In order to investigate the presence of such elements in the arginase gene we started the characterization of its mRNA.

A DNA fragment containing 4.3kb, 4.0kb upstream to the arginase ORF, was used in S1 nuclease assay and a 1.0 kb fragment was protected. This result delimited the *trans*-splicing site and provide the means to design oligonucleotides, around that site, to perform a primer-extension experiment. In fact, two oligonucleotides were synthesized at positions –540 and –647, in relation to the start codon of the gene. Both oligonucleotides rendered primer-extension products. Although a complex pattern of bands was obtained, probably due to the processed RNA products in *trans*-splicing event, the deduction of the 39 mini-exon nucleotide long, mapped in a polypyrimidine rich region. Each of those two oligonucleotides will be used in pair with an oligonucleotide derived from the mini-exon sequence in RT-PCR assay, using total RNA as template to precisely map the *trans*-splicing site.

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MB-52 – IDENTIFICATION OF TWO DIFFERENT MITOCHONDRIAL HSP 70 GENES FROM *LEISHMANIA (L.) CHAGASI*

Nascimento, M.G.M.V*, Ferraz Júnior, J.C.S, de Melo Neto, O. P.[+]
Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Av. Moraes Rego s/n, Caixa Postal 7472, Recife PE 50670-420. * Depto. de Genética, Universidade Federal de Pernambuco [+]*e-mail*: opmn@cpqam.fiocruz.br

Many studies have been directed towards the development of ways of inducing protective immunity against Leishmaniasis, a disease caused by protozoan parasites from the different species of *Leishmania*. One specif aim of these studies is to identify and characterize antigens and proteins that could be used in a vaccine capable of inducing an effective immune respose against the parasites. Whithin this framework, a protein fraction from *Leishmania (Viannia) braziliensis*, called LbbF2, whose components have their molecular weight ranging from 67 to 94 kDa, was identified (Frommel, 1988) and used in an immunotherapy assay (Monjour e cols, 1994) against cutaneous Leishmaniasis. It was observed that this protein fraction could stimulate healing of the lesions from afflicted patients. However very little is known about the (molecular) components of this protein fraction.

In previous studies we have reported the identification of 2 different clones from *Leishmania (Leishmania) chagasi* coding for mitochondrial Heat Shock Proteins 70 (mt HSP 70). These clones were obtained by the screening of a *L. chagasi* cDNA library with a rabbit anti-serum raised against the LbbF2 protein fraction from *L. braziliensis*. After the screening, the 2 clones were isolated and partial sequencing identified them as coding for mt HSP 70. One of the cloned inserts had 1.1 Kb and the other 2.2 Kb. We have now accomplished the subcloning of both clones using different restriction enzymes so as to have smaller fragments for posterior automatic sequencing. With more than 80% of the clones sequenced with both strands we have confirmed that they represent 2 different genes. The proteins encoded show a similarity in the N-terminal half of the protein of 94%, whilst in the C-terminal half the homology is 88%. No homology in the 3' untranslated region was seen between the two genes. Further studies will show whether the proteins encoded by these genes have different expression profiles, location and/or distribution within the parasite, as well as their role in the parasite's life cycle. To our knowledge this is the first description in kinetoplastid protozans of two different genes for mt HSP 70 from the same species.

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MB-53 – IDENTIFICATION OF TRANSCRIBED SEQUENCES FLANKING THE META 1 GENE IN *LEISHMANIA AMAZONENSIS*

Ramos CS, Praxedes RZ, Uliana SRB
Departamento de Parasitologia, Instituto de Ciências Biomédicas, USP, Caixa Postal 66208, 05389-970 São Paulo, SP, Brasil

The meta 1 gene of *Leishmania amazonensis* is upregulated in metacyclic promastigotes and encodes an 11.5 kDa protein with no significant similarities to other proteins in the existing databases. The overexpression of this protein renders the parasite more virulent to mice (Uliana et.al., Exp. Parasitol., in press) but the precise mechanisms underlying this effect remain undetermined. The characterization of the meta 1 gene in *L. amazonensis* was obtained initially by screening a cosmid library from which clones were selected representing 60 Kb of contiguous sequences. Characterizing this contig may provide information about the functional organization of this region regarding transcribed sequences and regulation of the gene expression. Southern blots of *Nco* I and *Xho* I digested cosmid DNA were probed with reverse transcribed metacyclic poly (A^+) RNA. The following hybridizing fragments were selected for further analysis: 7 Kb *Xho* I, 4 Kb *Nco* I and 3 Kb *Nco* I. These fragments were purified and subcloned and will be used to probe Northern blots of procyclic, metacyclic and amastigote RNA. Fragments of interest will be sequenced.

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MB-54 – CHARACTERIZATION OF THE INTERGENIC REGION OF *LEISHMANIA HOOGSTRAALI* RIBOSOMAL DNA

Orlando, T.C. & Floeter-Winter, L.M.
Departamento de Parasitologia - Instituto de Ciências Biomédicas - USP - C.P. 66208 São Paulo SP

Leishmania hoogstraali is a natural lizard-infecting parasite that belongs to the Trypanosomatid family. As other lizard *Leishmania*, the taxonomic position and phylogenetic relationship with other *Leishmania* spp remains uncertain. Our goal is to use the intergenic spacer of the rRNA genes, that evolves at a faster rate than the coding region, to study very closely related organisms and verify if this region can be used to establish the phylogenetic relationships among *Leishmania* species.

A clone containing a 9,0Kb fragment, encompassing the 3' end of the LSU subunit and the IGS/ETS region of the rRNA genes of *Leishmania hoogstraali*, was obtained from a partial genomic library and characterized. In the IGS region, the digestion with *Dde*I showed the presence of approximately 40 copies of a 63bp long repetitive element. The chromosomes of *L. hoogstraali* were separated by pulsed field gel electrophoresis (PFGE) and a segment containing the IGS/ETS region of the organism was mapped in the same chromosome that the fragment correspondent to the rRNA 18S, showing that these repeats are unique to the rDNA region. The nucleotide sequence of the 1186bp, located 5'from the 18S subunit was determined and compared to the corresponding region of *L. tarentolae* (Orlando e cols., Mem. Inst. Osw. Cruz, 93 (Suppl): 173, 1998), *L. amazonensis* (GenBank accession number U21687), *L. chagasi* (U42465) e *L. donovani*(L38572), showing between 69% and 97% of sequence identity. In the ETS region of both *L. hoogstraali* and *L. tarentolae*, the presence of a *Hin*dIII site and a unique nucleotide sequence seems to be characteristic of lizard *Leishmania.*

A fenogram was obtained with the alignment, using the Neighbor joining method and shows *L. hoogstraali* and *L.tarentolae* more closely related to *L. chagasi* and *L. donovani* than to *L. amazonensis.* The same topology was obtained when the repeated elements were used in the analysis. Taking all together, the results could indicate that lizard leishmanias branched from Old World leishmanias.

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MB-55 – SEARCHING FOR CENTROMERIC SEQUENCES OF *LEISHMANIA MAJOR*

[1]Casagrande,L, [2]Schwarz,J, [3]Beverley, SM & [1]Cruz, AK
[1]Depto de Bioquímica, FMRP/USP, Av. Bandeirantes, 3900, Ribeirão Preto, SP, Brasil.
[2]University of Missouri, Medical School, USA.
[3]Dept of Molecular Microbiology, WUSM, St Louis, MO, USA.

Centromeres are the structures that direct eukaryotic chromosome segregation in mitosis and meiosis and the site of formation of the kinetochore.

For the identification of discrete centromeric regions in *Leishmania* we are using 3 libraries: a genomic library and two sub-libraries representing chromosomes 2+3 and 6. These libraries were constructed utilizing vectors that can be rendered linear and behave as artificial chromosomes (LAC - *Leishmania* artificial chromosome) inside the parasite cell. The three libraries were mass transfected in *L. major* and transfectants obtained would be expected to be unstable in the absence of drug pressure. On the other hand, transfectants bearing sequences involved in accurate segregation should be more stable under the same conditions.

The study of the dynamics of maintenance, for the transfectants, was made simpler by the combination of two strategies: a modified limiting dilution assay, followed by an evaluation of cell growth under an Elisa reader. After ten passages in liquid culture under no drug pressure, transfectants were submitted to the assay in 96 well-microtiter dishes. A diluted culture of each transfectant is placed in 12 wells under no drug pressure. A replica of each dish is made into fresh media with and without drug.

Such approach decreases the number of clones to be analyzed and sheds light on the dynamics of maintenance of the exogenous chromosome. Such kinetics was expressed as "Percentage of LAC maintenance", which refers to the percentage of wells (cells) still resistant to the drug, after a second replica, relative to the total number of wells (cells) growing in the original plate under no drug pressure.

For both chromosomes 2 and 6, more than 78% of LACs were unstable, while the remainder transfectants showed varying degrees of stability ranging up to 46%. Four *Leishmania* transfectants bearing episomes with high percentage of LAC maintenance were used as template for amplification of episomal DNA. Using single-primer PCR and a subsequent hybridization experiment, two different clones were rescued from chromosome 2. A detailed restriction map of these two clones is under construction. It is noteworthy that they share common sequences. From each clone a variety of deletions are being produced to further test defined regions, through functional analysis, for centromeric function.

Financial Support: FAPESP

MB-56 – PHYSICAL AND TRANSCRIPTIONAL MAPS OF COSMIDS REPRESENTING EXTREME REGIONS OF THREE DISTINCT *LEISHMANIA* CHROMOSOMES

Pedrosa, A. L. & Cruz, A.K.
Laboratório de Parasitologia Molecular, Depto. de Bioquímica, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Av Bandeirantes, 3900, Ribeirão Preto, SP.

Telomeres are specialized structures located at the extremities of linear chromosomes of eukaryotic organisms. These structures are characterized by tandemly organized repeated sequences and have been implicated in genome plasticity and in mechanisms that led to antigenic variation in parasites protozoan parasites.

Aiming the structural characterization of *Leishmania* extremities, we have rescued several clones, from a *Leishmania major* LV39 genomic library, which bear the typical hexameric terminal repeat of telomeres. Three clones (008B01, 008B02 and 117E08) were further analyzed. Bal31 assays confirmed their endmost chromosomal location and PFGE Southern analysis (multi-strain blots) addressed them to chromosomes 3, 7 e 18, respectively. Detailed restriction and hybridization analysis revealed that common sequences of clones 008B02 and 117E08 are restricted to the very end (less than 4 Kb) of these chromosomes. Clone 008B01 showed the presence of reiterated sequences along over than 15 kb from its extremity.

Each one of the recombinant cosmids were transfected in *Leishmania* and maintained under high levels of drug pressure (50 mg/mL Hygromycin B) in order to detect expression of putative genes present in the inserts by hybridization of restriction fragments of the clones in northern blots. The designed strategy allowed the construction of a preliminary transcriptional map for the extreme 40 Kb of the extremities of the 3 chromosomes, even for that genes poorly expressed in the parental lineage. The size of the transcripts detected varied from 1 kb to over than 9 kb. Northern analysis using poly-A+ RNA will confirm whether these transcripts are correctly spliced and a subsequent focused sequence analysis will indicate gene classes present in these regions.

Financial Support: FAPESP.

MB-57 – SIMILAR RDNA PROMOTER REGIONS ARE DIFFERENTLY RECOGNIZED BY EACH *LEISHMANIA* TRANSCRIPTION FACTORS

Stempliuk, V.A. & Floeter-Winter, L.M.
Departamento de Parasitologia - Instituto de Ciências Biomédicas – USP - C. P. 66208 - São Paulo SP

The transcription of ribosomal RNA gene is a species-specific mechanism controlled by RNA polymerase I in association to transcription factors. The transcription initiates at the transcription start point (+1) located around the RNA polI promoter domains, the core domain (-35 to +5), the upstream domain (-150 to -130), and the 5'repetitive enhancer sequences (Sollner-Webb & Tower, 1986).

The *L. amazonensis*, *L. mexicana* and *L. major* rDNA promoter domains were PCR amplified, cloned and sequenced. The sequences were compared showing a high degree of similarity. Transient transfection experiments, using constructs containing different regions of promoter domains, showed three major sites of transcription control. The core promoter was responsible for basal transcription, the upstream domain suppressed this transcription, and the enhancer region increased the expression of the reporter gene. This increase was more effective in the heterologous host cells, despite the sequence similarity (Stempliuk, et al 1998, *Mem Inst Oswaldo Cruz 93*:182)

To study the influence of interaction between rDNA promoter and transcription factors in the regulation of rDNA transcription, we made primer extension assays for each *Leishmania* species. Although similar in sequence, each species mapped the tsp (+1) in a different nucleotide. This result suggests possible differences in transcription factors interaction for each *Leishmania* species. These differences in the recognition of promoter domains by different host cells could then be relied in the specificity of each factor for the promoter ambient instead for sequence composition itself. Gel shift mobility assays were then performed and the results showed that the promoter domains of each *Leishmania* species presented different affinities and mobilities patterns with homologous or heterologous transcription factors.

This results suggest that the complex of transcriptions factors may be different for each *Leishmania*, but they recognize the same sequence of the promoter domains and this recognition is sufficient to drive the transcription.

Financial Support: FAPESP and CNPq

MB-58 – VALIDATION OF THE USE OF POLYMERASE CHAIN REACTION FOR DIAGNOSING AMERICAN CUTANEOUS LEISHMANIASIS IN PERNAMBUCO STATE, BRAZIL

Rodrigues, E.H.G.[1]; Brito, M.E.F.[1]; Mendonça, M.G.[3]; Brandão-Filho, S.P.[1]; Jardim, M.L.[3]; Albuquerque, M.F.P.M.[2]; Fernandes, O.[4] & Abath, F.G.C.[1]
[1]Departamento de Imunologia e [2]Departamento de Saúde Coletiva, Centro de Pesquisas Aggeu Magalhães/FIOCRUZ, Recife-PE. [3]Departamento de Dermatologia, Universidade Federal de Pernambuco, Recife-PE. [4]Departamento de Medicina Tropical, Fundação Oswaldo Cruz, Rio de Janeiro-RJ

The leishmaniasis are caused by several species of the genus *Leishmania*. It is estimated that there are 12 million people infected in the world and approximately 350 million under risk of infection. In Northeast Brazil, American tegumental leishmaniasis is an important public health problem, and is predominantly caused by *Leishmania Viannia braziliensis*.

Two PCR based approaches targetting kDNA regions were used with the aim of evaluating the performance of the systems in the diagnosis of leishmaniasis patients from endemic areas of Pernambuco State, Brazil. One PCR system was specific for the subgenus *Viannia* (De Bruijn *et al.*, 1993, *Trop. Med. Parasitol.* 44:201-207) and the other genus specific, requiring additional hybridization for the identification of groups of *Leishmania* (Schubach *et al.*, 1998, *J. Infect. Dis.* 178:911-914). The detection limit was 10 fg and 1 pg of *L. V. braziliensis* (MHOM/BR/75/M-2903) genomic DNA, respectively for the first and second approaches. Ten µl of the amplification products were analysed by agarosis electrophoresis.

With regard to patients with active lesions 53/59 (90%) were positive using the subgenus *Viannia* specific PCR whereas 47/59 (79.6%) were positive using the genus specific PCR. One out of 4 patients with healed lesions were positive by the *Viannia* specific PCR. No amplification occurred in patients with cutaneous lesions caused by other organisms. We are currently comparing the results with other obtained through conventional diagnostic methods and evaluating if some atypical profiles obtained can be ascribed to intraspecific variation, since these endemic areas have not been extensively studied before.

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MB-59 – COMPARATIVE ANALYSIS OF TWO LEISHMANIA BRAZILIENSIS M2903 ISOLATES DIFFERING FOR THE PRESENCE OF A 245 KB CHROMOSOME

Maria da Conceicao Rocha Sampaio, Andra Figueiredo Barbosa, Maria da Gloria M. Este, Claude Pirmez, Sandra Maria, Pereira de Oliveira and Yara M. Traub-Cseko
Departamento de Bioquimica e Biologia Molecular, Departamento de Entomologia, Instituto Oswaldo Cruz, FIOCRUZ.

Genetic amplification is common in Leishmania, one example being the appearance of a small (245kb) linear chromosome in Leishmania braziliensis M2903, not associated to drug pressure. Pteridine limitation could be the driving force behind this amplification, since Moore & Beverley (1996) found the BT1 gene in this small chromosome. This gene codes for a pteridine transporter with preference for unconjugated pteridines such as biopterin, for which Leishmania are auxotrophic. We are evaluating possible biological and biochemical differences between these two isolates with (M2903+) and without (M2903-) the small chromosome. Metabolic differences were studied by growing both isolates in the presence of biopterin, tetrahydrobiopterin and methotrexate. M2903+ showed a higher resistance to methotrexate (EC50 8nM against 128nM of M2903-) which agrees with the role of BT1 in the development of resistance. Infectivity to macrophages and to the leishmaniasis sand fly vector Lutzomyia longipalpis were studied. Murine macrophages (strain J774) were exposed to the parasites (promastigotes) for 3, 6, and 24 hours then stained and observed with immersion objective. Infected macrophages (amastigotes present) were counted. M2903- were approximately twice more infective than M2903+. There was a slightly lower infection of sand flies with M2903- when these insects were artificially fed with blood mixed with either strain and dissected after 7 days, when bloodmeal digestion is completed. We are now infecting hamsters with the two isolates in order to observe possible differences in 'in vivo' infections.

MB-60 – N-ACETYL GLUCOSAMINE -1-PHOSPHATETRANSFERASE (NAGT) GENE POLYMORPHISM IN *LEISHMANIA* AS A MOLECULAR APPROACH FOR TAXONOMIC STUDIES

Fábio Aguiar Alves[1], Kwang-Poo Chang[2], Hooman Momen[3], Gabriel Grimaldi Jr[1]. and Elisa Cupolillo[1]
[1]Depto de Imunologia, [3]Depto. Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, RJ, Brasil; [2]University of Health Science, Chicago, USA.

Genetic variation in medically important *Leishmania* species and the mechanisms involved in such variation are currently the subject of much interest and controversy. A consequence of the genetic heterogeneity observed in natural populations of New World *Leishmania* (*Am J Trop Med Hyg 50*:296) is the production of different phenotypes (such as infectivity, virulence, antigenic variation, pathogenecity, and drug resistance) which may be associated with distinct clinical manifestations, and may have important epidemiologic implications as well.

Several methods (based on parasite-specific markers) have been proposed to identify isolates by comparison to reference strains. Isoenzymes and monoclonal antibodies are among the most frequently used molecular markers for the identification of *Leishmania*.

Here we have analyzed the variability of the NAGT gene that occurs in *Leishmania* to discriminate these parasites. PCR amplification products were obtained for the evolving conserved region (a 600-800 bp sequence) of this gene, from several reference strains. Amplified DNAs were digested with 4 endonucleases (*Alu* I, *Hae* III, *Msp* I and *Mbo* I), and fragment patterns compared after acrylamide gel electrophoresis. Higher levels of variation were observed in *the* subgenus *Viannia* compared with those belonging to the *subgenus Leishmania*. Intraspecific variability was higher among *Viannia* parasites, showing to be a polymorphic group. Intersting, *L. equatorensis* was not distinguished from *L. colombiensis* by comparing size variation in the DNA banding patterns. The restriction profiles for *Hae* III could discriminate dermotropic (*L.tropica, L.amazonensis*, and *L.major*) from viscerotropic (*L. donovani* and *L. chagasi*) species. In conclusion, this approach is useful for taxonomic studies of *Leishmania*.

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MB-61 – HIGH GENETIC POLYMORPHISM AND POLYPHYLETIC ORIGIN OF *TRYPANOSOMA* (*MEGATRYPANUM*) SPP. ISOLATED FROM DOMESTIC AND SYLVATIC MAMMALS DISCLOSED BY RIBOSOMAL AND RAPD MARKERS

Rodrigues, A. C.; Campaner, M.; Takata, C. S. A.; Ferreira. R. C.; Takeda, G. F.; and Teixeira, M. M. G*.
Department of Parasitology, University of São Paulo, SP, Brazil

Trypanosoma species have been classified as *Megatrypanum* considering exclusively host origin and morphological features of the largest trypomastigote blood forms in mammalian hosts. Almost all domestic and wild mammals are commonly infected by *Megatrypanum* spp. which are considered specific to their host species and worldwide widely dispersed. Aiming for a correct identification of *Megatrypanum* spp. we characterized known isolates previously classified into this subgenus according to morphological criteria and new isolates from domestic (*T. theileri* from cattle and buffalo) and wild animals. However, once large blood trypomastigotes are substituted in culture by epimastigotes indistinguishable from those observed in *Herpetosoma*, which could be also confused with *Schizotrypanum*, we analyzed mice, triatomines and mammalian cell cultures susceptibility to these isolates. According to morphological and behavioral criteria, all isolates from cattle and buffalo could be classified into subgenus *Megatrypanum*. However, isolates from 9 wild mammals, one (from monkey) revealed to be a *T. cruzi* stock, three (monkey, anteater, and sloth) could be classified as *Herpetosoma* and only 4 (rat, monkey, opossum, kangaroo) remained classified as *Megatrypanum*. Thus, morphological criterion is not sufficient to classify *Megatrypanum* from wild animals. To investigate the existence of correlation between morphological/behavioral classification and molecular markers, we evaluated, through RAPD and SSUrRNA, the polymorphism within *Megatrypanum* and the genetic relationship among trypanosomes of all Stercoraria subgenera. RAPD analysis disclosed a high genetic polymorphism among isolates from wild mammals while isolates from bovids were clustered together. Trypanosomes from wild animal previously classified as *Megatrypanum* were segregated in different clusters, some of them clustered with *T. cruzi* or with *T. rangeli*, corroborating the previous classification based on behavioral data. Although all isolates from bovids represented a characteristic monophyletic group, cattle and buffalo isolates were segregated into two subclusters, indicating that although very closely related, isolates from these host-species are not identical, corroborating the host species-specificity of these trypanosomes. When synapomorphic fragments generated for all isolates from bovids, or specifically for isolates from buffalo or from cattle were used as probes, hybridization occurred only with their respective cluster memberships, not hybridizing with any other *Trypanosoma* spp., including all *Megatrypanum* spp. from wild animals. Thus, RAPD data revealed clusters that are directly related to all previous morphological and behavioral analysis, reinforcing its suitability for analysis of phylogenetic relationship among trypanosomes. To better evaluate the evolutionary relationships among members of *Megatrypanum* and other trypanosomes, sequences of SSUrRNA were determined for our new isolates and aligned with those of other species from Gene Bank. The phylogenetic tree obtained showed grouping pattern similar to that generated for RAPD data. Moreover, Brazilian isolates from cattle and buffaloes clustered together with *T. theileri* from German and Japan and also with an isolate from deer, *T. cervi*, resulting in a very consistent clade composed by trypanosomes from the Artiodactyla order. Therefore, our data confirmed that the members of *Megatrypanum* are highly heterogeneous and presented a polyphyletic origin, and thus, is an artificial taxon as previously suggested for a small number of not very well characterized species (Stevens et al., 1999, Int. J. Parasitol.). However, isolates from Arctiodactyla order always originated a well-defined cluster of very closely related organisms. Once *T. theileri* is considered the type-species of this subgenus, we suggested that only species closely related to its clade should be classified as *Megatrypanum.*

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MB-62 – *TRYPANOSOMA (HERPETOSOMA) SPP*.: GENETIC DIVERSITY AND TAXONOMIC POSITION DEFINED BY RIBOSOMAL, SPLICED LEADER AND RAPD MARKERS

Silva, F. M.; Rodrigues, A. C.; Campaner, M; Ferreira, R. C.; Takeda, G. F. & Teixeira, M. M.G*.
Department of Parasitology, University of São Paulo, SP, Brazil

The subgenus *Herpetosoma* is constituted by two groups of nonpathogenic trypanosomes: *T.rangeli* and *T.lewisi*. Although displaying similar morphology of epimastigotes in culture, which is the only feature used to group these species within only one subgenus, organisms of these two groups differ in all other biological aspects. *T.rangeli* is widely distributed in Central and South America, where is sympatric with *T.cruzi*, infecting humans, domestic and silvatic animals, and triatomine vectors. *T.rangeli*-like organisms have been reported on distinct wild mammals and named as distinct species, although their relationship with *T. rangeli* is unclear. Although transmission of *T.rangeli* is by inoculation of infected saliva during bug-feeding, this species is considered as belonging to Stercoraria. In contrast, *T. lewisi*, the type-species of this subgenus and its correlated species are transmitted by fleas, infecting only wild mammals, specially rodents and with worldwide distribution. Morphological similarity of the behaviorally different trypanosomes within this subgenus has led considerable uncertainty regarding the taxonomic status of these species. These uncertainties are only beginning to be resolved by studies on SSU rRNA, which indicated a close evolutionary relationship between *T.rangeli* and *T.cruzi* but not with *T lewisi* and Salivaria trypanosomes (Stevens et al., 1999). To better evaluate the genetic diversity within this subgenus we employed RAPD, ribosomal (SSU and ITS) and spliced-leader (SL) gene markers to analyze reference-species and new isolates of both groups: *T.rangeli* (5 isolates from humans, 2 from triatomines and 4 from wild mammals) and *T.lewisi* (4 *T.lewisi* stocks and *T. blanchardi* and *T.rabinowitshae*). New isolates were previously classified at subgeneric level according to the traditional taxonomic parameters: morphology in culture; mice and triatomine infection and absence of invasion and multiplicati on within cells in vitro. The main criterion used to classify new isolates as *T.rangeli*-like was infection of haemolymph/salivary glands of triatomines. Analysis of polymorphism and genetic relatedness by RAPD, disclosed high similarity among members of *Herpetosoma*, which was clearly divided in two clusters, *T.rangeli* and *T.lewisi*. Moreover, synapomorphic fragments distinguished *T.rangeli* from all other trypanosomes and with distribution of *T.rangeli* isolates in at least two subclusters. Analysis of SL gene repeats also showed absence of close genetic relationship between members of different groups within *Herpetosoma*, segregating the organisms in the same groups defined by RAPD and also corroborating the existence of at least two subgroups of *T. rangeli* isolates. As previously demonstrated, analysis of SSU rRNA disclosed high similarity among all members of *T.rangeli* group, which showed to be not close related with *T.lewisi* and its related species. Comparison of ITS of rRNA resulted in identical grouping of organisms and also disclosed the previously observed partition of *T.rangeli* isolates. Taken together, data from morphological comparison, transmission experiments and molecular characterization supported the segregation of *Herpetosoma* spp. in at least two well-separated clusters composed by very closely related trypanosomes. Thus, results confirmed that *Herpetosoma* is a polyphyletic subgenus, reinforcing that the taxonomic position of its members needs to be reevaluated. Moreover, results from distinct molecular markers suggested a complex structure of *T. rangeli* clade, with intra-specific genetic variability that permitted separation of *T.rangeli* isolates in two main groups, one composed by isolates from humans and triatomines from Central and northern South America, and other composed by isolates from Amazonian wild animals.

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MB-63 – MINI-EXON GENE REVEALS THE POSSIBILITY OF EXISTENCE OF TWO DIFFERENT GROUPS AMONG *TRYPANOSOMA RANGELI* STRAINS

Grisard, E.C.[1,2,3]; Steindel, M.[1]; Campbell, D.A.[2] & Romanha, A.J.[3]
1- Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Catarina, Caixa postal 476, Trindade, Florianópolis, SC – Brazil, 88040-900, e-mail: grisard@ccb.ufsc.br; 2- Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA – USA and 3- Centro de Pesquisas René Rachou – FIOCRUZ, Belo Horizonte, MG – Brazil.

Trypanosoma rangeli is one of the two parasites of this genus that infects humans, domestic and wild animals in Central and South America. During the last years, *T. rangeli* infection in a wide variety of hosts, including human cases in northern of Brazil have been reported. *T. rangeli* is being considered as a problem for Chagas disease epidemiology due to their morphological, biological and biochemical similarities with *T. cruzi*, as well as sharing the same reservoirs and vectors. Studies on *T. rangeli* strains isolated from different reservoirs, vectors and geographical origins using biological, biochemical and molecular methods such as isoenzyme profiles, RAPD, kDNA structure and sequence and susceptibility of different triatomine vectors species have been carried out during the last years. In all studies, strains isolated in Santa Catarina State, the southernmost distribution of this parasite in South America, proved to be distinct from the others isolated in Venezuela, Colombia and Honduras. Interestingly, strain H8GS, isolated in Honduras, also revealed to be distinct from the others. Previous studies on the mini-exon gene sequence of some *T. cruzi* and *T. rangeli* strains isolated from different geographical regions, revealed two distinct groups, formed by each trypanosoma species. Despite its groupment, *T. rangeli* strain SC-58 revealed to be genetically distinct from the other *T. rangeli* strains. Together, the mini-exon gene analysis and previous biological, biochemical and molecular data, suggest the existence of two distinct groups or lineages among *T. rangeli* strains isolated throughout the American Continent, as recently described for *T. cruzi*. Further studies, including more strains and genetic markers, must be performed in order to confirm this hypothesis.

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MB-64 – EXPRESSION OF NEOMYCIN PHOSPHOTRANSFERASE AND GREEN FLUORESCENT PROTEIN (GFP) BY *TRYPANOSOMA RANGELI*

Grisard, E.C.[1,2,3]; Romanha, A.J.[2] & Campbell, D.A.[3]
1- Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Catarina, Caixa postal 476, Trindade, Florianópolis, SC – Brazil, 88040-900, e-mail: grisard@ccb.ufsc.br; 2- Centro de Pesquisas René Rachou – FIOCRUZ, Belo Horizonte, MG – Brazil and 3- Department of Microbiology and Immunology, UCLA School of Medicine, Los Angeles, CA – USA.

Trypanosoma rangeli is an hemoflagelate parasite that infects human as well as domestic and wild animals in Central and South America. During the past years, studies on *T. rangeli* life cycle in the vertebrate host have revealed controversial results. Due to its morphological, biological, and antigenic similarities with *T. cruzi*, the study of *T. rangeli* life cycle in its reservoirs and vectors is very importat for the Chagas disease epidemiology. In order to develop a new approach to study *T. rangeli* life cycle in both vertebrate and invertebrate hosts, the aim of our work was to obtain transfected lineages of the parasite expressing the green fluorescent protein (GFP). For that, we have made a new construct based on the 5.6Kb plasmid pTEX (*Nucleic Acids Res.*, 20: 3963-69, 1992), suscessfully tested in *T. cruzi* and *Leishmania* sp. This plasmid contains a neomycin phosphotransferase gene, confering resistance to neomycin analogs, such as G-418 and paromomycin sulfate (HumatinÒ) to transfected cells. The GFP gene used is a mutant which fluorescence is 50 x more intense than the original gene. This gene was inserted into pTEX, preserving the multiple cloning site of the plasmid. The new plasmid, named pTEX-GFP_{mut} (6.4Kb), was transfected into epimastigote forms of *T. rangeli* strains SC-58, isolated in Brazil, and Choachi, isolated in Colombia. Transfected parasites from both strains were able to grow in the presence of 15µg/ml of G-418 or 200µg/ml of paromomycin sulfate, revealing a strong and stable fluorescence distributed throughout the parasite cell. Both live and metanol fixed/Giemsa stained fluorescent parasites were seen just 72 hours post-transfection. Studies are being carried out in order to evaluate the plasmid replication form within the parasite cell and, to clone the transfected lineages prior their submission to "*in vivo*" assays. These assays will be performed independently in both mice and triatomine bugs, and later, through cyclical passages mice-triatomine-mice.

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MB-65 – CLONING AND EXPRESSION OF *T. CRUZI* FATTY ACYL-COA BINDING PROTEIN

Kenneth G. Milne & Michael A.J. Ferguson
Dept. of Biochemistry, Wellcome Trust Building, University of Dundee, Dundee DD1 5EH, Scotland, U.K.

African trypanosomes posses a coat of variant surface glycoprotein (VSG) that is attached to the cell surface by a glycosylphosphatidylinositol (GPI) anchor. The GPI biosynthetic pathway has been elucidated using a cell-free system and important aspects are the fatty acid remodelling and exchange reaction necessary to form the mature GPI precursor for anchoring the VSG. During these reactions the fatty acids attached to the glycerol moiety are removed and replaced with myristic acid that is incorporated from myristoyl-CoA. In other eukaryotes it has been shown that fatty-CoA's are bound to fatty acyl-CoA binding protein (ACBP) to prevent their metabolism and also shuttle them to the acylation machinery. This study involved identifying, expressing and assaying the effect of *T. brucei* ACBP upon the fatty acid remodelling reactions in the GPI biosynthetic pathway.

I 'blast" search databases with the human ACBP sequence and identified a *T. brucei* expressed sequence tag (EST) from the dbEST of GenBank. I used the EST sequence as a probe against a size selected library from which I cloned the full lenght gene and subsequently over expressed the recombinant protein with an N-terminal histidine tag in the baculovirus system. The purified recombinant protein was shown to bind myristoyl-CoA by electrospray mass spectrometry and its affinity for this ligand was determined to be 2×10^{-10} M by equilibrium dialysis. When the recombinant trypanosoma ACBP was added to the *T. brucei* cell-free system to study GPI biosynthesis I observed a greatly increased level of fatty acid remodelling. I am currently attempting to knock out the gene to determine if it is an essential gene for the parasite.

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MB-66 – BRAZILIAN STOCKS OF *TRYPANOSOMA EVANSI* FROM DOMESTIC AND WILD MAMMALS: MOLECULAR AND MORPHOLOGICAL ANALYSIS DEMONSTRATED TOTAL AKINETOPLASTY OF TRYPANOSOMES FROM BOTH LABORATORY STOCKS AND NATURALLY INFECTED ANIMALS

Ventura, R. M.; Takata, C. S. A.; Silva, R.A.M.S*; Takeda G. F. & Teixeira, M. M. G[1].
Department of Parasitology, University of São Paulo, SP, Brazil; *Embrapa, SC, Brazil.

T. evansi is the only species belonging to the subgenus *Trypanozoon* (Salivaria) existing in America being fatal for horses and dogs and also affecting several wild mammals. *T. evansi* differs from *T. brucei* in that it cannot complete cyclical development in the tsetse fly, but it is only mechanically transmitted by other blood sucking flies and vampire bats. Transmission through tsetse fly requires functional mitochondria and thus kDNA, the mitochondrial DNA of trypanosomes. In nature, all trypanosomatids present maxi and minicircles of kDNA, except *T. evansi* and *T. equiperdum*. All *T. evansi* stocks have aberrant kDNA network lacking completely maxicircles and although most exhibit minicircles (dk, dyskinetoplasty; dkDNA), some stocks can be totally disproved of both maxi and minicircles (ak, akinetoplasty; akDNA). It was suggested by Giemsa-staining that incidence of ak stocks is very low in Old World (0 to 8%), whereas in the New World the incidence of such forms is higher (4 to 100%). Despite the small number of isolates examined so far by molecular methods, both ak and dk have been described in South American isolates. Thus, due to the lack of more extensive molecular studies about kinetoplastic status of American stocks some questions remain to be elucidated: a) the prevalence and geographic distribution of akDNA stocks; b) the existence of both dkDNA and akDNA trypanosomes within one stock; c) the existence of minicircles in very small amounts or presenting very different conformation. To elucidate these questions we comparatively analyzed old and recently isolated Brazilian stocks, maintained in laboratory by passages in mice, and stocks from Old World. Giemsa-staining showed to be insufficient to ascertain the presence of kDNA once it was very difficult in several cases to distinguish between dye artifact and kinetoplast, whereas DAPI analysis showed negative results for all stocks. Stocks analyzed by electron microscopy showed to be disproved of typical kinetoplast, which was substituted by small clumps of electron-dense material surrounded by a double membrane. Despite using very sensitive methods, hybridization and kDNA-PCR assays, we could not detect minicircles in any Brazilian stock. In addition, our analysis also did not reveal kDNA in trypanosomes recovered directly from naturally infected blood of domestic animals (horses and dogs), frequently treated with antitrypanosomes drugs, or wild animals (capybaras and coatis), which are never drug-treated. For this purpose we standardized a duplex PCR assay to simultaneously amplify both kDNA and SL gene fragments of distinct size, using as DNA crude template preparations from blood smears. Results suggested that ak of Brazilian stocks are neither induced in laboratory by long period of time and successive passages in mice or by antitrypanocides, but might be the natural state of these stocks. Thus, these stocks must be indeed totally disproved of minicircles in contrast to stocks previously described from Colômbia, suggesting that distinct stocks could be introduced independently in different regions of South America. Therefore, kinetoplastic state of American *T. evansi* needs to be evaluated whenever stocks from new areas are available. Total ak of Brazilian stocks besides taxonomic and phylogenetic interest also provides valuable information for chemotherapy and diagnosis.

Supported by FAPESP. [1] e-mail mmgteix@icb.usp.br

MB-67 – DETECTION AND TYPING OF *TRYPANOSOMA VIVAX* BY PCR

Dávila A.M.R., Silva R.A.M.S., Traub-Cseko, Y. M.
[1] Lab. de Biologia Molecular de Tripanosomatídeos, Departamento de Bioquímica e Biologia Molecular, IOC/FIOCRUZ, Av. Brasil 4365, Rio de Janeiro, RJ. davila@gene.dbbm.fiocruz.br. [2] Embrapa-Centro Nacional de Pesquisas de Suínos e Aves.

Trypanosoma vivax belongs to the Salivaria section of the genus Trypanosoma (Hoare 1972) and is the causative agent of cattle trypanosomosis in most parts of Africa and Latin America. Estimated economic impact could be about 160 million dollars for the Pantanal-Brazil and Bolivian lowlands where more than 11 million cattle are under risk. Since most of South American isolates are not infective for laboratory rodents and cultivation *in vitro* is difficult, this trypanosome species has been poorly studied. Previous studies at the molecular level showed that strains from different places can be discriminated by RAPD, although little genetic polymorphism is found. Since RAPD and RFLP require parasite DNA free of host-DNA and there are limitations to obtain a high number of parasites, the use of PCR for specific amplification of regions of parasite genome is the best option to evaluate the polymorphism in samples containing both host and parasite DNA. We are evaluating different DNA extraction methods (phenol-clorophorm, Chelex-100 and DNAzol-BD) on 55 isolates of *T. vivax* collected from different places of the Brazilian Pantanal and the Bolivian lowlands. The integrity of DNAs extracted by different methods was evaluated, and only when DNAzol was used we could detect DNAs in agarose gels. The presence of the parasite is being confirmed by PCR using species-specific primers for the satellite DNA (Masiga et al 1992) and the genetical polymorphism will be evaluated by PCR-RFLP of the ITS gene of *T. vivax*. Because these primers are designed based on the conserved flanking regions of this gene, the ITS of other trypanosome species could be also amplified, particularly in samples presenting mixed infections. Our preliminary results show that *T. vivax* and *T. evansi* can be successfully discriminated by the size of the products obtained. We obtained ITS sizes of ~550bp and ~1.1Kb for *T. vivax* and *T. evansi*, respectively.

MB-68 – MECHANICALLY TRANSMITTED *TRYPANOSOMA VIVAX* FROM BRAZIL: CHARACTERIZATION OF SPLICED-LEADER GENE REPEAT AND DEVELOPMENT OF A SPECIES-SPECIFIC PCR ASSAY BASED ON INTERGENIC REGION

Ventura, R. M.; Paiva, F. ; Buck, G. A.* & Teixeira, M. M. G.
Department of Parasitology, University of São Paulo, SP, Brazil. * Medical College of Virginia, Virginia Commonwealth University, VA, USA.

Trypanosoma (*Dutonella*) *vivax* is a major pathogen of cattle in Africa and is distributed throughout the areas where tsetse occur and also in areas free of this insect because this trypanosome has adapted to a mechanically transmission by other fly species. *T. vivax* is also widespread in Central and South America, where it seems to have been introduced with cattle imported from West Africa. This species was first reported in Brazil in 1972, by Shaw and Lainson, in a water buffalo in Belém, Pará State. Nowadays, *T. vivax* is found infecting cattle in the north of Brazilian Pantanal and in its vicinities as in the lowlands of Bolivia, which are considered the most important livestock producing regions of these two countries. *T. vivax* in South America is transmitted mainly by tabanids and the rainy season represents the period of greatest risk of trypanosome transmission by these insects due to their abundance. In infected cattle from Brazil and Bolivia, this bovine trypanosomosis has been reported as a devasting disease. Diagnosis of *T. vivax* infections in cattle is done routinely by microhematocrit test and serological methods. However, these methods present low sensitivity and/or specificity and thus, molecular methods have been investigated using probes and PCR assays based on repetitive DNA sequences. Development of sensitive and specific diagnostic methods suitable to efficient screening *T. vivax* infections in cattle and wild reservoirs is very desirable to an earlier detection of this trypanosome, to prevent its further spreading and to estimate the livestock population at risk.

In contrast to *T. vivax* from Africa, occurrence of American stocks was only determined by morphology of blood trypanosomes, without molecular characterization and comparative studies with African stocks. Aiming both, to make a comparative analysis between Brazilian and African *T. vivax* stocks, and also to develop an alternative diagnostic method, we decided to characterize the spliced-leader (SL) gene repeats from a stock recovered from cattle of the Pantanal Region, Miranda city (MS), Brazil. SL gene sequence was obtained from a PCR-amplified SL repeat, using as template total DNA obtained from blood of experimentally infected animal. Alignment of this sequence with that from African *T. vivax* deposited in gene Bank, revealed high similarity, even within the intergenic region, while sequences of all other trypanosome species showed very different intergenic regions. Thus, to develop a *T. vivax* specific PCR assay we designed primers which are complementary to SL intergenic sequences in order to amplify a 210 bp of the spacer-region. We evaluated the specificity of SL-PCR assay by testing 12 *Trypanosoma* species, including Salivarian and Stercorarian species. Results showed that this method is species-specific, amplifying just *T. vivax* stocks either from Brazil or from West Africa. Furthermore, this PCR presented high sensitivity, showing the expected amplified fragment with DNA content of only one parasite. To verify if the *T. vivax* PCR could be applied in large field studies, avoiding the need of purified DNA from well preserved blood samples, we evaluated the feasibility to use crude preparations of DNA obtained from infected cattle blood samples. These samples were collected on both filter papers and glass-slide blood smears. Results obtained using these crude preparations indicated that the developed *T. vivax*-specific SL-PCR assay can be an useful tool to specifically detect this species in field-collected blood samples.

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MB-69 – IDENTIFICATION OF SEQUENCES RELATED TO THE META 1 GENE IN TRYPANOSOMATIDS

Serezani, C.H.C, Uliana, S.R.B.
Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Caixa Postal 66208, 05389-970 São Paulo, SP

The meta 1 is a stage-regulated gene, initially identified in *Leishmania major,* that encodes an 11.5 kDa protein expressed predominantly in metacyclic promastigotes. Very low levels of expression are observed in procyclic promastigotes and amastigotes. The *Leishmania* meta 1 sequences share no significant similarities to other proteins in the existing databases. While the precise function of the protein remains unknown, meta 1 overexpressing promastigotes show increased virulence in the mouse model of infection (Uliana *et al* . Exp. Parasitol., in press). Taking into account the high degree of conservation in the nucleotide sequences of meta 1 genes in both New and Old World *Leishmania* species, we have designed PCR primers to verify whether similar sequences are present in other members of the Trypanosomatidae family. The expected size amplified products were obtained when PCR reactions were performed with *T. cruzi*, *C. fasciculata* and *L. tarentolae* genomic DNA as template. The PCR products were purified by electroelution and ligated into pMOS (Amershampharmacia). Sequence determination of the PCR products will provide data to allow comparisons with *Leishmania* meta 1 sequences to be made.

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MB-70 – PCR AMPLIFICATION OF THE SPLICED LEADER GENE FOR THE DIAGNOSIS OF TRYPANOSOMATID PARASITES OF PLANTS AND INSECTS IN METHANOL-FIXED SMEARS

Serrano, M.G.[1], Campaner, M.[1], Buck, G.A.[2], Teixeira, M.M.G.[1] & Camargo, E.P[1].
[1]Departamento de Parasitologia, Instituto de CiÃncias Biom¾dicas, Universidade de S±o Paulo, 05508-900 S±o Paulo, SP, Brasil, [2]Department of Microbiology and Immunology, Medical College of Virginia Campus, Box 980678, Virginia Commonwealth University, Richmond, Virginia 23298-0678, U.S.A.

The identification of trypanosomatids belonging to the *Phytomonas* genus is challenging despite of development of several serological, biochemical and molecular methods. Most of these methods require parasites in culture which is not feasible for all isolates and may not reflect the original parasite population in the host. Thus, to facilitate the detection and elucidate the diversity and distribution of *Phytomonas*, we analyzed DNA from methanol-fixed smears of insects and plants parasitized by tryapanosomatids using a well-defined PCR assay of the spliced leader gene (SL-PCR). The PCR-based method was adapted from the reaction previously described for the discrimination of true *Phytomonas* from other genera of kinetoplastid protozoa (Serrano et al., 1999). To perform the SL-PCR, we employed smears of methanol-fixed parasites on glass slides as templates with different annealing conditions; i.e., those which permit amplification from all trypanosomatids (Try-SLPCR) or those which amplify exclusively DNA from *Phytomonas* (Phy-SLPCR). Amplicons were hybridized with an oligonucleotide probe (SL3') specific for *Phytomonas* and SL201 probe, which detects DNA from any genus of trypanosomatid. The amplified PCR products were cloned in pMOS Blue blunt-ended cloning kit and sequences were determined in an automated ALF DNA sequencer. The DNA sequences of seven (7) amplicons were consistent with existence of several different species/ strains of *Phytomonas* circulating among diseased palms and fruits. Our method obviates the requirement for cultivation of parasites, many of which are very fastidious or non-cultivable. The technique was applied to archival glass slides and to newly collected material. It proved to specific for *Phytomonas* spp., permitting efficient detection in plants and insects.

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MB-71 – RANDOMLY AMPLIFIED POLYMORPHIC DNA ANALYSES AS TOOL FOR CLUSTERING *CRITHIDIA* AND *HERPETOMONAS* SPECIES

Pires MQ, Esparis CM & Pacheco RS
Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz- FIOCRUZ

Previous reports (Pacheco *et al.* 1994 *J Protozool Res* 4: 71-82; Pires *et al* 1997 *Mem Inst Oswaldo Cruz* 92: 187) have shown the high genetic variability among species belonging to the genus *Crithidia* by using restriction fragment length polymorphisms of kDNA analyses (RFLP) and hybridization with mini-exon gene probes.

In this study randomly amplified polymorphic DNA analyses (RAPD) were used as molecular markers to investigate 20 different monogenetic trypanosomatids from the genera *Crithidia* (14) and *Herpetomonas* (6) providing further information to previous data already reported and also adding some new insight to the genus *Herpetomonas.* Fourteen distinct *Crithidia* species were randomly amplified using the primer OPA-10 (Operon Technologies - 5'GTGATCGCAC 3'). The amplification cycles were as follows: denaturation at 95 ^{0}C for 2 min., followed by 34 cycles at 95 ^{0}C for 1 min, annealing at 37 ^{0}C for 1 min. and for extension the temperatures of 72 ^{0}C was used for 2 min. The final extension was carried out during 10 min at 72 ^{0}C. *Crithidia* and *Herpetomonas* species studied revealed genetic polymorphisms confirming the status of different taxa. The RAPD data were evaluated by phenetic analyses using Dice and Simple Matching coefficient of similarities. The matrix of similarity was transformed into a dendrogram showing different degree of similarities. Among *Crithidia* species, a cluster was composed by *C, fasciculata, C. guilhermei*, *C. luciliae* and *C. desouzai*.. *C. guilhermei* seems to be more close related to *C. fasciculata* displaying a coefficient of similarity of 0.95. Two isolates of *C. fasciculata* (ATCC 11745 e ATCC 12857) showed 100% of similarity between then. *C. luciliae* and *C. desouzai*, although belonging to the same cluster, appear to be more distant displaying coefficient of similarities of 0.70 and 0.40 respectively. This result corroborated previous data using *C. fasciculata* mini-exon gene as probe were a strong sequence homology was detected with *C. guilhermei* . The second cluster was formed by *C. oncopelti* and *C. harmosa* separated from the first one by a coefficient of 0.30, both species showed 50% of similarity. The third cluster, composed by *C. luciliae thermophila, Crithidia sp* (isolated from *Zellus leucogramus*), *C.hutneri, C. deanei, C. flexonema* and *C. mellificae*, revealed more polymorphisms at nuclear level. The same was observed at the mitochondrial level when RFLP analyses were performed. This last cluster was found to be detached by a coefficient of 0.30 from the previous ones. On the other hand, C. acanthocephali displayed less of 20% of similarity when compared with the latter groups. Six *Herpetomonas* species were randomly amplified using the primer OPA-09 (5'GGGTAACGCC 3') according to the same protocol. RAPD and phenetic analyses have allowed to group *H. muscarum ingenoplastis* and *H. mariadeanei* into the same cluster showing 87% of similarity. *H. anglusteri* and *H. roitmani* were grouped into a second cluster with 85% of similarity. Both clusters are linked by a coefficient of similarity of 0.82. The species *H. megaseliae* and *H. samuelpessoai* are separated displaying 78% and 70% of similarity with the prior clusters, respectively. By this criterion, the most separated specie in this genus is *H. samuelpessoai*.. Fewer polymorphisms were found among *Herpetomonas* when compared with *Crithidia* species.

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MB-72 – PURIFICATION AND CHARACTERIZATION OF POSSIBLE HOMOLOGUES OF THE TRANSLATIONAL INITIATION FACTOR EIF4E FROM TRYPANOSOMATIDS

Dhalia, R., Standart, N.* and de Melo Neto, O. P.$^+$
Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Av. Moraes Rego s/n, Caixa Postal 7472, Recife PE 50670-420. * Depto. of Bochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge UK CB2 1GA$^+$*e-mail*: opmn@cpqam.fiocruz.br

Trypanosomatids are primitive eukaryotes which are characterized by very peculiar processes involved in the formation of mature RNAs, such as RNA editing and *trans*-splicing. Through *trans*-splicing, a compulsory mechanism required for mRNA maturation, pre-mRNAs are processed by the addition of a 39 nt sequence (the spliced leader - SL) to the 5' end of the mature mRNAs. The first 4 nt of the SL sequence are subjected to various modifications including several methylations. To its 5'end a 7 methyl-GTP nucleotide, identical to the cap of higher eukaryotes, is added. The cap signals the beggining of the mRNA in other eukariotes and the complex of 5 nucleotides in the 5' end of the SL sequence, and consequently all parasite mRNAs, is denominated cap4. It may or may not constitute a different signal from the simpler cap of higher eukariotes, whereas it functions as a binding site for the eIF4E translational initiation factor (also known as cap binding protein). The eIF4E acts mediating the recruitment of other translation factors (the cap binding complex) and of the smallest ribosomal subunit to the mRNA, allowing the start of translation. In this work, we proposed to investigate the presence of homologues of eIF4E in trypanosomatids, which might be involved in cap4 recognition for translation. First we purified by affinity chromatography using the resin 7-methyl GTP sepharose (Pharmacia) 2 polypeptides from *Crithidia* sp. extracts. These proteins, of 47 and 80kDa, are capable of binding the resin in high salt concentration and are eluted in conditions similar to those observed for the elution of mammalian eIF4E. They were then purified and used to immunize mice, in order to produce specific antibodies. Sera which specifically recognizes the 80 kDa protein has now been obtained. Lack of cross-reactivity with the 47 kDa protein suggests that they are not related. At the present moment we are carrying out new assays in order to confirm the identity of these proteins and their involvement in the specific recognition of the cap. Further on amino-terminal sequencing of the two proteins will be performed and screening of cDNA expression libraries might be tried with the available antibody for the 80 kDa protein. Any clones obtained will be used for further homology analysis with know parasite proteins and eIF4E from higher eukaryotes.

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MB-73 – HOMOLOGY MODELING OF *PLASMODIUM FALCIPARUM* DIHYDROFOLATE REDUCTASE. A TOOL FOR THE DESIGN OF ANTIMALARIAL DRUGS

Santos Filho, O. A.[1] ; Figueroa-Villar, J. D.[1]* and Alencastro, R. B.[2]
1.Departamento de Química do Instituto Militar de Engenharia, Praça general Tibúrcio 80, 22290-270, Rio de Janeiro, RJ, Brasil;
2. Instituto de Química da Universidade Federal de Rio de Janeiro, RJ, Brasil.

The 2,4-diamino pyrimidines, such as pyrimetamine, have been extensively used as antimalarial drugs. The mechanism of action of this family of drugs is related to their capacity to inhibit *P. falciparum* dihydrofolate reductase (DHFR, 5,6,7,8-tetrahydrofolate-NADP $^{+}$ oxidoreductase, E.C. 1.5.1.3), being extraordinarily active against the erythrocytic parasites as well as non toxic for the mammalian host. Unfortunately, drug resistance for pyrimetamine appeared only two years after its introduction and is now a wideworld problem. As for the 2,4-diaminopyrimidines, other DHFR inhibiting drugs, like proguanyl, have had their efficiency in the prophylaxis of malaria seriously decreased by drug resistance. Although the resistance to DHFR inhibiting antimalarial drugs have been related to mutations in the aminoacid sequence, the structure of this enzyme have not been determined. The DHFR of *P. falciparum* exist as a domain of a bifunctional enzyme, DHFR (aminoacid residues 1 to 228) linked to thymidylate synthase (TS; 5,10-methylenetetrahydrofolate: dUMP C-methyltransferase, E.C. 2.1.1.45, aminoacid residues 323 to 608), linked by a long junctional sequence of 94 amino acids (Bzik *et al., Proc. Natl. Acad. Sci. USA*, 1987, 84, 8360-8364).

To the best of our knowledge we have obtained the first low resolution structure of the native *P. falciparum* DHFR and its active site. This was accomplished by homology modeling of the protozoan enzyme using as template the 2.0 Å resolution chicken liver DHFR. The template was chosen after a careful analysis of the degree of homology between the DHFRs reported in the Protein Data Bank and the sequence of aminoacids of the malarial enzyme using multiple alingment procedures with the Swiss-PdbViewer program. The greater homology was obtained with the chicken liver enzyme (35.43%). The first crude structure of the protozoan enzyme was gerated by mutation of the aminoacids of the template enzyme followed by the introduction of the loops and deletion of aminoacids until the whole sequence of the malarial enzyme was obtained. The new loops were modeled using the database of the Swiss-PdbViewer program and the whole structure refined using the AMBER force field to 10 kcal. mol^{-1}. $Å^{-1}$ and a medium with a dielectric constant of 78.58 (water). The final structure possesses 5 α-helices, 8 strand-twisted β-sheets and several extended loops. The Ramachandran plot generated using the PROCHECK program showed that 98% of the aminoacids have conformations in the allowed regions of the plot for the whole enzyme. The aminoacids of the active site were defined by comparison with the literature information on substrate and inhibitor binding for several DHFRs. The aminoacids involved in the propposed active site are: F9, D10, I11, L40 to F58, E71 to R77, N100 to W109, I112 to N124, S128, R129, T130, K132, I143, K145, V146, I168 to E176, T189, R190, I191, D198, V199. The Ramachandran plot for these residues indicate that 100% of them are in favorable regions, indicating that our model is reliable.

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MB-74 – DEVELOPMENT OF A POLYMERASE CHAIN REACTION (PCR) FOR DIAGNOSIS OF MALARIA AND COMPARASION WITH OTHER DIAGNOSTIC METHODS

Schindler, H. C[1] Montenegro, L. L[1]., Montenegro, R. A[1]., Nascimento, E. J. M[1]., Jaureguiberry, G[2]. & Abath, F. G.C[1].
1.Departamento de Imunologia, Centro de Pesquisas Aggeu Magalhães/FIOCRUZ, Cidade Universitária, Recife, PE.
2.Museum National D'Histoires Naturelle, Laboratorie de Biologie Parasitaire et Chimiotherapie, EP CNRS 1790, Paris France.

Malaria is one of the major of public health problems in Brasil, with more than two million cases diagnosed in the last three years. Difficulties to diagnose patients with low parasitemia actively interfere in the control of the disease. Among the conventional parasitologic tests, the direct microscopic exams of thick smear and blood film have shown low sensitivity. With the aim it developing a sensitive PCR method for simultaneous identification of *Plasmodium falciparum* and *Plasmodium vivax*, we used oligonucleotids designed from a 18S SSU rRNA sequence of *P. falciparum.* The sensitivities and specificities of three variations of the Polimerase Chain Reaction (PCR) technique were compared (simple PCR, PCR with digoxigenin labelling and double PCR) using direct microscopy as gold standard. 402 blood samples were studied, from patients distributed in 5 groups as following: Group 1- 156 symptomatic patients, from endemic area with positive parasitemia in thick smear and blood film; Group 2- 39 symptomatic patients with negative parasitemia by direct microscopy; Group 3- 144 asymotomatic patients, from endemic area with negative parasitemia by direct microscopy, 70 of them being blood donors; Group 4- 33 asymptomatic patients of non-endemic area and with negative microscopy and Group 5- 30 patients with other protozoan diseases, from non-endemic area, being 10 with chagas disease, 10 with visceral leishmaniosis and 10 with cutaneous leishmaniosis. Double PCR was able to detect 0,01 pg of purified DNA from *P. falciparum*, wich represents a detection capacity 10000 times higher in comparison to simple PCR and 1000 times higher than PCR-DIG. The QBC test showed a sensitivity of 91,7% and specificity of 83,7% using microscopic exams as gold standard. Sensitivity of double PCR (97,4%) was significantly higher when compared with QBC, simple PCR and PCR-DIG. All variations of PCR shown a 100% specificity, regarding normal individuals living in non endemic areas. There was no difference between sensitivity of PCR tests when the two species of Plasmodium (*P. falciparum* and *P. vivax*) were individually considered. Among the 70 blood donors, 17 (24,3%) were positive by double PCR. Theses results suggest that double PCR can detect asymptomatic carriers of malaria, in endemic areas, in situations where the microscopic exams were unable to detect the parasite.

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MB-75 – DIAGNOSIS OF MALARIA IN AN ENDEMIC AREA OF BRASIL USING A HEMI-NESTED PCR METHOD

Montenegro, L. L[1]., Montenegro, R. A[1]., Nascimento, E. J. M[1]., Jaureguiberry, G[2]., Schindler, H. C[1]. & Abath, F.G.C[1].
1.Departamento de Imunologia, Instituto Aggeu Magalhães/FIOCRUZ, Cidade Universitária, Recife-PE, Brasil. 2.Museum National D'Histoires Naturelle, Laboratorie de Biologie Parasitaire et Chimiotherapie, EP CNRS 1790, Paris France.

The malaria is a parasitic illness infecting about 300 the 500 million people over the world. The transmission through blood transfusion is relevant in regions of the world where the illness is endemic thus, it is essential the diagnosis of low parasitemia patients. The amplification of the 18S rRNA gene by PCR is being used in our laboratory as a diagnostic tool for malaria. To improve the sensitivity of the assay a primer was designed to be used in a hemi-nested PCR. The detection limit of the techinque was established analysing the detection of known amounts of purified *P. falciparum* DNA. One hundred samples from individuals living in endemic areas and 30 blood samples from individuals living in non endemic areas were processed by lisis with saponin 0.04%. The samples were analised by hemi-nested PCR. The first PCR (Primers GJ_1 and HR842) allowed the amplification of a 746 bp product and the second PCR (primers GJ_1 and GJ_2) amplified a 213 bp fragment. The detection limit of the first and second PCR was 10pg and 0.01pg, respectively. The amplificons were analysed by 1.5% agarose gel eletrofophoresis and visualized by ultraviolet transillumination. Fifty out of 130 samples were positive by conventional microscopy, while 80 were negative. Using the conventional microscopy as gold standard, the simple and the hemi-nested PCR presented sensitivities of 30% and 94%, respectively. Respecting the specificity, the PCR assays were 100% specific in normal individuals living in non endemic areas. In conclusion, the results indicate that the double (hemi-nested) PCR was very sensitive being able to detect as little as 0.01pg DNA.

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MB-76 – STANDARTIZATION OF A BLOT-ELISA FOR SIMULTANEOUS DETECTION OF CS PROTEIN FROM *PLASMODIUM FALCIPARUM* AND *P. VIVAX* IN ANOPHELES MOSQUITOES

José Bento Pereira Lima (1, 3), Izidro Bendet (1), Miguel Quintana (1), Robert A Wirtz (2), Ricardo Lourenço-de-Oliveira (3)
(1) USA Medical Research Unit, Instituto de Biologia do Exército, Rio de Janeiro, RJ, Brasil; (2) Centers for Diseases Control and Prevention, Atlanta, USA; (3) Departamento de Entomologia / FIOCRUZ-RJ.

In order to develop a BLOT-ELISA capture method for the simultaneous detection of *Plasmodium falciparum* (Pf) and *Plasmodium vivax* (Pv) in infected mosquitoes, nitrocellulose membrane (NCM) was used as a solid phase binding support for the malaria species-specific monoclonal antibodies (mAbs) Pf2A10 and PvNSV3. To sensitize the NCM, the diluted monoclonal antibodies were applied using a computer controlled pen plotter to produce parallel lines. The appearance of a dark line in the NCM indicated the presence of the recombinant circumsporozoite protein. The BLOT-ELISA sensitivity was 6pg for both PfR32tet32 and PvNS1v20 RCS proteins. In order to evaluate the BLOT-ELISA specificity, an ELISA test was carried in parallel, using triturated mosquitoes infected with Pf or Pv parasites. Cross-reaction between the recombinant CS proteins or with *Plasmodium gallinaceum* esporozoites was not observed. Cross-reaction with blood feeding source was only observed when using mAbs against Pf and mosquitoes recently fed (10 min) with rabbit blood. The advantages of BLOT-ELISA method here developed include the possibility of simultaneous detection of different Plasmodia species in the same nitrocellulose strip, the possibility of visual detection of CS protein, without need of reader equipment, easy procedure, and the reduction of total reaction time.

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MB-77 – GENETIC POLYMORPHISM OF MSP-2 ANTIGEN IN *PLASMODIUM FALCIPARUM* FIELD ISOLATES FROM THE BRAZILIAN AMAZON

Sallenave-Sales, S[1]; Ferreira-da-Cruz, MF[1]; Faria, CP[1]; Sanfins, CS[1];Durlacher, R[2]; Pang, L[2]; Daniel-Ribeiro, CT[1] & Zalis, MG[3].
Department of Immunology, Fiocruz, RJ-Brazil[1]; USA Medical Research Unit, RJ-Brazil[2] & IBCCF, UFRJ, RJ-Brazil[3]

The extent polymorphism of *P. falciparum* antigens and the implications of this characteristic in relation to the immune response have stimulated epidemiological studies in order to understand the mechanisms of this variability. In the present work, we verify the genetic polymorphism of MSP-2 antigen (Merozoite Surface Antigen 2) in 105 *P.falciparum* isolates from the Brazilian endemic area of Peixoto de Azevedo (MT-Brazil). A hundred fifty two fragments were amplified by PCR (Polimerase Chain Reaction) using specific primers to the central variable region of MSP-2. The PCR fragments were first analyzed in a 2% agarose gel, and then by typing using specific probes corresponding to the two main allelic families of this antigen: FC27 and 3D7. Four different allelic variants were detected: 520bp (52%) and 500bp (5%) - that hybridized with FC27 probe - and 620bp (41%) and 600bp (2%) - that hybridized with 3D7 probe. In order to detected sequence microheterogeneities between the allelic groups, 118 out of 150 fragments (24% of them were detected only by hybridization) were analyzed by SSCP technique (Single Strand Conformational Polymorphism). Each fragment was digested with RsaI restriction enzyme, denatured and analyzed in a 10% silver stained polyacrilamide gel. Differences between the SSCP patterns observed between the fragments of groups I (520bp) and III (620bp), suggest a small sequence microheterogenities not detected by hybridization. The sequencing analysis of these samples and the evaluation of humoral response of the individuals could provide a better evaluation of MSP-2 polymorphism in the studied area.

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MB-78 – DRUG RESISTANCE MOLECULAR MARKERS IN *PLASMODIUM FALCIPARUM* ISOLATES FROM THE BRAZILIAN AND COLOMBIAN AMAZON REGION - EVIDENCE OF CLONAL EXPANSION OF CHLOROQUINE RESISTANT PARASITE

Pedro-Paulo Vieira, , Christiane Marques,Luis Giraldo Rivera, Mariano G. Zalis
Laboratório de Biologia da Malária, Instituto de Biofísica Carlos Chagas Filho, UFRJ
Instituto de Biologia do Exército - Rio de Janeiro
Centro de Internacional de Investigaciones Medicas - CIDEIM, Cali, Colombia

The first reports 40 years ago describing chloroquine resistance in *P. falciparum* pointed to an independent appearance in two foci. Strains appearing in Southeast Asia spreading to Africa on the one hand, and in South America on the other. Circumstantial evidence led to the belief that the overexpression or mutations of a P-glycoprotein (pfmdr1 gene) was the cause of chloroquine resistance. However, genetic cross analysis between resistant and sensitive strains did not show segregation of mutations in the pfmdr1 loci. A recently published paper demonstrates a strong correlation between a specific set of polymorphism in the cg2 gene of *P. falciparum* strains from Southeast Asia and Africa, and chloroquine resistance. The data on the only South American strain presented in this paper shows a different set of polymorphism. Analysis of 18 fresh cultured-stable *P. falciparum* isolates from the Brazilian Amazon region showed in vitro resistance to Chloroquine and Quinine. The cg2 repeats and mutation pattern were similar to the South American strain 7G8. This data could indicate that chloroquine resistance in the Brazilian Amazon is a clonal expansion of a single resistant parasite and could point to a different mechanism of resistance in South American strains. A new marker called *Plasmodium falciparum* Transporter Linked to Chloroquine resistance (pftcr) was also investigated (Christopher Plowe, unpublished data).

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MB-79 – MOLECULAR CLONING, SEQUENCING AND EXPRESSION OF A SERINE PROTEINASE INHIBITOR FROM *TOXOPLASMA GONDII*

Pszenny V., Angel, S.O., Ledesma, B., Guarnera, E., Ruiz, A.E. & Bontempi, E.
Instituto Nacional de Parasitología 'Dr. M. Fatala Chaben', A.N.L.I.S. 'Dr. Carlos G. Malbrán', Av. Vélez Sarsfield 563 (1281), Buenos Aires, Argentina.

A huge number of serine proteinase inhibitors (serpins) have been described in a wide range of organisms from virus to mammals. The Kazal inhibitor family is one of numerous families of serine proteinase inhibitors. The basic structure of such type of inhibitor has been well characterized and the mechanism of interaction of serpin with their cognate enzymes is known in considerable detail. (Laskowski Jr. *et al.*, 1980, *Ann. Rev. Biochem.* 49:593-626). At our knowledge there are not described serpin proteins in protozoa parasites. *Toxoplasma gondii* is an obligate intracellular parasite, member of the phylum Apicomplexa, closely related to other important parasites as *Plasmodium* and *Eimeria*. *T.gondii* is known to cause transplacental infections that can lead to abortion or to severe neonatal malformations. Recently, *T. gondii* has emerged as an opportunistic pathogen of major importance in immunocompromised individuals. Here, we isolated a cDNA clone from *T.gondi*i tachyzoites cDNA λZap library encoding a KAZAL-type non classical serin proteinase inhibitor. The cDNA clone contains an open reading frame (ORF) of 882 bp, encoding a protein of 294 amino acids including a putative 23 amino acids signal peptide with a predicted molecular weight of 30,190 Da and a pI of 4.86. This protein displays an internal sequence homology of residues 30-66, 114-150, 181-217 and 247-283 indicating a four-domain structure. Based on the amino acid sequence, the four domains exhibit high homology to serine proteinase inhibitors belonging to the non-classical Kazal-type family. The gene apparently exists as a single copy in the tachyzoite haploid genome of RH strain as it is suggested by Southern blot analysis. The genomic version was picked up by PCR. From its sequence it was clearly demonstrated the presence of several introns. For expression, a fragment encoding the deduced mature *Toxoplasma* serpin protein was subcloned into pQE30, wich adds 6-His residues to the N-terminal end of the protein. Expressed protein was purified by metal affinity chromatography and analized by SDS-PAGE. Using the recombinant protein several functional assays will be performed. Some hypothetical functions of this protein could be: to protect the parasite from the intestinal enzymes, to act as immunosubversive protein (McFadden *et al.*, 1995, *J. Leukoc. Biol.* 57:731-738), and/or to participate in a regulatory mechanisms blocking the invasion as recently described for other proteinase inhibitors. (Conseil *et al.*, 1999, *Antimicrob. Agents Chemother.* 43:1358-1361).

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MB-80 – DIFFERENTIAL DIAGNOSIS OF SPECIES AND STRAINS OF *EIMERIA* SPP. OF DOMESTIC FOWL BY RAPD AND PCR AMPLIFICATION OF THE RRNA INTERNAL TRANSCRIBED SPACER 1

Fernandez, S.; Katsuyama, A.M and Gruber, A.
Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, USP, São Paulo, SP, Brasil

Seven different species of the genus *Eimeria* can cause a severe intestinal disease that leads to important economic losses in poultry production. Species identification is actually achieved using some imprecise parameters, including oocyst shape and size, prepatent period and site of infection. Strain discrimination is even more difficult, since intra-specific variations can only be detected by more elaborated methods such as isoenzymatic analyses. The present study aimed at discriminating *Eimeria* species and strains isolated in Brazil using the randomly amplified polymorphic DNA (RAPD) as well as a specific PCR assay. A total of 165 decamer oligonucleotides (Operon Technologies, Inc.) were screened as primers for the RAPD method and 56 resulted in multiband profiles allowing for a high discrimination of the 7 species. In addition, 36 primers generated molecular markers associated with intraspecific variations of 6 different strains of *E. acervulina*, 5 of *E. tenella* and 2 of *E. maxima*. Recently, a specific PCR reaction for *Eimeria* species discrimination was described by others (Schniztler *et al*., 1998; 1999). This assay, directed towards the internal transcribed spacer 1 (ITS1) of the ribosomal genomic cistron, employed 7 primer pairs, specific for each one of the species. In the present work, a new set of primers was designed, using one common upper primer located in a conserved region of the 18S ribosomal subunit, plus seven lower primers directed to species-specific priming sites in the variable ITS1 region. The resulting amplified products differ in circa of 100 bp from each other and range from 147 to 711 bp. The PCR reactions were standardized employing isolated and mixed samples, and a specific amplified product was observed for each species. A multiplex PCR assay using the 8 primers in a single tube reaction and allowing for the discrimination of the seven pathogenic *Eimeria* species from field samples is under development.

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MB-81 – HIGH RESOLUTION SSU RDNA RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP) AS A TOOL FOR ASSESSING GENETIC RELATIONSHIPS IN GENUS *ACANTHAMOEBA*

Alves, J.M.P., Teixeira, M.M.G. & Affonso, M.H.T.
Depto. de Parasitologia, Instituto de Ciências Biomédicas - USP, 05508-900, São Paulo - SP.

Although organisms of the genus *Acanthamoeba* are the causative agents of a severe keratitis (closely associated with contact lens use) and a fatal granulomatous amebic encephalitis (GAE), and despite the many decades of using them as models in cellular, biochemical and molecular studies, species assignment, phylogeny and diagnosis are still poorly defined. The classical morphological criteria have already shown inadequate for such purposes, by being inaccurate, slow and subjective. Therefore, there have lately been several attempts of using molecular methods to solve biological questions concerning *Acanthamoeba*, and to improve diagnosis. Of the several available molecular methods, those dealing with SSU rDNA sequences are the most promising in terms of phylogeny and identification. Therefore, we are attempting to use SSU rDNA to know more about the relationships between different Brazilian keratitis and ATCC strains as well as to search for diagnostic sequences for genus or species.

PCR amplified SSU rDNA genes of keratitis and ATCC reference strains were analyzed by RFLP technique in silver stained polyacrylamide gels, which provide much more sharp bands and well resolved patterns than ethidium bromide stained agarose gels. This allowed us to clearly define some groupings of more genetically related isolates. As expected for *Acanthamoeba*, the obtained patterns revealed an extensive divergence between the various species and isolates of the genus. Beside that, reference species *A. polyphaga* and *A. castellanii* were decidedly polyphyletic, and therefore those strains are misplaced, confirming the need of reviewing classification and species definition in the genus.

Our results show that this approach can be used as a fast and sensitive indicative of genetic variability within the SSU rDNA gene of *Acanthamoeba*, and therefore indicates which isolates would be the best candidates for sequencing and posterior phylogenetic studies. This is another very advantageous point of our technique, since such an a priori indication could save a lot of time that would be spent in unnecessary sequencing.

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MB-82 – IDENTIFICATION OF RELATED PROTEINS TO INVASIVE PHENOTYPES IN *ENTAMOEBA HISTOLYTICA* STRAINS

Valle, P.R[1]., Souza, M.B.G[1]., Pires, E.M[1]., Pesquero, J.L[2]., Silva, E.F[1]. & Gomes, M.A[1].
Departamento de [1]Parasitologia, [2]Fisiologia e Biofísica, ICB-UFMG, Av. Antônio Carlos, 6627, Belo Horizonte, MG, Brasil

The clinical forms of amoebiasis, a disease caused by the protozoan parasite *Entamoeba histolytica*, are very variable. It is verified from serious symptomatic cases, as hepatic abscess, to asymptomatic ones. One of the explanations for this fact is the variability of the pathogenic potential among the countless strains. However, the pathogenic potential itself, as an important factor in the determination of the virulence of the parasite, is closely related with the pattern of protein expression. So, variations in the pattern of gene expression of a certain strain can alter its behavior in the infection. The strains HM1 and CSP, used in this work, were isolated in Mexico and Brazil, respectively, of patients with dysenteric colitis. Both possessed great invasive capacity *in vivo*, producing intense tissue destruction in the experimentation animals at the time of its isolation. Its maintenance in a long axenic cultivation left them nonvirulent. The inoculation in animals, in which didn't cause anymore any lesions, reestablished its invasive capacity. The protein expression in the two forms of the strains, virulent and not, was evaluated through the technique of RAP-PCR. Of the 40 used primers, one made possible the detection of differences in the intensity of expression of two fragments among the virulent strains and the nonvirulent ones, suggesting the importance of these in the determination of an invasive phenotype. The analysis of those sequences is being made and it can supply important information regarding the establishment of the disease in man.

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