XXX ANNUAL MEETING ON BASIC RESEARCH IN CHAGAS DISEASE - XIX MEETING OF THE BRAZILIAN SOCIETY OF PROTOZOOLOGY - HOTEL GLÓRIA, CAXAMBU, MG, BRASIL - 10-12 NOVEMBER 2003. **Rev. Inst. Med. trop. S. Paulo, 45**(Suppl. 13), November, 2003.

BM1 - PROMOTER REGIONS OF *PLASMODIUM VIVAX* ARE UNABLE TO RECRUIT THE TRANSCRIPTIONAL MACHINERY OF P. FALCIPARUM.

Azevedo, M.F. & del Portillo, H.A.

Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo SP, Brasil.

Stable and transient transfection in *Plasmodium* has been use to study gene function and gene regulation, becoming a powerful tool to better understand the biology of this parasite. This technique has helped to elucidate the mechanisms involved in virulence, imune evasion, drug resistance, cell invasion, stage differentiation and others. *Plasmodium vivax*, the parasite responsible for about 80% of Brazilian malaria cases, and which causes great socio-economical burden for this region cannot be cultured continuously *in vitro* precluding the use of transfection technology to carry functional studies of this human malaria parasite. To functionally characterize promoters and elements responsible for the control of gene expression in *P. vivax*, we constructed vectors having the luciferase reporter gene under the control of the promoter regions of the *P. vivax msp1* and *dhfr* genes and used them in heterologous transient transfections in *P. falciparum*. Strikingly, none of the constructs were able to drive expression of the luciferase gene unlike positive controls which included promoter regions from *P. berghei* and *P. chabaudi*. These data seem to indicate that unlike other *Plasmodia spp*, *P. vivax* promoters are unable to recruit the transcriptional machinery of *P. falciparum*. As the AT-content of the *pvmsp1* and *pvdhfr* promoter regions is GC-rich, present efforts are guided to construct a luciferase reporter plasmids with an AT-rich promoter region from *P. vivax* which closer resembles promoter regions of *P. falciparum*.

Supported by FAPESP and CNPq

BM2 - *PLASMODIUM VIVAX*: PRIMARY STRUCTURE AND ALLELE POLYMORPHISM OF THE *PVMDR1* GENE AMONG ISOLATES FROM BRAZIL, PAPUA AND MONKEY-ADAPTED STRAINS.

Martha-Sá J+, Nomura T++, Wellems TE++ & del Portillo HA+@.

+Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo SP, Brazil.
++ National Institute of Allergy and Infectious Diseases, NIH, USA
@correspondence (hernando@icb.usp.br).

Despite intensive efforts at eradication and control, malaria remains a major public health problem. Yearly, it is estimated that 300-500 million people worldwide are afflicted with a death toll of 1.7-2.4 millions mostly in children below five years old. The emergence of *Plasmodium falciparum* resistance to widely used antimalarial drugs such as chloroquine as worsened this scenario and made malaria control and treatment even more difficult. Drug resistance is also now emerging in *Plasmodium vivax*, the most widely distributed human malaria and responsible for 70-80 million clinical yearly cases. Multidrug resistance genes (mdr) from *Plasmodium* are proposed to be involved in certain forms of drug resistance, including resistance to mefloquine, chloroquine and quinine. These genes have been reported and analyze for *P. falciparum* but not for *P. vivax*.

To identify *P. vivax* mdr genes, degenerate oligonucleotides were used in PCR amplifications of *P. vivax* DNA and a unique 4.5 kB fragment cloned and sequenced. *In silico* analysis revealed that this sequence shared 70% similarity with the pfmdr1 gene and that it displayed conserved domains with two highly conserved ATP bidding cassette (ABC) sites and two conserved transmembrane domains. This same fragment was amplified cloned and sequenced from different chloroquine sensitive and resistant isolates including 4 patients from the Brazilian Amazon, 4 from Papua and 2 from *P. vivax* monkey-adapted strains. Similarity and dendrogram analyses revealed that sequences could be grouped according to their geographical origin and that within each geographical group resistant samples branched independently. Interestingly, all samples from Papua and the monkey-adapted chloroquine resistant strain revealed the presence of an in frame stop codon that resulted in a truncated MDR protein. The identification and characterization of the *P. vivax mdr1* gene opens new avenues to further studies of drug resistance in this human malaria parasite.

Supported by FAPESP and CNPq

BM3 - STUDIES ON THE POLYMORPHISM OF CYTOADHERENT DOMAINS OF VARIANT SURFACE ANTIGENS IN FIELD ISOLATES OF *PLASMODIUM FALCIPARUM* FROM THE BRAZILIAN AMAZON.

Albrecht, L., Tumilasci, V. F., Wunderlich, G.

Department of Parasitology – ICB – USP.

The infection of humans with the apicomplexan parasite *Plasmodium falciparum* causes the most serious form of Malaria. An important virulence factor is a variant surface antigen, located on the surface of the infected red blood cell (iRBC), and named *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1). Different allelic forms of this protein mediate the cytoadherence to several different endothelial receptors. PfEMP1s are encoded by the large multigene family named *var*. Recent studies indicate that the majority of PfEMP1s show cytoadherence to the CD36 receptor. The responsible CD36 binding domains are highly conserved in their three-dimensional structure, and can be used as components of an anti-adherent vaccine. Apparently the same occurs for cytoadherence of PfEMP1/s to CSA, a major complication in primigravid women. In the current study, *P. falciparum* field samples are being collected and selected *in vitro* for cytoadherence to the ICAM1 receptor, implied by different authors to be one of the most important receptors for cytoadherence of iRBC in brain capillaries, which itself possibly results in cerebral malaria. The corresponding *var* gene transcripts are being characterized from *P. falciparum* cultures selected for ICAM1 cytoadherence. The repertoire of *var* gene transcripts associated to ICAM1-cytoadherence in different *P. falciparum* isolates will provide data on the viability of an anti-adherence vaccine, by determining similarities or differences between the corresponding transcribed *var* genes. This is the first study concerning the repertoire of ICAM1-cytoadherent domains of PfEMP1s in field isolates. The first *var* gene sequences from 5 different ICAM1-cytoadherent field samples will be presented.

Financial support: CNPq and FAPESP.

BM4 - *PLASMODIUM FALCIPARUM VAR* GENES TRANSCRIPTION AND SILENCING IN A CENTRAL *VAR* GENE CLUSTER DEPENDS ON THE COMBINATION OF SPECIFIC *VAR* INTRON AND PROMOTER SEQUENCES.

Author(s): Gölnitz, U., Tumilasci, V.F., Wunderlich G.

Institution: University of São Paulo, Institute of Biomedical Science, Department of Parasitology, Laboratory of Molecular Biology of *Plasmodium falciparum*

Text: The *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1), encoded by the multigene family named *var*, is responsible for the cytoadherence of infected erythrocytes in malarial infections. Approximately 50 *var* genes exist per parasite genome, which are mostly located in subtelomeric

regions of all chromosomes, but are also found as clusters in central chromosomal regions. It was shown that almost all *var* transcripts are detectable in ring stage whereas in trophozoite stage one or only a few genes are transcribed while the rest of the family remains transcriptionally downregulated. Recent data published by Deitsch et al. indicate that *var* gene silencing requires the presence of a *var* intron and elements within it and an upstream element in the promoter. In the present study we selected a parasite adhesion phenotype by multiple panning procedures on E-selectin and identified a transcribed *var* gene in a centromeric/ central cluster of 4 *var* genes and 1 *rif* gene on chromosome 4. In order to describe factors, which were important for *var* transcription of one *var* gene or silencing of an adjacent one in the cluster, we compared the promoter and intron sequences and the *var* gene sequences itself, and tested the presence of transcripts. Interestingly, an adjacent 5' localized *var* gene containing exactly the same promoter sequence, but a different intron, appeared not to be transcribed. The same was true for a copy of another *var* gene localized in 3´-postion of the transcribed *var* gene, which contained the same intron, but a different promoter compared to the transcribed *var* gene. All promoters localized in the cluster were of the previously described 5B1-type. Our data indicate that the combination of *var* promoters and *var* introns are critical for silencing and transcription of the respective *var* genes.

Financial support: FAPESP

BM5 - SEQUENCE DIVERSITY, ANTIBODY RECOGNITION AND EVOLUTION OF THE MALARIA VACCINE CANDIDATE ANTIGEN MEROZOITE SURFACE PROTEIN-2 (MSP-2) OF *PLASMODIUM FALCIPARUM*

Tonon, A.P.*, Hoffmann, E.H.E.*, Silveira, L.A. *, Ribeiro, A. G. *,. Gonçalves, C. R. S*, Ribolla, P. E. M.†, Wunderlich, G. *, and Ferreira, M. U. *

*Departamento de Parasitologia, Instituto de Ciências Biomédicas da Universidade de São Paulo, Brazil, and †Departamento de Parasitologia, Instituto de Biociências de Botucatu da Universidade Estadual Paulista, Brazil.

The malaria-vaccine candidate Ag merozoite surface protein-2 (MSP-2) of *Plasmodium falciparum* comprises polymorphic central repeats flanked by dimorphic domains that define the allelic families FC27 and IC1. We have examined temporal patterns of sequence variation in the *MSP-2* gene of *P. falciparum* populations in Brazil and its impact on MSP-2 Ab recognition by local patients. DNA sequence analysis of 61 isolates revealed only 25 unique *MSP-2* alleles, many of them differing by single-nucleotide replacements and insertion/deletion events. Identical *MSP-2* alleles were found in genetically unrelated parasites collected 6-13 years apart, indicating their independent origin by homoplasy in the absence of major selective pressure exerted by variant-specific immunity. To examine Ab cross-reactivity patterns by ELISA, recombinant Ags derived from both locally prevalent and foreign MSP-2 variants were used. Ab recognition of FC27-type Ags and local IC1-type variants correlated with cumulative malaria exposure, but was frequently IgM-restricted. Foreign IC1-type variants, such as 3D7 (included in an experimental vaccine currently under field trial), were poorly recognized. Abs were able to discriminate between local and foreign IC1-type variants, but cross-recognized structurally different IC1-type Ags that were prevalent in local parasites. In contrast, cross-reactive Abs to local and foreign FC27-type variants were more frequently found. The IgG1:IgG3 subclass balance of MSP-2 Abs was exposure-dependent, but no clear-cut bias towards IgG3 was found in heavily exposed patients. We suggest that consensus or ancestral *MSP-2* sequences derived from evolutionary models could be used in vaccine prototypes to minimize the genetic difference between local parasites and vaccine Ags.

Financial support FAPESP, CNPq and TDR/WHO

BM6 - *PLASMODIUM FALCIPARUM:* GENETIC DIVERSITY OF THE MEROZOITE SURFACE PROTEIN-2 (MSP-2) IN ISOLATES FROM ENDEMIC AREAS OF PARÁ AND RONDÔNIA STATES, BRAZIL

FARIA, CP[1]; SALLENAVE-SALES, S[1]; SOUZA, JM[2]; FIALHO, RR[3]; SANTOS, F[4];DANIEL-RIBEIRO, CT[1] & FERREIRA-DA-CRUZ, MF[1].

Departamento de Imunologia, IOC/Fiocruz, RJ[1]; Instituto Evandro Chagas, Fundação Nacional de Saúde, PA[2], Fundação Nacional de Saúde, DF[3] & Fundação Nacional de Saúde, RO[4].

Plasmodium falciparum had been shown to present an extensive genetic polymorphism, and a given isolate can be constituted by different clonal populations. In the present work, we have evaluated the genetic polymorphism of the MSP-2 antigen in eighteen *P. falciparum* isolates from an endemic area of Pará (Paragominas) and seventy-eight from Rondônia (Porto Velho), Brazil. Forty fragments from Pará and one hundred nineteen from Rondônia isolates were amplified by nested-PCR using specific primers for the central variable region of MSP-2. The PCR fragments were analyzed in a 2% agarose gel. In Pará, seven different alleles were detected: 600bp (12.5%), 570bp (12.5%), 550bp (42.5%), 490bp (2.5%), 470bp (2.5%), 450bp (25%) and 400bp (2.5%); in Rondônia, ten different alleles were detected: 620bp (1%), 600bp(1%), 550bp (43%), 520bp (4%), 500bp (2%), 490bp (1%), 470bp (2%), 450bp (38%), 400bp (5%) and 340bp (3%). In order to detect sequence microheterogenities, thirty-four fragments from Pará were also analyzed by the SSCP (Single Strand Conformational Polymorphism) technique. Each fragment was digested with *RsaI* restriction enzyme, denatured and analyzed in 10% silver stained polyacrilamide gel. In this case, no sequence polymorphism was observed in any of the analyzed fragments, showing a sequence similarity between these alleles. We concluded that the isolates from Pará and Rondônia presented a limited genetic polymorphism when compared to those observed in areas of high endemicity. The allelic typing and sequencing of the samples will provide a better evaluation of MSP-2 polymorphism in the studied areas.

Supported by CNPq, Fiocruz

BM7 - PROKARIOTIC EXPRESSION AND PURIFICATION OF *PLASMODIUM GALLINACEUM* CS N-TERMINAL AND C-TERMINAL AND PRELIMINARY ANALYSIS OF CROSS-REACTIVITY WITH SERA OF *P. FALCIPARUM* INFECTED PATIENTS

Mansur, S. B. E., Alves-Brito, C. F. and Krettli, A. U.

Laboratório de Malária, Centro de Pesquisas René Rachou -FIOCRUZ, Belo Horizonte/MG

The circumsporozoite (CS) protein is the predominant surface antigen of *Plasmodium* sporozoites and the major target recognized by the host immune system. CS proteins have been characterized from many *Plasmodium* and contain a secretory signal sequence, a N-terminal region and a C-terminal region, regions of charged amino acids, and an anchor sequence. Phylogenetic analysis using CS gene suggested that the *P. gallinaceum* is more closely related to the *P. falciparum* than other species of human malaria *Plasmodium*. Therefore, the *P. gallinaceum* antigens showed a strong cross-reactivity with antibodies against *Plasmodium falciparum*. Thus, the *P. gallinaceum* has been used as a model to study the immunity against *P. falciparum* infections. In this study, the N-terminal (CSA) and C-terminal (CSC) regions of *P. gallinaceum* CS protein were subcloned in the vector pET32a (Novagen). The expressions of CSA and CSC proteins were induced by addition of 1mM IPTG and recombinant proteins purified using Ni-NTA resin and electroelution. The proteins were used as antigens to coat microplates and specific antibodies were search in sera

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of patients infected with *P. falciparum* in ELISA. Significant levels of antibodies anti-CSA were detected in sera of *P. falciparum* infected patients. The antibody levels were detected independent of the number of malaria infections and the last malaria was *P. falciparum* or *P. vivax*.

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BM8 - EVALUATION OF POLYMORPHISM OF THE P126 *P. FALCIPARUM* PROTEIN AND ITS POSSIBLE ROLE IN DEVELOPMENT OF SPECIFIC IMMUNE RESPONSE

Pratt-Riccio, L.R.[1], Lima-Junior, J.C.[1], Sallenave-Sales, S.L.[1], Zalis, M.G.[2], Guimarães, M.L.[1], Ferreira-da Cruz, M.F.[1], Camus, D.[3], Daniel-Ribeiro, C.T.[1] & Banic, D.M.[1]

1. Fundação Oswaldo Cruz, Rio de Janeiro, Brazil, 2. Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; 3 Inserm U42, France.

The *Plasmodium falciparum* P126 antigen is one of a number of erythrocytic-stage proteins being studied as potential malaria vaccine components. The P126 amino-terminal portion, containing 6-octamer repeat, has been shown to be involved in the induction of protection against *P. falciparum* challenge in monkeys. However, a polymophism present in some isolates that contained 5- instead of 6-octamer repeats was observed. In this study we evaluated the genetic polymorphism of N-terminal region of the P126 protein in *P. falciparum* isolates and its possible role in development of specific immune response in individuals living in Brazilian endemic areas. The frequency of polymorphism was verified by SSCP-PCR in 83 isolates from Porto Velho (RO) and 92 isolates from Peixoto de Azevedo (MT). The humoral immune response was analyzed by ELISA using the synthetic peptide Nt47, corresponding the N-terminal region of the protein. Only two different allelic fragments were detected in each area studied: I (199pb) and II (175pb). In Porto Velho, the allele I was detected in a higher frequency (92%) than allele II (8%). In Peixoto de Azevedo the alleles I and II were observed in similar frequencies, 59% and 41%, respectively. Analysis by SSCP does not revealed microheterogeneities of sequences between fragments with same size and only one SSCP pattern was observed for each fragment identified. It was not observed associations between allelic fragments and the humoral immune response against Nt47. However, a positive correlation between cytophylic response (IgG1+IgG3) and the presence of fragment I in individuals living in Porto Velho was verified. The data here presented showed a limited genetic polymorphism of the P126 in *P. falciparum* obtained from infected individuals living in Porto Velho and Peixoto de Azevedo. This allelic polymorphism seem does not influence the development of specific humoral immune response.

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BM9 - EXPLORING THE GENOME OF *TRYPANOSOMA VIVAX*: TOWARDS A COMPARATIVE GENOMICS APPROACH

Dávila, A.M.R.[1], Guerreiro, L.T.A.[1], Souza, S.S.[1], Hall, N.[2]

1 DBBM, Instituto Oswaldo Cruz, Fiocruz. Av. Brasil 4365, Rio de Janeiro, RJ, CEP 21045-900. Email: davila@fiocruz.br; 2 The Sanger Institute, Hinxton, Cambridge, CB10 1SA, UK.

Trypanosoma vivax is a hemoparasite affecting livestock industry in South America and Africa. Despite the high economic relevance of the disease caused by *T. vivax*, few researches on its molecular characterization has been done to date compared with human trypanosomes as *T. brucei* spp and *T. cruzi*. The main reason is the difficulty to grow the parasite into laboratory rodents and "in vitro". These characteristics have limited the research on *T. vivax* during the last decades, consequently very few markers have been described for its molecular characterization. A search in Genbank showed that there are only 22 entries for *T. vivax* confronted with nearly 98319, 38686, 23586 for *T. brucei*, *T. cruzi* and *Leishmania*, respectively. *T. vivax* (molecular) biology is also little understood, even considering major differences as mechanical transmission in South America and both cyclical and mechanical transmission in Africa. In order to identify new molecular markers for *T. vivax*, a small-insert genomic library was constructed into the BamHI site of pUC18 using the cloned stock ILDat2160. The libray was semi-normalized by hybridization with known repetitive regions (rDNA, satellite DNA, mini-exon,etc). Negative colonies were selected, then inserts ranging from 1.5 – 3 Kb chosen for sequencing. From the 501 chromatograms obtained, quality evaluation, vector removing and assembly were performed using the Phred/Phrap/Consed package. The resulting 288 sequences were used as queries for the Standalone Blast software (NCBI) using TblastX, BlastN and BlastX in combination with the following databases: Kineto (EBI), NR, NT (NCBI), RepBase (GIRI), *T. vivax* and *T. congolense* (Sanger). Several scripts in Perl and Bioperl were written to obtain frequencies of hits and parse the Blast results. The Blast survey shows that our data has 39.58%, 60.76, 48.61% and 86.81% of "no hits" in Kineto, Repbase, NT and *T. congolense* databases using TblastX. Moreover, using BlastN, our data has 82,64%, 45.14%, 76.38% and 93.06% of "no hits" in RepBase, *T. vivax*, NT and *T. congolense* databases. The BlastX search with the NR database showed 77.78% of "no hits". The most abundant hits were those presenting high similarity (e-values better than 1e-8) to Histone H4, Dynein, Protein kinase, Actin-like protein, Phosphoglycerate mutase, INGI retroelement, *T. brucei* – MVAT, protein kinase, *T. brucei* - GRESAG, ubiquitin and P-glycoprotein. While we have identified a number of new markers for *T. vivax*, in general most of our data represent new genomic regions that should explored as species-specific markers, especially those with "no hits" in the databases. Finally, all regions presenting high similarity to kinetoplastid databases can be further explored in a comparative genomics approach.

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BM10 - PROT-OGIM: A NOVEL SOFTWARE TO IDENTIFY MOTIFS IN ORPHAN GENES FROM PROTOZOAN GENOMES PROGRAM.

Cestari, I.S, Carvalho, R.B., Silva, R.L. And Ramirez, M.I.

Depto. de Imunologia, Centro de Pesquisas Aggeu Magalhães-FIOCRUZ. Av. Professor Moraes Rego SN, Recife, PE.

Parasite genome programs has been showing a high percent of unknown genes when submit to GeneBank Database. It was notified in recents reports from *P. falciparum*, *C. elegans* and others organism a big amount of unknown genes(higher than 30%). These genes called orphan genes represent a challenger to basic research to find functions and to discover the involvement of different genes in cellular mechanism. Results obtained from data-mining analyses in genome program follow general rules and is necessary to dissect the information for specialists.

Trypanosomatidae is a family with a very complex gene regulation and cell biology. Orphan genes could be a putative chimioterapic target specially when associated with protozoan organell not present in mamalian cell. Using mathematics approach with regular expression including especific protozoan and others eucariotic motifs and domains, we have develop a new software, PROT-OGIM (orphan gene identification motifs from protozoan) able to renamed orphan genes from all informations available in tripanosomatids genomics program. We are defining new genes to analise function through heterologous and homologous transfection in order to understand the cell biology of the parasite and to elucidate new stool as vaccine and drug candidates to control the disease.

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BM11 - A SURVEY OF THE *LEISHMANIA (V.) BRAZILIENSIS* GENOME

Laurentino, E. C., Ruiz, J.C, Tosi, L.R.O. Cruz, A.K.

Faculdade de Medicina de Ribeirao Preto – USP, Departamento de Biologia Celular e Molecular, Ribeirao Preto, Sao Paulo, Brasil.

The current status of genome projects of various organisms is encouraging. Such progresses strongly support research efforts on comparative analysis of content and organisation of the genomes from different organisms.

To expand the available information for *Leishmania* ssp., our aim is to generate sequences and proceed comparative and functional analyses on *L. braziliensis* genome. For this purpose, two different strategies have been pursued. The first one is centered on the development of a systematic gene trapping strategy using a Tn5 transposon system associated with the functional study of delimited regions of the genome. Clones from a *L. (V.) braziliensis* genomic library (into cosmids) corresponding to the chromosomes 2, 5, 14 and 35 were chosen as target for the transposition reactions. After transfection into *Leishmania* parasites, the selected clones will allow to localize and conduct functional studies of trapped genes. The second strategy aims to generate a suitable coverage of *L. (V.) braziliensis* genome and has been carried out using a shotgun genomic library. Eleven thousand and two hundred recombinant clones (about 2 kb inserts) were sequenced corresponding to 5 Mb, approximately 14% of the *L. (V.) braziliensis* haploid genome. Database comparisons showed that 81.02% of the genome survey sequences (GSSs) lacked high score similarity to any protein sequences in GenBank. Nineteen percent of the sequences matched with deposited genes in public databank and were classified according to functional groups such as metabolism, signal transduction, information pathways and electron transport, among others. It is intriguing the low level of similarity found between *L. (V.) braziliensis* GSSs and the *L. (L.) major* databank (54.8%). We are currently investigating the meaning of this finding.

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BM12 - GENEDB: A KINETOPLASTID DATA RESOURCE

Christopher Peacock

Wellcome Trust Sanger Institute

GeneDB is a generic database resource for prokaryotic and eukaryotic organisms. The resource is designed to display genomic and post-genomic data integrated with information from scientific literature and community sources. GeneDB has an easy to use, intuitive interface that is consistent between organisms. For some organisms, including some Kinetoplastid and *Plasmodium* species, full time biological staff not only maintain and curate the database but act as a means of interaction between the community and the genome projects. This will improve not only the service to the user but also facilitate the contribution of unpublished information to GeneDB. Facilities currently available include simple querying, complex querying using Boolean operators, browsable lists, chromosome maps, sequence searching using BLAST and OMNIBLAST, motif searching, bulk data downloads, graphical gene and protein feature maps, sequence genome browser and comprehensive links to and from other data resources. Consistent annotation, use of controlled vocabulary and complex querying across species, will greatly improve comparative studies between related organisms. Currently, the datasets in GeneDB include three Kinetoplastid organisms (*Leishmania (L.) major, Trypanosoma brucei* and *Trypanosoma cruzi*) with a further three planned for addition in the near future (*Trypanosoma vivax, Trypanososma congolensi* and *Leishmania (L.) infantum*).

BM13 - EFFECT OF GAMMA RADIATION ON *TRYPANOSOMA CRUZI* IS ASSOCIATED WITH PARTIAL LOSS OF CHROMOSOMES AND INDUCTION OF RAD51 GENE EXPRESSION

Luiz Augusto-Pinto, Carlos G. R. Silva, Jorge M. Freitas, Márcio P. Tadeu,, Sérgio D. J. Pena, Andréa Mara Macedo, Santuza M. R. Teixeira, Carlos Renato Machado.

Universidade Federal de Minas Gerais,Rua Antônio Carlos 6627, Departamento de Bioquímica ICB, Pampulha, Belo Horizonte, Minas Gerais, Brasil, CEP:31270-901. augusto@icb.ufmg.br

Trypanosoma cruzi is the human parasite of Chagas' disease. Genes involved in DNA repair have been described in this species, however the DNA breaks repair by homologous recombination has not been depicted yet. The *Rad51* gene product is one of the major proteins involved in homologous recombination processes in eukaryotic cells. Here, we have isolated and characterized the *Rad51* gene from *T. cruzi* (*TcRad51*). Using PCR strategy, we cloned the *TcRad51* open reading frame. Remarkably, this gene is present in two copies in the *T. cruzi* genome as revealed by Southern blot. However, analysis of eleven *T. cruzi* strains by 5´-terminal sequencing of the two *TcRad51* copies demonstrated a high degree of conservation in this gene. To investigate whether conservation of *TcRAD51* gene could be associated with the importance of DNA breaks repair by homologous recombination for parasite survival, we submitted three strains from distantly related lineages to gamma irradiation. These strains showed the same high radiation resistance, since motility of the parasite was not abolished by dosages as high as 1 KGy. Additionally, an enhancement in the *TcRAD51* gene expression was observed after irradiation. Also, one of these strains, Cl Brener, showed a different chromosome pattern in a "Pulse Field Gel Electrophoresis" (PFGE) after irradiation, suggesting a likely recombination event or a possible loss of an unessential chromosome. The high gamma radiation resistance of *T. cruzi* is a highly unexpected phenomenon for which we provided the first molecular evidences of its association with a DNA repair gene.

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BM14 - ISOLATION AND CHARACTERIZATION OF POLYMORPHIC MICROSATELLITES OF TRINUCLEOTIDE REPEATS FROM *TRYPANOSOMA CRUZI* GENOME

Valadares, H.M.S[1], Macedo, A.M.[2], Pena, S.D.J.[2], and Oliveira, R.P.[1]

[1]Departamento de Ciências Biológicas, ICEB, UFOP, Ouro Preto, MG, Brasil.
[2]Departamento de Bioquímica e Imunologia, ICB, UFMG, Belo Horizonte, MG, Brasil

The genetic variability in the nature populations of *T. cruzi* has been demonstrated by a great number of biochemical and molecular techniques. In 1998, a new aproach to characterize *T. cruzi* samples emerged with the discovery of polymorphic microsatellites of CA repeats in the *T. cruzi* genome. We now characterize new polymorphic microsatellite loci for *T. cruzi* consisting of trinucleotide repeats to avoid the problem with inaccurate size alleles due to *Taq* DNA polymerase slippage observed for dinucleotide repeats. Using the software Tandem Repeats Finder, we identified 634 trinucleotide microsatellite loci with the number of repeats greater or equal to 7 on total of 32, 607 *T. cruzi* sequences deposited on GenBank release 128. A most frequent repeats found were $(AAT)_n$ where 7 £ n £ 39 (44%) and $(AAC)_n$ where 7 £ n £ 16 (22%). We selected 9 markers for primer design using the program Oligos v.9.5. In addition to the search for repetitive motifs in DNA databases, we constructed an genomic library enriched 3 times for $(AAC)_n$ repeats. We obtained 36 recombinant colonies and after hybrization with the $(AAC)_n$ probe, 24 colonies (67%) were positive

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and all of them were selected for sequencing. Eleven colonies (45%) showed 8 different $(AAC)_n$ repeats with 5 £ n £ 10 and four of them were selected for PCR amplification. To test the variability of the thirteen selected microsatellite loci, we performed a rough screening to evaluate the degree of polymorphism. We typed four genetically distant *T. cruzi* isolates that have been previously characterized by rRNA and $(CA)_n$ microsatellites. Six loci showed accentuated size polymorphism (ACC8, GTT8, AAC16, AAT18, AAT21 and CAA737), three loci displayed discrete size polymorphism (CAA739, CAA9P and CAA9IM), two loci were apparently monomorphic (CTC7 and AAC10) and finally two loci amplified fragments with size different from expected (AGC19 and TTA25).

Apoio financeiro: CAPES/WHO

BM14 - EXTENSIVE HETEROZYGOSITY IN TWO HYBRID GROUPS OF *TRYPANOSOMA CRUZI*

Scott J. Westenberger, Nancy R. Sturm, David A. Campbell

Department of Microbiology, Immunology, and Molecular Genetics. David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA 90095, USA

Trypanosoma cruzi had been divided previously into two major lineages, or three zymodemes. The species was further subdivided into six groups; I, IIa, IIb, IIc, IId, and IIe by Brisse, et al. (2000). We sought to clarify the evolutionary relationships among these subgroups through the use of molecular markers. Here we provide evidence of group-specific single nucleotide polymorphisms in protein coding genes and intergenic regions between single-copy and tandemly-arrayed multicopy genes. Hybrid groups IId and IIe display heterozygosity at nine loci on at least six different pairs of chromosomes. Markers analyzed include the intergenic regions of the tandemly-arrayed, multicopy genes histone H1, histone H3, histone H2B, histone H2A, and heat shock protein 60. Single nucleotide polymorphisms in protein-coding genes of TcMSH2 (a DNA mismatch repair gene) and glucose phosphate isomerase and the intergenic region between the single copy genes tcp17 (an unidentified ORF) and tcpgp2 (an ATP binding cassette transporter) were identified. Group-specific polymorphisms were identified by sequencing representative strains from each group. Genotyping of all strains was performed by PCR-RFLP. Previous analysis of rRNA and protein coding genes suggested that groups IIa and IIc were hybrids of *T. cruzi* I and *T. cruzi* II. Groups IId and IIe were also shown to be hybrids, but their position relative to the other *T. cruzi* II subgroups was unclear due to the presence of two genotypes at several loci. Our phylogenetic analyses suggest that strains from groups IIb and IIc were the parental lines that contributed genetic material to create groups IId and IIe. The two genotypes present in these hybrid strains may represent two distinct gene loci, or two alleles of the same gene. Our real-world observations are consistent with the study by Gaunt et al. (2003) which found that laboratory-generated hybrid *T. cruzi* strains retain both of the parental genotypes. Furthermore, some of the loci in specific IId and IIe strains have undergone further recombination between the two parental sequences and now display “mosaic” genotypes. The sequence variation supports distinction of four clades of *T. cruzi.* The evidence implies that mechanistic assortment or loss of chromosomes in formation of hybrids is not a random process. *T. cruzi* IId and IIe may represent examples of heterozygote advantage.

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BM15 - A NEW SPECIES OF TRYPANOSOMATID HARBORING BACTERIAL ENDOSYMBIONT: MORPHOLOGY AND PHYLOGENETIC RELATIONSHIPS WITH OTHER SYMBIONT-CONTAINING TRYPANOSOMATIDS.

Santos, M.A.*; Nunes, V.L.B.[&]; Takata, C.L.A*; Milder, R.V.*; Camargo, E.P*. & Teixeira, M.M.G*.

* Department of Parasitology, University of São Paulo, SP, Brazil [&] UNIDERP, Campo Grande, MS, Brazil.

Trypanosomatids are parasites of insects, plants and several species vertebrates and invertebrates and also species that are hosts for intracellular bacterial and viral symbionts. Only 5 species harboring bacteria-like endosymbionts are available in culture, all isolated from insects: *C. deanei*, *C. oncopelti*, *C. desouzai*, *H. roitmani* and *B. culicis*. Trypanosomatids isolated from other insect species and from fruit (*Morus* sp) showing endosymbionts revealed to be very similar to *C. deanei* by several molecular markers. Despite isolated from different hosts and distributed into three genera, trypanosomatids harboring bacterial symbionts shared several morphological, biochemical and molecular features. Among these species, only *B. culicis* presented epimastigotes whereas all others presented choanomastigote forms. In this study, we characterized a symbiont-containing trypanosomatid (TryCC219) isolated from a phlebotomine in the Mato Grosso do Sul State, Brazil. Giemsa-stained culture cells of this isolate also show choanomastigotes, although larger than that of other related-species, and also presented choanomastigotes with postnuclear-located kinetoplast called opisthomorphs, typical of symbiont-containing choanomastigotes. Transmission electron microscopy of TryCC219 showed morphological characteristics shared exclusively by trypanosomatids harboring bacterial-symbionts as the absence of paraxial rod and opposition of mitochondrial branches to plasma membrane portions lacking subpellicular microtubules. However, TryCC219 symbionts differed from the commonly described in trypanosomatids, presenting longer and more irregular shape. Moreover, its kDNA can be arranged either in a looser, as in other species, or in a tightly network. As for all other species, the presence of symbiont in the isolate TryCC219 enlarged the biosynthetic capability of the trypanosomatid-host, which could be easily cultivated in very simple culture media, with no source of hemin.

Several studies about the phylogenetic relationships inferred among these species showed that they form a monophyletic group, distant from *Crithidia*, *Blastocrithidia* and *Herpetomonas* species lacking symbionts, thus requiring a taxonomic revision and deserving separated status. However, despite the small number of trypanosomatids (3-5) included in these studies, there is a significant heterogeneity within this group, which need to be better evaluated. With this purpose, we are comparing 12 endosymbiont-harboring trypanosomatids by several approaches. All new isolates included in our study were clustered in this group by SSUrDNA sequence analysis, whereas no endosymbiont-free trypanosomatids were positioned in this group despite we have analyzed several new species/isolates of *Crithidia* (40) and *Herpetomonas* (13). However, among the new isolates with symbionts, only TryCC219 showed high sequence divergence from all other previously described species when we compared RAPD-patterns, classes of kDNA-minicircles, SSUrDNA sequences and length of ITSrDNA. Moreover, ribosomal sequences of the TryCC219-symbiont although close-related to those from other trypanosomatid-symbionts showed significant ITS sequence divergence. Therefore, all data permitted to classify the isolate TryCC219 as a new species of trypanosomatid harboring bacterium-endosymbiont.

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BM16 - COULD *TRYPANOSOMA CRUZI* CYCLES OF TRANSMISSION BE INFLUENCED BY HOSTS' PREVIOUS NATURAL EXPOSURE TO LOW INFECTIVE LINEAGES?

Diosque P.[a,b], Padilla A.M.[a,b], Barnabé C.[c], Tibayrenc M.[c] and Basombrío M.A.[a,b].

[a]Laboratorio de Patología Experimental, Facultad de Ciencias de la Salud, Universidad Nacional de Salta, Argentina. [b]Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina. [c]Génétique des Maladies Infectieuses, Unité Mixte de Recherche Centre National de la Recherche Scientifique (CNRS)/ Institut de Recherche pour le Développement (IRD) n° 9926, IRD, BP 64501, 34394 Montpellier Cedex 05, France.

For the two past decades our laboratory has been working on immunization studies using an attenuated *Trypanosoma cruzi* strain as immunogen against virulent parasites in different animal models (mice, guinea pigs and dogs). We have proved different degrees of protection in laboratory experiments and field studies (Basombrío et al.: Infect Immun 1982, 342-350; J Parasitol, 1987, 73, 1, 236-238; Am J Trop Med Hyg, 1987, 37, 1, 57-62; Am J Trop Med Hyg, 1993, 49, 1, 143-151). Our actual knowledge on *T. cruzi* phylogeny allows a re-interpretation of previous experiments. Now, we present the results of the characterization of these strains by Multilocus Enzyme Electrophoresis Analysis (MLEE) at 15 loci and Random Amplified Polymorphic DNA (RAPD) with five primers, using *T. cruzi* I, IIa, IId and IIe reference strains as control. We found that the attenuated strain belongs to *T. cruzi* I lineage, and the virulent strain most frequently used belongs to *T. cruzi* IIe. Moreover, in this work we show an extensive cross-resistance in hosts immunized with *T. cruzi* I and challenged with *T. cruzi* II lineage. Simultaneously, in a geographically restricted area in Argentina -located in the same region where field immunization studies in dogs were carried out by us- we have evidence that different lineages circulate in domestic cycle (*T. cruzi* I, *T. cruzi* IId and *T. cruzi* IIe). There we found evidence of natural selection on distinct lineages by different mammal species, including humans, suggesting some degree of specificity of particular lineages for specific hosts (Diosque et al., Int J Parasitol, in press). Low virulence had been associated with *T. cruzi* I by different authors, while natural resistance to certain strains in some mammals had been demonstrated by others. In the epidemiolgy of Chagas' disease both -genetic characteristics of parasites and hosts- have been implicated in infectivity and, therefore, in the determination of transmission patterns. We hypothesize that interactions among different parasite lineages of *T. cruzi* by natural non-infective first contact with some hosts, could play a protective role in the transmission dynamic of the parasite in areas where different lineages circulate. We think that these possible "natural immunizations" could be considered, in addition to host and parasite genotype and others epidemiological features, as another variable that could play an important role in the transmission dynamic of Chagas' disease.

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BM17 - *TRYPANOSOMA RANGELI* STRAINS REVEALED LIMITED SEQUENCE VARIABILITY IN BOTH INTERNAL TRANSCRIBED SPACERS (ITS) FLANKING THE 5.8S RRNA GENE

Beltrame-Botelho I.T.[1], Gaspar-Silva D.[1], Steindel M.[1], Dávila A.M.R.[2], Grisard E.C.[1]

[1]Departamento de Microbiologia e Parasitologia, Universidade Federal de Santa Catarina, Caixa postal 476, Florianópolis, SC, Brazil, 88040-900, and [2]Departamento de Bioquímica e Biologia Molecular, Fiocruz, Rio de Janeiro, Brazil. E-mail: grisard@ccb.ufsc.br

Trypanosoma rangeli is a hemoflagellate protozoan parasite of a variety of mammalian species, including humans, in a wide geographical area in both Central and South America. Despite the apparently non-pathogenic nature of *T. rangeli*, this parasite infection allows the occurrence of serological cross-reactivity with *T. cruzi*, turning difficult the specific diagnosis of Chagas disease. Studies on biological and molecular aspects of these parasites, such as evolution and behavior in mammalian hosts and triatomine vectors, revealed important intra and inter-specific heterogeneity. In this study, we have comparatively evaluated the ITS-1 and ITS-2 spacers flanking the 5' and 3' ends of the 5.8S subunit of the ribosomal RNA gene (rRNA) among *T. rangeli* strains isolated from distinct hosts and geographical origins. Confirming the PCR-RFLP patterns obtained for ITS-1, cloning and sequencing of the whole ITS repeat (ITS-1/5.8S rRNA gene/ITS-2) revealed a low-level variability of both spacers among the studied strains and the presence of single nucleotide polymorphisms (SNP's) with variable motifs and length within the 5.8S subunit of the rRNA gene. Despite the detected variability, no correlation with epidemiological inferences such as hosts or geographical origins was possible. Intra-specific sequence analyses showed that ITS-1 spacer is less polymorphic than ITS-2 spacer. The inclusion of homologous sequences from *T. cruzi* and *Leishmania* spp. strains obtained from the GenBank allowed a clear inter-specific differentiation, confirming the feasibility of the ITS repeat (ITS-1/5.8S rRNA gene/ITS-2) as a marker for pathogenic trypanosomatids differentiation. The SNP's contained within the 5.8S rRNA gene of distinct strains are under study.

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BM18 - COMPARATIVE STUDIES OF AMASTIN GENE SEQUENCES IN DIFFERENT *TRYPANOSOMA CRUZI* STRAINS

Freitas-Silva, D. M.; Cerqueira, G. C.; Silva, R.A.; Campos, P. C., Machado, C.R.; Teixeira, S. M. R.

Universidade Federal de Minas Gerais, Instituto de Ciências Biológicas, Departamento de Bioquímica e Imunologia, Laboratório de Imunologia e Biologia Celular de Parasitas.

The amastin gene occurs in multiple copies in the *T. cruzi* genome and codifies a 174 aminoacids, highly hydrophobic membrane protein. In spite of the constitutive transcription, the steady state levels of amastin genes are 60-fold higher in amastigotes compared to epimastigote and trypomastigote forms. Several molecular markers corroborate the existence of two major lineages for *Trypanosoma cruzi*, denominated *T. cruzi* I and *T. cruzi* II, which are associated with the domestic (*T. cruzi* II) and sylvatic (*T. cruzi* I) life cycle of the parasite. More recently, based on the sequence of the MSH2 gene, microsatellite markers and mitochondrial sequences analyses, a classification of strains into three groups termed haplogroups denominated A, B and C have been proposed. The aim of this work is to extend these studies by analyzing sequence variability of the amastin gene among *T.cruzi* strains. We selected two strains representative from each haplogroup, which are: Colombiana and D7 (haplogroup A); JG and 239 (C); Cl-Brener, 167 and 182 (B). To investigate genotypic variation, amastin genes were amplified from DNA isolated from these strains, and the 760 nucleotides PCR products were cloned into the TOPO-TA vector (Invitrogen). Sequencing of the plasmid inserts were performed with the Mega BACE sequencer and analyzed using these programs: Multialin (http://prodes.toulouse. inra.fr/multalin/multalin.html), Blast (http://www.ncbi.nlm.nih.gov/BLAST/), Bioedit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and Boxshade (http://www.ch. embnet.org/software/BOX_form.html). A filogenetic tree was constructed using program Mega based on the obtained data. The results suggest the amastin sequences can be divided into three haplogroups and also that increased variability of the amastin gene are found within strains belonging to haplogroups C and B (*T. cruzi* II) when compared to strains from haplogroup A (*T. cruzi* I).

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BM20 - CHROMOSOMAL SIZE VARIATION IN *TRYPANOSOMA CRUZI* ISOLATES AND EVOLUTIONARY INFORMATION ON PUTATIVE HYBRID STRAINS

Pedroso, A.[1], Silva, MN[1], Cupolillo, E.[2] and Zingales, B[1].

[1]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, C.P. 26.077, São Paulo, CEP 05513-970, SP, Brasil. [2]Departamento de Imunologia, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brasil

Trypanosoma cruzi is divided into two major phylogenetic lineages, named as *T. cruzi* I and *T. cruzi* II. An Expert Committee recommended that the classification of isolates typed as Zymodeme 3, rDNA group 1/2 and isoenzyme group 39 should await further studies. Despite the prevalence of clonal evolution in *T. cruzi*, convergent lines of evidence indicate that some *T. cruzi* genotypes are the result of ancient hybridization events. Among the putative hybrid strains CL Brener and rDNA group 1/2 isolates are included. We have evaluated whether chromosome size polymorphism would give evolutionary information on *T. cruzi* hybrid isolates. For this purpose, we have mapped 23 probes on chromosomes of four isolates representative of heterozygotic genotypes (CL Brener and three clones of rDNA group 1/2); three isolates of *T. cruzi* I and two, of *T. cruzi* II groups. These genetic markers cover almost all CL Brener chromosomes ranging from 600 to 3,300 kb. The relationships among the stocks were calculated by the aCSDI formula for the analysis of chromosomal size variation (Dujardin et al., 1995) and compared with those obtained from RFLP data originated from the hybridization of genomic DNA with the same probes. Both phenetic analyses identified three clusters, corresponding, respectively, to *T. cruzi* I, *T. cruzi* II and rDNA group 1/2 strains. Depending on the nature of the analyzed probes, CL Brener was included either in *T. cruzi* II or group 1/2 clusters. Recently, we have started to analyze by the by aCSDI method the phylogenetic relationship of four isolates classified as Zymodeme 3 (kindly provided by Prof. J.R. Coura and Dr. O. Fernandes, FIOCRUZ). Preliminary aCSDI analysis from 12 probes indicates that three stocks of Zymodeme 3 are closer to *T. cruzi* II group and one stock is included in the same branch that clusters CL Brener and group 1/2 strains. A larger number of genetic markers will be analyzed to elucidate the evolutionary association among Zymodeme 3 isolates and stocks from Bambuí (Minas Gerais), which belong to rDNA group 1/2 (kindly provided by Drb. E. Chiari, UFMG).

Support: FAPESP and CNPq.

BM21 - IDENTIFICATION OF *T. CRUZI* GENOTYPES FROM SYLVATIC CYCLES AND GENETIC DIVERSITY REVEALED BY RAPD ANALYSIS.

Marcili. A.[1], Pinto, P.L.S.[2], Junqueira, A.C.V.[1,3], Campaner, M.[1] & Teixeira. M.M.G.[1]

1. Departament of Parasitology, University of São Paulo, Brazil; 2. Institute Adolpho Lutz, São Paulo, Brazil; 3.Institute Oswaldo Cruz, Rio de Janeiro. Brazil.

The genetic variability of *T. cruzi* has been widely explored. Considerably heterogeneity has been demonstrated among isolates from both the domestic and sylvatic cycles, although contrasting to domestic cycles, little is known about the genetic polymorphism among *T. cruzi* isolates in sylvatic cycles. *T. cruzi* transmission cycles can be broadly classed as enzootic-cycles. To analyze the genetic diversity and population structure of Brazilian *T. cruzi* isolates from sylvatic mammals from areas of different transmission cycles we compared isolates from: a) Amazon Basin, where *T. cruzi* is enzootic, with few human cases of Chaga's Disease despite widespread sylvatic cycle involving several sylvatic mammal and triatomine species, b) São Paulo, a former endemic area where intradomiciliary transmission is presently considered controlled whereas the sylvatic cycle is very active, specially in areas of Atlantic Coast Rainforest.

Isolates from Amazon Basin (15) and São Paulo (17) were obtained from marsupials, non-human primates, bats and rodents. All new isolates (obtained harmocultures or xenodiagnosis) were classified as *T. cruzi* by tradicional criteria of morphology, growth features, infectivity for mice, behavior in triatomines and development in mammalian cultures cells. Behavior in mice (parasitemia and virulence) and% of metacyclic forms in cultures disclosed high heterogeneity among the isolates.

All isolates were molecularly diagnosed to separate *T. cruzi* from *T. rangeli* and *T. cruzi*-like and mini-exon typed into the three major lineages using human and triatomine isolates previously typed as *T. cruzi* I (TC1), *T. cruzi* II (TC2) or Z3 as contrals. In the Amazon Region it was found TC1 and Z3-lineages, as previously reported for human, sylvatic mammal and triatomine isolates. In this region, while these both lineages were recovered from non human primates, enlarged the sylvatic mammalian order host-range of Z3-lineage so far restricted mainly to armadillos, all isolates from marsupials and bats were TC1. Isolates from São Paulo were typed as TC1 or TC2. All TC2 isolates are from marsupials and rodents of Atlantic Coast, where people live close to forest whereas TC1 are from a former endemic area that is characterized by human cases associated to TC2 and domestic cycle. Thus, typing of isolates from these two areas is in agreement to their transmission cycles. RAPD analyses revealed high genetic polymorphism among isolates of different lineages and significant intra-lineages heterogeneity. Mini-exon typing and RAPD-dendogram branching-pattern suggested some association of lineages and/or sub-lineages with geographic region and mammalian species. A phylogenetic relationships among isolates from sylvatic mammals and triatomines of distinct transmission cycles are being inferred using SSUrDNA sequences.

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BM22 - CHARACTERIZATION OF TWO STRAINS OF *TRYPANOSOMA CRUZI* CHAGAS, 1909 (KINETOPLASTIDA, TRYPANOSOMATIDAE) ISOLATED FROM SPECIMENS OF *TRIATOMA RUBROVARIA* (BLANCHARD, 1843) (HEMIPTERA, REDUVIIDAE, TRIATOMINAE) COLLECTED IN WILD ENVIRONMENT.

Martinez, I.[*], Rosa, J.A. da[**]

[*] Departamento de Análises Clínicas [**] Departamento de Ciências Biológicas, Faculdade de Ciências Farmacêuticas-UNESP - Rodovia Araraquara-Jaú Km 01, CEP 14801-902 – Araraquara – SP – Brasil. e-mail: martinez@fcfar.unesp.br; rosaja@fcfar.unesp.br

The flagellate protozoan, *T. cruzi* is the etiological agent of Chagas's disease, a serious medical and social problem affecting approximately 18 million people in Latin America. The large heterogeneity of the parasite that circulate among human, different species of Triatominae and sylvatic and domestic animals it makes with that it presents great diversity of behavior, leading the different forms of clinical presentation of the disease in the man. Two *T. cruzi* strains, denominated QG1 and QG2, isolated from faeces of fifth instar nymphs of *Triatoma rubrovaria* collected in the Rio Grande do Sul state have been studied. In order to the characterization of *T. cruzi* strains maintened in Swiss mice, morphology of the bloodstream forms, the curve parasitemic, pre patent period, histiotropism, and the mortality rate in relation to the infection of Swiss mice were recorded. The strains have been maintenance in LIT medium. The molecular characterization was carried out by the technique of amplification of 24Sá rRNA gene sequences. The two strains, QG1 and QG2, have been classified as Type or Biodeme III and Lineage 2 indicating that both belong to the now defined group *T. cruzi* I.

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BM23 - ANURAN TRYPANOSOMES: MORPHOLOGICAL, BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF BRAZILIAN ISOLATES.

Ferreira, R.C; Campaner, M.; Takata, C.L.A.; Takeda, G.F. & Teixeira M.M.G.

Department of Parasitology, University of São Paulo, SP, Brazil.

Several species of Anura belonging to distinct families have been described harboring trypanosomes around the world. Most descriptions of anuran trypanosomes have been based on bloodstream forms, few isolates are available in culture and there are no cultivable isolates from South America. There are few data about development of trypanosomes in both anurans and vectors (leeches and dipterans). There are more than 60 trypanosome species described in anurans. Species classification is based on morphology and morphometry of blood trypomastigotes, host-species and geographic origin. The high polymorphism of trypanosomes in blood and the existence of morphologically similar flagellates in different host-species and geographic regions proved that these parameters are not suitable for taxonomy. There are no studies about anuran trypanosomes in Brazil. Isoenzymes, riboprinting and RAPD patterns distinguish the anuran species. Phylogenetic studies based on SSUrDNA clustered together all anuran trypanosomes from North America, Europe and Africa. To evaluate the prevalence, morphology, diversity and phylogenetic relationships among trypanosomes from Brazilian anurans we examined 237 specimens, of 49 species of Anura, from different ecosystems: Amazon; Pantanal; Atlantic Forest and Cerrado.

Blood trypanosomes were investigated by microhaematocrit, revealing 42% of infected animals (138 specimens) and by haemocultures, resulting in 43% of cultures (from 237 animals). A total of 107 anuran specimens were found harboring trypanosomes, ranging from 27% in Atlantic Forest to 69% in Pantanal (average of 48,5% in all regions). From 101 positive haemocultures, 70 isolates from 21 anuran species were established in culture. Morphological analysis of Giemsa-stained blood smears revealed a high polymorphism of size and shape among trypanosomes from distinct hosts and even within the same animal. It was observed very large trypomastigotes, long or rounded, showing a well-developed undulating membrane and small kinetoplast. Although epimastigotes from culture were more homogeneous than blood forms, inter and intra-isolate polymorphisms were detected, showing three major morphological patterns.

Analysis of polymorphisms of kDNA minicircle classes, length of ITS of rDNA and RAPD patterns showed high genetic variability among Brazilian isolates. These analyses distinguished isolates from different host-species as well as isolates of the same host-species. On the other hand, high homogeneity was observed among some isolates from different genera and families of Anura. In addition, isolates from distinct geographic regions showed similar patterns of molecular markers. Therefore, morphology, host-species and geographic origin are not enough to classify anuran trypanosomes.

Despite the high polymorphism, the association of molecular markers distributed the isolates in several groups. However, these grouping patterns could not be associated to either, host species or geographic origin. Phylogenetic analysis based on SSUrDNA is being inferred to define the taxonomic position of these groups as well as the phylogenetic relationships among them and with anuran trypanosomes from other countries.

Supported by FAPESP

BM24 - GENES ENCODING CATHEPSIN-B LIKE CYSTEINE PROTEASES OF TRYPANOSOMES: ISOLATION, CHARACTERIZATION AND PHYLOGENETIC ANALYSIS.

Cortez, A. P.; Maia-Silva, F.; Campaner, M. and Teixeira, M. M. G.

Department of Parasitology, University of São Paulo, SP, Brazil.

Cysteine-proteases are one of the most important families of enzymes that includes cathepsin L (cat-L) and B (cat-B), involved in a wide spectrum of biological processes and implicated in host-parasite relationship and in pathogenicity of parasites. These enzymes have been used as target for drugs, vaccines and diagnosis. In contrast to cat-L, there are few studies about cat-B of trypanosomatids and they are restricted to *Leishmania* and to the pathogenic trypanosomes *T. brucei* and *T. cruzi*. Characterization and inference of relationships of cat-B genes from trypanosomes of diverged phylogenetic groups, patoghenic or not, and with different biological cycles and behavior in their vertebrate and invertebrate are important for studying molecular evolution of both cysteine-proteases and trypanosomes themselves. Moreover, these data could provide a basis to further understanding the mechanism of enzyme action in a variety of organisms and to compare gene structure and function.

With this purpose we decided to compare sequences of cat-B genes of the non-pathogenic species *T. lewisi* (plus the allied species *T. rabinowitschae*) and *T. rangeli* with those from pathogenic species of *T. cruzi* and *T. brucei.* DNA fragments containing the catalytic domain of cat-B genes (~612bp) were isolated by PCR using degenerated primers, cloned and sequenced. Cat-B sequences differed among most trypanosome species on RFLP patterns of genomic DNA through cross-hybridization analysis using the amplified cat-B sequences as probes. Results suggested that *T. lewisi* (and probably *T. brucei*) has more than one copy of cat-B gene whereas *T. rangeli* apparently has a single copy similarly to described for *T. cruzi* and *Leishmania* spp. Northern blot analysis revealed transcripts of cat-B variable in size and sequence according to species of trypanosomes. To infer phylogenetic relationship among cat-B genes within *Trypanosoma* we constructed gene trees based on aligned sequences from the following species: *T. lewisi, T. rabinowitschae,* two isolates of *T. rangeli* belonging to distinct genetic groups (plus one sequence of an isolate of another group from GeneBank), 3 isolates of *T. cruzi* typed as *T. cruzi* 1, *T. cruzi* 2 and Z3 and *T. brucei* (sequence obtained from *T. brucei* genome data base). Analysis of sequence divergence revealed high genetic variability among cat-B from trypanosomes. Exception was *T. lewisi* and *T. rabinowitschae* that shared very similar cat-B genes. In contrast to the low sequence divergence among *T. cruzi* isolates significant intraespecific polymorphism was detected among isolates of *T. rangeli*. The branching patterns of the inferred trees constructed using nucleotides or predicted amino acids sequences were not identical, although, in both analysis *T. brucei* was positioned in a separated branch and *Leishmania* spp. were always positioned as outgroup of *Trypanosoma*. The Cat-L derived phylogenetic trees were compared with those obtained using cat-L and SSUrRNA sequences.

Supported by FAPESP and Capes.

BM25 - ISOLATION AND SEQUENCING OF CATEPSINA L–LIKE CISTEINE-PROTEINASE GENES OF *TRYPANOSOMA VIVAX* STOCKS REVEALED HIGH POLYMORPHISM AND A NEW MARKER FOR DIAGNOSIS

Cortez, A. P.; Ventura, R. M. & Teixeira, M. M. G.

Department of Parasitology, University of São Paulo, Brazil.

T. vivax, T. congolense and *T. brucei* are the major pathogens of cattle in Africa. *T. vivax* is also widespread in Central and South America. In contrast to African stocks, which are cyclically transmitted by tsetse flies, American stocks of *T. vivax* are mechanically transmitted by biting flies. Stocks from different geographical regions differ in morphology, pathogenicity, zymodemes and DNA sequences, separating stocks from West Africa and South America (mild/assymptomatic infections) from East African stocks (severe disease).

Cathepsin L-like (cat-L) cysteine proteases have been implicated with development in vector insects and with pathogenicity of *T. cruzi*, *T. brucei* and *T. congolense.* Cat-L genes are grouped in arrays of tandem repeats consisting

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of conserved catalytic domain and sequences variable according to species, stocks, and genes within the same isolate. Multiple copies and different degrees of conservation makes this gene excellent for diagnosis and evaluation of genetic relatedness within African trypanosomes and among *T. vivax* stocks differing in pathogenicity, as well as to compare stocks mechanically and cyclically transmitted.

We characterized cat-L gene sequences of *T. vivax* stocks from South America (Brazil), West Africa (Nigeria) and East Africa (Kenya). Sequences containing the catalytic domain were amplified by PCR, cloned, sequenced and nucleotide and amino acids sequences were aligned and compared to other trypanosomes. The high degree of similarity among sequences from all members of the subgenus *Trypanozoon* (*T. evansi, T. equiperdum* and *T. brucei* ssp.) contrasted with the high divergence detected among stocks of *T. vivax.* In addition, significant polymorphisms were observed among sequences of the same *T. vivax* stock, suggesting the existence of isoforms of cat-L genes, as described for *T. cruzi* and *T. congolense.* Phylogenetic analysis showed similar topologies of cat-L gene trees using either nucleotides or amino acid sequences clearly segregating sequences according to trypanosome species: a) sequences from *Trypanozoon* (Trypanopains) tightly clustered together; b) despite high genetic distance sequences among *T. vivax* stocks (Vivaxpain) they always clustered; c) sequences from *T. congolense* (Congopain 1 and 2) were separated in another clade. Cat-L genes from *T. cruzi* (Cruzipain 1 and 2) were positioned as outgroup of African species.

We developed a *T. vivax* specific PCR based on cat-L gene sequence able to detect all tested stocks, from South America, West and East Africa. No amplified products were generated for *T. congolense*, *T. theileri*, *T. evansi* and *T. b. brucei*. This method proved to be enough sensitive to detect cryptic *T. vivax* infection using crude preparations of field-collected blood samples. A multiplex PCR assays based on cat-L sequences are being currently improved to simultaneous detection of all cattle trypanosomes.

Supported by FAPESP and Capes.

BM26 - BIOLOGICAL AND MORPHOLOGICAL CHARACTERIZATION AND PHYLOGENETIC RELATIONSHIP OF BAT TRYPANOSOMES

Cavazzana, M.; Marcili, A; Campaner, M.; Veludo, H. H., Takata, C.L.A.; Paiva, F. Takeda, G.F. & Teixeira M.M.G.

Department of Parasitology, University of São Paulo, SP, Brazil

Trypanosomes have been recorded from numerous species of bats throughout world. Four subgenera are recognizing as occurring in bats: *Schizotrypanum, Megatrypanum, Herpetosoma* and *Trypanozoon*. Chiroptera are the only hosts of *Schizotrypanum* in the Old World, excepting *T. cruzi*, which is restricted to Americas and infects all mammalian orders. Despite *T.c.cruzi*, all other species classified in this subgenus are *T. cruzi*-like species restricted to bats: *T. cruzi marinkellei* (Central and South America), *T. dionisii* and *T. vespertilionis* (New and Old Word). Data about bat trypanosomes are mostly restricted to morphology of blood forms. Most cultivable species are of *Schizotrypanum* and showed significant genetic heterogeneity. Due to misclassification and to the lack of large number of isolates from distinct bat species and geographic regions, classification and phylogeny of *Schizotrypanum* are still controversy and the validity of several species is questionable.

To evaluate the prevalence and the genetic diversity among trypanosomes from different bat species and geographic regions of Brazil we investigated trypanosomes in bats from different and distant Brazilian ecosystems, Amazonia, Pantanal and Atlantic Forest, including bats belonging to 37 distinct species. From 460 bats examined, blood trypanosomes were detected in 129 by microhematocrit. Blood trypanosomes revealed significant polymorphism, although most were trypomastigotes typical of *Schizotrypanum*. It was obtained 57 cultures from Amazonia, Pantanal and Atlantic Forest, with no significant differences in prevalence of bat trypanosome infection in the studied regions. Isolates were obtained from 13 species of bats: 9 from insectivorous, 2 from frugivorous and 2 from hematophagous. Infectivity for mice and triatomines were used to distinguish *T.c.cruzi* from all other species. Most isolates multiply as amastigotes within mammalian cells in vitro. Differences in growth and morphological features (light and TEM) were also detected. Together, biological and morphological data suggested four major groups of isolates.

To investigate the taxonomic position of Brazilian bat trypanosomes, phylogenetic relationships among 46 isolates were inferred based on SSUrDNA sequences. Phylogenetic trees showed that isolates from Brazilian bats is polyphyletic and segregated into two distant branches. Most isolates were clustered into a major monophyletyc branch containing all *Schizotrypanum* species and were distributed into three heterogeneous groups: A) *T. c. marinkellei*, lacking infectivity for mice and only transiently infecting triatomines; B) *T. dionisii*, incapable to infect mice and triatomines; C) *T. cruzi,* infective for mice and triatomines, which members were segregated into two branches (*T. cruzi* 1 or Z3). Group D were positioned far from *Schizotrypanum,* differ in growth and morphology from all other groups and lacks infectivity for mice, triatomines and cell cultures. The grouping of isolates are in agreement with biological and morphological data and are independently of host-species and geographical origin, except the group D which was constituted only by isolates of *Pteronotus parnelli* from Amazon Region.

Supported by FAPESP and CNPq

BM27 - BRAZILIAN ISOLATES OF *TRYPANOSOMA* (*MEGATRYPANUM*) *THEILERI*: DIAGNOSIS AND DIFFERENTIATION OF ISOLATES FROM CATTLE AND WATER BUFFALO BASED ON BIOLOGICAL CHARACTERISTICS AND RANDOMLY-AMPLIFIED DNA SEQUENCES

A. C. Rodrigues, M. Campaner, C. S. A. Takata, A. Dell' Porto, R. V. Milder, G. F. Takeda and M. M. G. Teixeira

Departamento de Parasitologia, ICB II / USP, São Paulo, S. P.

Trypanosoma (*Megatrypanum*) *theileri* is the type-species of the subgenus *Megatrypanum* and is considered a cosmopolitan parasite of cattle with a worldwide distribution. In contrast to *T. theileri* in cattle, there are few records of *T. theileri*-like in buffaloes. More recently, *T. theileri*-like was recorded in water buffaloes from Brazil. Despite the ubiquity of *T. theileri* and in contrast to pathogenic salivarian trypanosomes of bovids (*T. vivax*, *T. brucei brucei*, *T. evansi* and *T. congolense*), few data exist about the vertebrate cycle, pathogenicity, biochemical and molecular characteristics of *T. theileri*. Moreover, there are no studies comparing morphological, growth and molecular features of isolates from cattle, buffaloes and other species of Bovidae. So far, no convincing data are available to tell whether *T. theileri*-like from distinct species of bovids belongs to only one species or whether trypanosomes from these hosts constitute different species. Thus, classification of these organisms at specific and infraspecific level needs additional taxonomic parameters.

In this study we detected and cultivated isolates of *Trypanosoma* (*Megatrypanum*) *theileri* from cattle and water buffaloes in São Paulo State, Southeastern Brazil, which were characterized by comparing morphological, growth and molecular features. Although isolates from cattle and water buffalo were morphologically indistinguishable, differences in growth characteristics separated them. Our RAPD analysis showed that, although *T. theileri* isolates were all from the same geographic region and always clustered together, organisms from this cluster were segregated into two subclusters according to their host species. These data demonstrated that *T. theileri* trypanosomes from cattle and water buffalo are not identical, supporting the idea of the host specificity of

Megatrypanum spp. The trypanosomes from water buffalo reported here are the first *T. theileri*-like isolates from the Asiatic buffalo (*Bubalus bubalis*) to be continuously cultured and biologically and molecularly compared with cattle isolates. In our RAPD analysis, *Megatrypanum* from wild mammals were segregated into different branches. The sequence of a synapomorphic RAPD-derived DNA fragment, which was shared by all *T. theileri*-trypanosomes from cattle and buffalo but not detected in any of 13 other trypanosome species, was used as target for a conventional *T. theileri*-specific PCR assay. We also defined RAPD fragments that permitted to distinguish cattle from buffalo isolates. Therefore, distinct growth features and genetic variability distinguished between isolates from cattle and water buffaloes of the same geographic origin, suggesting an association of these isolates with their host-species. However, these results did not exclude the existence of genetic variability among isolates of the same host species from different geographic regions. We are currently carrying out further studies to better evaluate the diversity among isolates from bovids from other areas of Brazil.

Supported by FAPESP.

BM28 - REPTILIAN TRYPANOSOMES: MORPHOLOGICAL, BIOLOGICAL AND MOLECULAR CHARACTERIZATION OF BRAZILIAN TRYPANOSOMES FROM LIZARDS, SNAKES AND ALLIGATORS

Viola, L. B.; Campaner, M.; Takata, C.L.A.; Paiva, F. Takeda, G.F. & Teixeira M.M.G.

Department of Parasitology, University of São Paulo, SP, Brazil

Reptiles can be infected by species of *Leishmania* in the Old World and by species of *Trypanosoma* in New and Old World. Although it was described several species of trypanosomes in lizards, chelonians, crocodilians and snakes, most data are restricted to morphology of blood forms and many species must be synonyms as well as new species can be underestimated. The only adopted taxonomic parameters for species classification are morphology, host-species and geographic origin. Leeches, ticks and dipterans can transmit reptilian trypanosomes. Although there are few data about life cycle of these trypanosomes in both vertebrate and invertebrate, they are considered non-pathogenic from their hosts and most are reported as highly species-restricted. Few isolates from reptiles are available in culture and there are only inconclusive data about prevalence, genetic diversity and phylogenetic relationships. Studies based on few isolates showed partition of isolates from lizards into two distant clades, together with isolates from fishes, anuran and chelonians (aquatic clade) or with avian isolates. There are no molecular studies regarding South American trypanosomes of reptiles.

Aiming to isolate in culture and to study the genetic diversity among Brazilian trypanosomes from reptiles we examined blood samples from lizards and snakes (several species) and from alligators. Few blood samples of lizards (2,85% of 140 animals) and snakes (2,75% of 400 animals) were positive for trypanosomes, in contrast to 32% of 37 alligators examined by microhaematocrit. Morphological analysis of Giemsa-stained smears revealed a high polymorphism of size and shape among blood trypomastigotes from distinct hosts. Most forms were large trypomastigotes, showing a well-developed undulating membrane and a very small kinetoplast. It was obtained 14 isolates by haemocultures: 4 from lizards (*Tropidurus plica* from Amazonian Region); 7 from snakes (*Crotalus durissus terrificus* from Southern and Southeast Brazil) and 3 from alligators (*Caiman yacare* from Pantanal Region). Different growth and morphological features were observed among the isolates. When cultivated at 25°C, most culture forms were large epi- and tripomastigotas, differing according to host-species.

Comparative analysis showed large size variability of kDNA minicircles among the reptilian trypanosomes, with patterns shared for isolates of the same host-species. Genetic polymorphism evaluated by analysis of ITS of rDNA amplified by PCR disclosed length variability. In addition, cross-hybridization analysis of ITS indicated significant sequence heterogeneity. These data permitted to separate the isolates into three major groups, according to their host species, despite small heterogeneity among alligator isolates. Analysis of ssUrDNA genes, amplified by PCR, cloned and sequenced, also revealed high polymorphism among the reptilian isolates. We are currently inferring phylogenetic trees based on ssUrDNA among our isolates aligned with those from reptiles (GeneBank) and other hosts in order to define their taxonomic position and phylogenetic relationships.

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BM29 - MOLECULAR DIFFERENTIAL DIAGNOSIS BASED ON ITS1 RDNA: OPTIMIZATION OF THE PCR ASSAY FOR FIELD STUDIES.

Souza, S. S.[1], Njiru, Z.K.[2], Traub-Cseko, Y.M.[1], Dávila A.M.R.[1]

1. DBBM-IOC, Fiocruz, Av. Brasil 4365 Manguinhos, Rio de Janeiro, RJ, Brasil, 21045-900. Email: davila@fiocruz.br; 2. Kenya Trypanosomiasis Research Institute (KETRI), Nairobi, Kenya.

Trypanosomosis is one of the most intractable protozoan diseases of livestock caused by more than five species of trypanosomes. There is a general lack of species-specific PCR-based assays for livestock trypanosome infection since there are reports showing that some *T. vivax* infection were not detected by PCR in West Africa and Latin America. More recently Dávila (2002) developed a PCR assay based on the ITS1 rDNA (Tryps-B primers) to detect several livestock trypanosome infections in a single reaction using only one pair of primers. The assay was shown to be efficient and will be tested in the field in the following enzootic countries: Bolivia, Brazil, Burkina Faso, Uganda and South Africa, within a consortium funded by IAEA. Before the assay is tested in the field, some extra optimization was needed in order to identify the best PCR cocktail able to detect as many trypanosome species as possible. PCR cocktails with different concentrations of dNTP's, $MgCl_2$, KCl and adjuncts were tested in combination with two "DNA mix" as templates in 25µl reactions. DNA Mix A: *T. vivax, T. evansi, T. congolense* Savanna, *T. congolense* Kilifi, and *T. simiae,* DNA Mix B: *T. vivax, T. evansi, T. congolense* Savanna, *T. congolense* Kilifi. dNTPs were tested in the following final concentrations: 72.5mM, 108.75mM, 145mM, 181.25mM and 217.5mM. $MgCl_2$ was tested in the following final concentrations 0.5mM, 1mM, 1.5mM, 2mM and 2.5mM. KCl was tested in the following final concentrations: 25mM, 50mM and 75mM. The following adjuncts were also tested according to the manufacturer indications of the PCR Optimization kit II â Sigma: Formamide, Ammonium sulfate, Betaine, BSA, Glycerol and DMSO. The size of the products obtained individually were: *T. vivax* - 230bp, *T. evansi*-550bp, *T. congolense* Savanna -710bp and *T. congolense* Kilifi - 650bp, and *T. simiae* - 400pb. Our results show that 2 PCR cocktails containing higher concentrations of dNTPs and KCl worked better amplifying several trypanosome species. Cocktail K: 2mM MgCl2, 50mM KCl, 217.5mM dNTPs, 45µg/ml BSA. Cocktail L: MgCl2 2mM, KCl 75mM, 217.5 mM dNTPs, 5% DMSO. With either DNA mix A or B, only 4 different species of trypanosomes were amplified. We do not consider this a limitation for the assay, as when an animal is found infected with any of the four species, it will have to be treated anyway and this will help to avoid the use and costs of 4 different reactions for each animal. Based on these results the assay based on ITS1 rDNA showed to be ready to be tested in the field. The optimization of the assay was useful to establish the best PCR cocktail in order to detect unequivocally as many species as possible.

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BM30 - ANALYSIS OF *TRYPANOSOMA RANGELI* RDNA LOCUS: SEQUENCE POLYMORPHISM OF THE INTERGENIC SPACER IN DIFFERENT GEOGRAPHICAL STRAINS

Guevara P*., Feo O. and Abreu M. T.

Laboratorio de Genética Molecular, Instituto de Biología Experimental, Universidad Central de Venezuela, Apartado Postal 48162,Caracas 1041A, Venezuela. *palmiragt@hotmail.com

Trypanosoma cruzi and *Trypanosoma rangeli* are the only two known American trypanosomes of man. In the insect vector both trypanosomes divide and develop infective forms but *T. rangeli* affects feeding mechanism and survival. Although both parasites occur in the same mammalian hosts and share Triatomine vectors, *T. cruzi* causes Chagas' disease and *T. rangeli* is considered non pathogenic to humans This overlapping complicates the diagnosis and epidemiology of the Chagas disease, because both parasites have similar surface antigens. We have undertaken the development of *T. rangeli* species specific PCR assays based on the rDNA locus. The analysis of the rDNA intergenic spacer (IGS) by long PCR amplification of previously typed *T. rangeli* strains from different geographical locations in Colombia and Venezuela revealed size polymorphisms. Sequencing of four cloned IGS from *T. rangeli* San Agustin (Colombia) demonstrated a high sequence conservation within this isolate. Based on the IGS San Agustin sequences we have designed an amplification assay with a 500 bp product. When five *T. rangeli* DNAs from different geographical areas of Venezuela were tested, this PCR assay resulted in the specific amplification of only the homologous San Agustin strain. This data suggests high heterogeneity within the *T. rangeli* strains tested as judged by this criteria introducing a new aspect to consider in the already complex epidemiology of mixed infections with *T. cruzi*. We are currently testing an increasing number of *T. rangeli* strains from Central America to Brazil. Supported by FONACIT grant S1-98002681 to GP. AMT has a fellowship from FONACIT and UNU/BIOLAC.

BM31 - CHAGAS' DISEASE MOLECULAR SEROEPIDEMIOLOGY IN TWO RURAL POPULATIONS AT SUCRE STATE, VENEZUELA

Figuera, L.[1]; Jorquera, A[2]., González, R[2]., Ledezma, E[2]., Martínez, C[3]., Carrasco, H[3]., y Marchán, E[1].

[1]Instituto de Investigaciones en Biomedicina y Ciencias Aplicadas (IIBCA), [2]Centro de Investigaciones en Ciencias de la Salud (CICS)/Universidad de Oriente. [3]Instituto de Medicina Tropical. Universidad Central de Venezuela. figueralourdes@hotmail.com

Chagas' disease is a serious public health problem in Latin America (WHO, World Health Report, 1998). In Sucre state located at Northeastern of Venezuela, we selected two rurals populations: Ribero municipality with high endemicity records in 1982 of *Rhodnius prolixus* peridomestic infection of 49.3% and domestic 7.2% with *T. cruzi* parasite 10.8% and 4.9% respectively, and Arismendi municipality with low endemicity records of *Rhodnius prolixus* peridomestic infection of 1.2% and domestic 0.1% both without *T. cruzi* parasite (source: Sucre Rurals Endemics Division dead files). However, 2000 latest official report by the Chagas' Disease Venezuelan Control Programme (CDVCP) showed no seroprevalence at Sucre state, which is surprising regarding the gradual state of abandon of epidemiological control and surveillance imposed by economics limitations. In this sense, by active search we carried out a molecular seroepidemiologic study at Catuaro village, Ribero municipality (10°24'00'' LN and 63°29'50''LW) and San Juan de las Galdonas village (10°42'46'' LN and 62°50'20'' LW) with the propose to determine Chagas' disease seroprevalence. We studied 239 random selected individuals during 2000 june-october. Inmunodiagnostic was realized using quantitative ELISA IgG anti *T. cruzi* (Bioschile, IGSA) and direct agglutination test (DAT) with crude antigen of *T. cruzi*, YBM strain, HOM zimodem. PCR standardization was carried out with S35/S36 primers on serum samples of seropositives individuals confirmed by the immunological tests before mentioned. DNA purification were made by two methods: conventional procedure with Guanidine HCl/EDTA and Wizard commercial kit (Promega). Seroprevalence of Catuaro was 21.3% (2.7 higher in relation to national prevalence, CDVCP, 2000). San Juan de las Galdonas showed similar national results (7.3% vs. 8.1%), highlighting between 20 years old individuals, 50.0% of them were seropositives suggesting active transmission of the Chagas' disease. By PCR, the sensibility was 100% with DNA purified by Wizard kit showing direct correlation between the ethydium bromide bands intensity of the amplified DNA fragments and the optics densities of ELISA, whilst the conventional method sensibility was 40%. These findings showed the highest seroprevalence observed in Venezuela likely associated with the abandonment of control, prevention and epidemiologic surveillance at Sucre state. In addition is important pointing out that purification of DNA serum samples with Wizard kit represent a new valuable molecular tool for detection of *T. cruzi* in seroepidemiological studies.

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BM32 - MULTICLONAL *LEISHMANIA (V.) BRAZILIENSIS* POPULATION STRUCTURE AND ITS CLINICAL IMPLICATION IN A REGION OF ENDEMIC AMERICAN TEGUMENTARY LEISHMANIASIS (ATL).

A. Schriefer[1], A. L. F. Schriefer[1], A. Góes-Neto[2], L. H. Gimarães[1], L. P. Carvalho[1], R. P. Almeida[1], P. R. Machado[1], H. A. Lessa[1], A. Ribeiro de Jesus[1], L.W. Riley[3] and E. M. Carvalho[1]

[1]Serviço de Imunologia, Hospital Universitário Professor Edgard Santos, Universidade Federal da Bahia; [2]Laboratório de Pesquisa em Microbiologia, Departamento de Ciências Biológicas, Universidade Estadual de Feira de Santana; [3]Division of Infectious Disease, School of Public Health, UC-Berkeley.

In Corte de Pedra (CP), northeastern Brazil, *Leishmania braziliensis* causes three distinct forms of American tegumentary leishmaniasis (ATL). To test the hypothesis that strain polymorphism may be involved in this disease spectrum and accurately characterize the parasite population structure in CP, we compared one *L. (L.) major*, two non CP *L. (V.) braziliensis*, one CP *L. (L.) amazonensis* and 45 CP *L. (V.) braziliensis* isolates, obtained over a ten-year period from localized cutaneous, mucosal and disseminated leishmaniasis patients, using randomly amplified polymorphic DNA (RAPD). Electrophorectic profiles were mostly unique across species. All typing protocols revealed polymorphism among the 45 CP *L. (V.) braziliensis* isolates, which displayed 8 different RAPD patterns and greater than 80% overall fingerprint identity. The UPGMA dendrogram based on the sum of RAPD profiles of each isolate unveiled nine discrete typing units clustered into five clades. Global positioning showed extensive overlap of these clades in CP, precluding geographic sequestration as the mechanism of the observed structuralization. Finally, all forms of ATL presented a statistically significant difference in their frequencies among the clades, suggesting that *L. (V.) braziliensis* genotypes may be accompanied by specific disease manifestation after infection.

BM33 - A NEW ENZYMATIC VARIANT OF *LEISHMANIA (VIANNIA) GUYANENSIS* FOUND IN ISOLATES FROM PATIENTS IN SANTARÉM, PARÁ STATE, BRAZIL

Jennings, Yara L. L.; Ishikawa, Edna A. Y.; de Souza, A.A. Adelson; Lainson, Ralph; Silveira, Fernando T.

XXX ANNUAL MEETING ON BASIC RESEARCH IN CHAGAS DISEASE - XIX MEETING OF THE BRAZILIAN SOCIETY OF PROTOZOOLOGY - HOTEL GLÓRIA, CAXAMBU, MG, BRASIL - 10-12 NOVEMBER 2003. **Rev. Inst. Med. trop. S. Paulo, 45**(Suppl. 13), November, 2003.

Instituto Evandro Chagas (SVS) Belém, Pa.

Introduction: Among the various dermotropic species of *Leishmania* in the Amazon region, *Leishmania (V.) guyanensis* is essentially silvatic and predominantly distributed north of the river Amazonas. Antigenic variants in populations of the parasite have been found, related to its reaction with the monoclonal antibody B19 (Grimaldi *et al*., 1991; Romero *et al.*, 2002). Till now, enzyme electrophoresis, however, has indicated that strains from north Brazil are represented by populations homogenous and identical to the reference-strain of *L.(V.)guyanensis* (MHOM/BR/1975/M4147 – Monte Dourado, Pará). This communication records the first finding of intraspecific variation observed by enzyme electrophoretic characterization among isolates of this parasite. Objetives: To study the phenotypic characters of strains of *L.(V.) guyanensis* by their monoclonal antibody and enzyme electrophoretic profiles. Materials and methods: Thirteen samples of *Leishmania* isolated from patients from three municipalities of the lower amazon region (Santarém: 9; Óbidos: 3; Almerim: 1) in the west of Pará State were studied by a panel of 23 monoclonal antibodies (biotine-avidine system) and enzymatic electrophoresis in starch-gel with the six enzymes 6PGDH, G6PD, PGM, MPI, ASAT and ALAT. Profiles of the samples were compared with the reference-strains of all the know *Leishmania* species from the Amazon region. Results: All thirteen isolates reacted with the monoclonal antibody B19, which is species-specific for *L.(V.) guyanensis*, and thus showed a reaction comparable with that of serodeme II recorded by Shaw *et al*. (1984). With regards enzyme electrophoresis, two different profiles were detected: one (6 samples) agreeing with that of the reference-strain of *L.(V.) guyanensis* and another (7 samples) differing in the motility of the enzymes 6PGDH and PGM. Conclusions: The present study records, for the first time, the presence of *L.(V.) guyanensis* south of the Amazon river, in an area of the Santarém municipality, Pará. This extends the recorded distribution in Amazonian Brazil, although it is geographically very close to the major endemic area, north of the Amazon river. On the other hand, while all thirteen isolates showed the same serodeme profile as that of the reference-strain of *L.(V.) guyanensis*, seven showed a enzymatic variant of the reference-strain in mobility of the enzymes 6PGDH and PGM. This contrasts with the results of Romero *et al.* (2002) who indicated the presence of a different serodeme in a population of *L.(V.) guyanensis* from Manaus, Amazonas State, whereas the enzyme electrophoretic profiles were compatible with that of the reference-strain of that parasite.

BM34 - AUTOCHTHONOUS VISCERAL LEISHMANIASIS IN DOGS OF EMBU-DAS-ARTES, SÃO PAULO.

Savani, E.S.M.M.[1]; Neves, E.[2]; D'Áuria, S.R.N.[1]; Zampieri, R.A.[3]; Ishikawa, E.[4]; Camargo, M.C.G.O.[1]; Shaw, J.J[3]. & Floeter-Winter, L.M.[3]

1. Centro de Controle de Zoonoses do Município de São Paulo; 2. Prefeitura da Estância Turística de Embu das Artes; 3. Departamento de Parasitologia ICB - USP; 4. Instituto Evandro Chagas - Belém Para.

Visceral leishmaniasis is caused by *Leishmania (Leishmania) chagasi* and the geographical distribution of its vector, *Lutzomyia longipalpis*, is considered as a major limiting factor for the dispersion of the disease. During recent years we have witnessed an increase in the distribution of both the disease and the vector. Classically the disease is endemic in north-eastern Brazil and parts of Amazônia but there has been a steady increase in the number of recorded cases outside these areas in such States as São Paulo. Cases of canine visceral leishmaniasis were recorded in the northern region of São Paulo state in 1998 and in 1999 autochthonous human cases of visceral leishmaniasis were diagnosed in the city of Araçatuba which is situated in this same region.

A serological survey was initiated in dogs of Embu das Artes, a region near to the capital of São Paulo State. 462 serum samples were examined at the Centro de Controle de Zoonoses de São Paulo, using the indirect immunofluorescent reaction, between February and May 2003. Of these 412 were non-reactive, although 8 had titers of 1:20 dilution they were considered as negative. The other 42 samples has titers ranging from 1:40 to 1:1280. Fragments of spleen, liver, bone marrow aspirate and lymph nodes were taken from 13 serologically positive animals that had been sacrificed. Amastigotes were seen in Giemsa stained tissues smears of 7 animals. Promastigotes were isolated in culture from two samples of dogs in which parasites had not been seen in Giemsa smears. In another sacrificed dog, amastigotes were seen in cytological preparations of spleen, liver, bone marrow aspirate and skin lesion. Promastigotes were obtained from culture of fragments of liver, bone marrow and skin lesion. Serology was not performed on this animal. Promastigotes of three isolates were identified as *L. (L.) chagasi* by monoclonal antibody typing and/or an SSU-based PCR test.

This is the first time that this species of *Leishmania* has been detected in dogs in a region that is so close to the city of São Paulo. In the absence of records of human cases from this region the present findings of *L. (L.) chagasi* is a complete surprise. An entomological survey is under way but so far no specimens of L. longipalpis have been found. Further studies in neighbouring counties are needed to assess the potential risk of visceral leishmaniasis becoming endemic in Latin Americas largest city, São Paulo.

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BM35 - THE FIRST RECORD IN THE AMERICAS OF AN AUTOCHTHONOUS CASE OF *LEISHMANIA* (*LEISHMANIA*) *INFANTUM CHAGASI* IN A DOMESTIC CAT (*FELIS CATUS*) FROM COTIA COUNTY, SÃO PAULO STATE, BRAZIL

Savani,E.S.M.M.[a]; Camargo, M.C.G.O.[a]; Carvalho, M.R.[b]; Zampieri, R.A.[c]; dos Santos, M.G.[c]; D'Áuria, S.R.N.[a]; Shaw, J.J.[c] & Floeter- Winter, L.M.[c]

[a] Centro de Controle de Zoonoses do Município de São Paulo. [b] Clínica Veterinária Zoofarma. [c] Departamento de Parasitologia Instituto de Ciências Biomédicas da Universidade de São Paulo.

Leishmaniasis in domestic cats (*Felis catus*) has been reported sporadically in various parts of the world. These observations suggest that the cat is an accidental host, but its true role as a reservoir of the disease is unknown. The majority of cases in the literature refer to cutaneous lesions and rarely related visceral infections. The species incriminated in these cases, when identification was performed, was either *Leishmania* (*Leishmania*) *infantum* or a *Leishmania*(*Viannia*) species.

In July 2000, a two year old domestic male cat that had been born and reared in Cotia Municipality, São Paulo State, Brazil, was taken to a local veterinary clinic because of a nodular lesion on its nose. The animal had lost both weight and musculature, was severely dehydrated and had enlarged lymphatic ganglion.

The indirect fluorescence test for leishmaniasis of a serum sample was positive at a 1:80 dilution. Serology for feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) was negative, but a positive result (1:400) was obtained for feline infectious peritonitis (FIP). So, apparently, the animal was not immunodepressed because a viral infection.

Both intra and extracellular amastigotes were abundant in Giemsa stained smears of the cutaneous nodule. The animal was sacrificed and Giemsa stained cytological preparations were made from both the spleen and liver. Attempts to isolate the parasite from tissue samples in blood agar base culture medium failed. DNA extracted from a spleen fragment was used in a rDNA based PCR assay and resulted in the amplification of a 520 bp fragment. This fragment was purified, cloned and sequenced. A comparison of the obtained nucleotide sequence with those described by Uliana *et al.*(1994), detected presence of a C in position 1714 and a G in position 1721, identifying the parasite as *Leishmania (L.) chagasi*.

The present finding is the first record of a natural infection of *L. (L.) i.*

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chagasi in the domestic cat (*F. catus*) in Brazil and in the Americas. The cases of feline visceral leishmaniasis described in the literature are all from cats living in endemic visceral leishmaniasis areas of the Old World. What is even more surprising, in respect to the present report, is that the cat was a native of an area where so far there have been no reports of either autochthonous human or canine visceral leishmaniasis.

Financial support: FAPESP, CNPq and FUNASA.

BM36 - FURTHER EVIDENCE SUPPORTING THE WATER RAT, *NECTOMYS SQUAMIPES*, AS A PRIMARY RESERVOIR OF *LEISHMANIA (VIANNIA) BRAZILIENSIS* IN AN ENDEMIC REGION OF CUTANEOUS LEISHMANIASIS IN PERNAMBUCO STATE, BRAZIL.

Brandão-Filho, S. P.; Carvalho, F.G.; Brito, M.E.F.; Alves, I.C.; Ishikawa, E.; Cupolillo, E.; Floeter-Winter, L.M. & Shaw, J.J.

Centro de Pesquisas Aggeu Magalhães/FIOCRUZ, Recife; Instituto Evandro Chagas, Belém; Instituto Oswaldo Cruz/FIOCRUZ, Rio de Janeiro; Instituto de Ciências Biomédicas/USP, São Paulo.

There is an accentuated incidence of cutaneous leishmaniasis (CL) in the State of Pernambuco, especially in the region that corresponds to that of the Atlantic rainforest (“Zona da Mata”) which accounts for 60% of the recorded cases. A multidisciplinary study of the ecoepidemiology of CL has been developed in the Amaraji, locality that is situated in the “Zona da Mata”, some 90Km from the state capital, Recife. One of the principal objectives has been to find natural infections of *Leishmania*, especially *Leishmania (Viannia) braziliensis* in both wild and sinanthropic mammals. Monthly collections of small wild mammals were made using Tomahawk wire traps.

A *Leishmania* (MNEC/BR/2003/CPqAM191) was isolated from a hamster in July 2003 that had been inoculated with the skin and spleen cells collected from a water rat, *Nectomys squamipes*. The animal had been captured in October 2001 in an area of swamp land surrounded by sugar cane and pasture. The isolation was made in Blood Agar base (Difco B45) and the strain was identified as *L. (V.) braziliensis* using the specific G6PD PCR, according the method described by Castilho *et al*., 2003, *J. Clin. Microbiol*. *41*: 540-546. It was also identified as *L. (V.) braziliensis* serodeme 1, using a panel of monoclonal antibodies. The present result adds further weight to the hypothesis that rodents are the principal sylvatic reservoirs of *L. (V.) braziliensis* (Brandão-Filho *et al*., 2003, *Trans. R. Soc. Trop. Med. Hyg. 97*). The brown rat, *Rattus rattus*, is perhaps a reservoir in the peridomestic habitat. *Lutzomyia whitmani* is the principal vector in the zoonotic cycle and other species such as *Psychodopygus complexus (Lu. complexa)* a possibly involved in the enzootic cycle.

Financial Support: CPqAM/ FIOCRUZ, FAPESP, FACEPE and CNPq.

BM37 - MOLECULAR CHARACTERIZATION OF *LEISHMANIA* ISOLATED FROM THE CUTANEOUS LESIONS OF PATIENTS FROM RONDÔNIA-BRAZIL

Duarte, E.C., Faria, D.L.*, Camargo, L.M.A.**, Shaw, J.J. & Floeter-Winter, L.M.

Departamento de Parasitologia – Instituto de Ciências Biomédicas – USP, * Instituto Evandro Chagas – FUNASA - Belém - Pará, ** Campus Avançado - Departamento de Parasitologia – Instituto de Ciências Biomédicas – USP - Monte Negro – Rondônia.

The leishmaniases are caused by many species of *Leishmania* that are respectively classified as belonging to either the subgenera *L.* (*Leishmania*) or *L.* (*Viannia*). Different markers have been used in taxonomic studies, such as rDNA, kDNA, isoenzymes and monoclonal. In the present study, 10 *Leishmania* isolates of patients from Rondônia-Brazil were analyzed by amplification and sequencing using distinct targets, such as the small subunit ribosomal RNA (SSU rRNA) and the glucose-6-phosphate dehydrogenase coding gene (*G6PD*). G6PD presents eight isoforms and is widely used in multilocus enzyme electrophoresis to identify *Leishmania*.

In the PCR assays, one set of paired primers were used in the SSU rRNA based assay and three pairs for G6PD based assay (Uliana *et al.,* 1994 and Castilho *et al.*, 2003). The amplified fragments were cloned and sequenced.

Comparisons of SSU rDNA variable regions sequences of the isolates with *L. (L.) amazonensis, L. (L.) mexicana, L. (L.) donovani, L. (L.) chagasi, L. (L.) major, L. (V.) braziliensis* and *L. (V.) guyanensis* grouped all 10 isolated into subgenera *L.* (*Viannia*).

The alignment of the deduced G6PD amino acid sequence of the isolates showed a similarity of 94% to 100%, when compared to particular G6PD N-terminal region of the named species of the subgenera *L. (Viannia)*. Isolate MHOM/BR/1998/M16726 was the most divergent having a similarity of 96% with *L. (V.) guyanensis, L (V.) panamensis, L. (V.) shawi* and *L. (V.) lainsoni*, a similarity of 94% with *L. (V.) braziliensis* and a similarity of around 61% with *L.* (*Leishmania*) isolates. These results, associated with the analysis of other markers will contribute to determining the species diversity of the *Leishmania* of this geographical region of Brazil.

Supported by FAPESP, PRONEX and CNPq.

BM38 - EXTRACTION AND AMPLIFICATION OF *LEISHMANIA* DNA FROM ARCHIVED GIEMSA STAINED SLIDES (1965-2000) FOR THE DIAGNOSIS OF AMERICAN CUTANEOUS LEISHMANIASIS (ACL) IN BRAZIL

Volpini Â. C., Marques M. J., Lopes dos Santos S., Machado-Coelho G. L., Mayrink W., Romanha A. J.

Centro de Pesquisas René Rachou/FIOCRUZ – MG, UFMG, UFJF e UFOP

In the present work, we have used the polymerase chain reaction coupled to restriction fragment length polymorphism of the conserved region of minicircles kDNA from *Leishmania* (PCR-RFLP mkDNA) for specific *Leishmania* identification, directly from Giemsa stained slides containing biopsy imprints from ACL human lesions. The slides had been prepared for routine parasitological examination. This material allowed to perform a 35 years retrospective study in the ACL endemic region of Vale do Rio Doce – MG. This area has been regarded as an ACL endemic region since the 60s and *L. (V.) braziliensis* and *L. (L.) amazonensis* were described as the aethiological agents of leishmaniasis there. 475 out of 3,652 Giemsa stained slides from ACL patients were selected. An area of two imprints was scrapped from each slide, resuspended in water and the DNA extracted by heating. The preparation was centrifuged and the supernatant used as DNA source for PCR. Firstly, a specific PCR amplification was performed using primers that amplified the conserved region of the *Leishmania* kDNA minicircles. Then, RFLP was done using the endonucleases HaeIII and ApaLI. 395 (83.2%) out of 475 slides, were PCR-positive, showing the expected DNA amplified fragment of 120 bp. All 395 PCR-positive samples were digested by the enzymes and they showed RFLP profiles identical to those from reference strains of *L. (V.) braziliensis*. PCR-RFLP mkDNA was able to identify *L. (V.) braziliensis* as the only causative agent of ACL in Vale do Rio Doce-MG, in samples of slides archived from 1965 to 2000. The predominance of phlebotomine sand flies of the species *Lutzomyia whitmani* suggests this diptera as the likely vector of *L. (V.) braziliensis* in that region and in the State of Minas Gerais. On the other hand, the information on the absence of *Lu. flaviscutellata* the vector of *(L.) (L.) amazonensis* and cases of diffuse cutaneous leishmaniasis in the region are coincident. Our results show

that archived slides are useful for both DNA extraction and species identification of the aethiological agent of ACL.

Financial Support: Centro de Pesquisas René Rachou/FIOCRUZ – MG

BM39 - A POLYMERASE CHAIN REACTION MULTIPLEX ASSAY IN THE DIAGNOSIS OF NATURALLY INFECTED *LUTZOMYIA* PHLEBOTOMINES BY *LEISHMANIA* SPP.

Pita-Pereira D[1], Souza MB[2], Britto C[1], Cardoso MAB[1], Pontes CS[2], Feder F[1], Caetano-Araújo DF[1] & Alves CR[1]

[1]Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz; [2]Departamento de Ciências Biológicas, Escola Nacional de Saúde Pública, FIOCRUZ, Av. Brasil, 4365 Manguinhos, CP 926, 21045-900, Rio de Janeiro, Brasil. Corresponding author: C. R Alves (calves@ioc.fiocruz.br)

It is known that *Lutzomyia* transmits leishmaniasis in endemic areas of the New World and the rate of natural infection of these sandflies is low. Insects infected with *Leishmania* can be diagnosed by microscopy, culture and the polymerase chain reaction (PCR) test. Herein, we propose a PCR-multiplex assay for a better performance of the molecular diagnosis of natural infections of *Lutzomyia sp* with *Leishmania* in an endemic area of the Rio de Janeiro State. We have developed a PCR test that is able to amplify concomitantly the minicircle kDNA sequences from *Leishmania sp* -120 bp (Passos et al., 1996) and the *Lutzomyia sp* cacophony gene - 220bp (Lins et al., 2002). The insects captured in peri-domiciliar area were transported to the laboratory for taxonomic identification of male and female. Between May/2002 and July/2004 we have got predominantely *Lu. intermedia* (2498♂, 808♀), *Lu. longipalpis (*74♂, 18♀*)*, *Lu. migonei* (114♂, 75♀), *Lu. fischeri* (0♂, 57♀), *Lu. firmataoi* (21♂, 5♀), *Lu. schreiberi* (10♂, 0♀), *Lu. quinquefer* (0♂, 2♀) and *Lu. pelloni* (2♂, 0♀). Some of these species were pooled in sets of 10 insects in 100 mL of buffer 10 mM Tris-HCl pH 9.2 containing 10 mM EDTA. These pools were treated with 1mg/mL of proteinase K and the DNA recovered by using the phenol-chloroform extraction method. The expected amplification products occur in 11 out of the 24 female pools assayed, accordingly to the *Lutzomyia* spp.: *Lu. intermedia* (n=6), *Lu. migonei* (n=4) and *Lu. fischeri* (n=1). Curiously, we have not detected *Leishmania* parasites in a previous analysis of individual female sandflies by using the mid-gut dissection method. Furthermore, the hybridization of these amplicons with a specie specific probe will be necessary in order to check which *Leishmania* spp. is infecting those phlebotomines. The sandfly male pools were included in the study as negative controls of the *Leishmania* PCR assay detection method and also as positive controls for *Phlebotomus* genes. In this last case, the assay is able to detect only a single 220bp band. Our strategy help us in the identification of true negative samples for *Leishmania*, avoiding false negative diagnostic results that may occur by the presence of PCR inhibitors during the DNA sample processing. We also demonstrate that PCR-multiplex is a suitable approach to the establishment of the *Leishmania*-sandfly relationship and for epidemiological studies of leishmaniasis.

	Lu. intermedia	*Lu. migonei*	*Lu. fischeri*	*Lu. longipalpis*	*Lu. firmatoi*	*Lu. schreiberi*	*Lu. quinquefer*	*Lu. pelloni*	Total
Areas	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀	♂/♀
Piabas	2/4	32/6	0/0	0/0	21/5	3/0	0/0	0/0	58/15
Colônia	1563/606	8/0	0/0	0/0	0/0	1/0	0/0	0/0	1572/606
P. Fome	402/97	56/7	0/18	74/18	0/0	6/0	0/0	0/0	538/140
V.Grande	191/23	3/0	0/0	0/0	0/0	0/0	0/0	2/0	196/23
V.Pequena	338/55	63/8	0/6	0/0	0/0	0/0	0/0	0/0	401/69
R.Bonito	2/2	4/0	0/0	0/0	0/0	0/0	0/0	0/0	6/2
G.Funda	0/0	0/0	0/0	0/0	0/0	0/0	0/2	0/0	0/2
Grumari	0/21	1/0	0/0	0/0	0/0	0/0	0/0	0/0	1/21
Total	2498/808	167/21	0/24	74/18	21/5	10/0	0/2	2/0	2772/878

Consolidated of colleted Phlebotomies in Jacarepaguá area: May/2002 until July/2004

Supported by CNPq and Centro de Referência de Leishmaniose

BM40 - PCR STANDARDIZATION TO IDENTIFY CYSTS OF *ENTAMOEBA HISTOLYTICA* AND *E. DISPAR* FROM FECAL SPECIMENS

Vianna, E.N.[1], Benneton,M.L.F.N[2]., Moraes M.S[3]., Freitas M.A.R[1]., Silva, E.F[1], Gomes, M. A[1].

[1]*Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG,* [2] *Universidade Federal do Amazonas, Manaus, Amazonas,* [3]*Instituto Evandro Chagas, Belém do Pará,Pará*

The correct diagnosis of the infection caused by the protozoan *Entamoeba* has critical significance for treatment due to the morphologic similarities between cysts and trofozoítos of *Entamoeba histolytica* and the nonpathogenic one *E. dispar*. The examination of fecal samples by optic microscope is not able to differentiate these two species. It reaches about 60% sensitivity in detention of cysts and can give false-positives since it can not distinguish trofozoítos of other species of *Entamoeba*. The PCR in the diagnosis of ameba reveals, therefore, as an important tool for the differentiation between *E. histolytica* and *E. dispar*. Some protocols had been described and used for the detention of the two species, amount them the amplification of fragments of genes of rDNA that have shown more sensible than the methods of antigen detention. However, in all the protocols the DNA is obtained through laborious and delayed techniques that usually results in low yield. Good yield and quality of DNA from human feces are obtained through kits that are very expensive. In this work we present a new method for the extration of DNA from human feces. This method showed to be so sensible as the commercial kit DNA Stool minikit QIAamp® and was used to extract DNA from fecal samples of individuals with and without amebiasis. The extracted DNA was used to standardize a specific PCR with primers for the two species of *Entamoeba*. It were used fecal samples from the Amazon region and southeastern of Brazil. Samples were fixed in formalin 10%, concentrated by the sedimentation method in formol–ether, examined by optic microscope and later the DNA was extracted through a modified alkaline lise protocol. The PCR was carried out using specific primers able to amplify a different ribossomal DNA fragment of 310 bp from each ameba. The PCR products were loaded in a 4% gel polyacrylamide, submitted to electrophoresis and visualized through silver nitrate. Of 150 analyzed samples, 93 were positive being 27 specific for *E. histolytica* and 66 for *E. dispar*. The DNA extration method demonstrated to be simple, effective and of low cost and the diagnosis through the PCR showed also to be sensible, fast and specific for the differentiation of *E. histolytica* from the non-pathogenic one *E. dispar*.

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BM41 - A SINGLE STEP DUPLEX PCR TO DISTINGUISH *ENTAMOEBA HISTOLYTICA* FROM *ENTAMOEBA DISPAR*

M. A. R. FREITAS[1], E. N. VIANNA[1], A S. MARTINS[2], E. F. SILVA[1], J. L. PESQUERO[2,3] *and* M. A. GOMES[1*]

[1]Departamento de Parasitologia, [2]Fisiologia e Biofísica, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos, 6627, CEP: 31270-901 Belo Horizonte, MG, Brasil, [3]Universidade de Mogi das Cruzes, São Paulo, SP, Brasil.

In this study, a single step duplex polymerase chain reaction procedure was

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developed for a rapid, specific and sensitive identification of *E. histolytica* and for its diagnostic differentiation from *E. dispar.*

Two gene sequences were targeted simultaneously in this PCR procedure. One comprises an internal segment of 242 bp of the cysteine proteinase 5 (EhCP5) gene (Bruchhaus et al., 1996), present only in *E. histolytica* (EhCP5), and the oligonucleotide primers designed were EhCP6F, and the other with 300 bp sequence within the actin gene, and the oligonucleotide primers designed were Act3, common to both *E. histolytica* and *E. dispar* (Edman, Meza & Agabian, 1987; Huber *et al*. 1987), in order that two amplicons will identify the first and one amplicon identifies the last one, respectively.The PCR products were submitted to electrophoresis in 4% polyacrylamide gel and amplicons visualized by silver staining. The PCR developed in the present work was specific and efficient to identify and differentiate these parasites from each other in either cultured or from stool material.

Good correlation between zymodeme and PCR methods was observed. Additionally, the results showed that this duplex PCR approach may be applied also for stool samples confirming either positives or negatives as reported by the optical microscopy technique with sensitivity to detect just one cyst (wells 12 and 13). In conclusion, these results altogether might facilitate now to distinguish these two parasites in positive samples and bring about a tool for important diagnostical differentiation.

Finnancial support: Fapemig

BM42 - GENETIC VARIABILITY OF *TOXOPLASMA GONDII* STRAINS FROM BRAZIL DETECTED BY RANDOM AMPLIFIED POLYMORPHIC DNA – POLIMERASE CHAIN REACTION (RAPD-PCR) AND SIMPLE SEQUENCE REPEAT ANCHORED – PCR (SSR-PCR)

Ferreira AM, Vitor RWA, Carneiro ACAV, Brandão GP, Melo MN

Departamento de Parasitologia do Instituto de Ciências Biológicas da Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil. e-mail: drimelo@icb.ufmg.br

Toxoplasma gondii is currently considered to be the only species in the genus *Toxoplasma*. However, strains of this parasite have been subdivided into two or three major groups based on their virulence or lethality for mice during acute infections. Genetic diversity among *T. gondii* strains from different parts of the world, mainly Europe and North America, has been demonstrated by several molecular techniques. Our aim was to study the genetic variability of 19 *T. gondii* strains isolated from humans and animals in Brazil using two methods for analyzing DNA polymorphism: Random Amplified Polymorphic DNA-PCR (RAPD-PCR) and Simple Sequence Repeat anchored-PCR (SSR-PCR). It is the first time that Brazilian *T. gondii* strains are studying under RAPD and SSR, PCR-based methods. Two reference strains, RH (highly virulent) and ME49 (avirulent), were submitted to both assays. *Besnoitia* sp, *Plasmodium falciparum* and *Babesia bigemina* were used as out-groups. Purified *T. gondii* tachyzoites were obtained from peritoneal cavities of Swiss or C57BL/6 INF-g knockout mice, depending on the growth rate. DNA was prepared by proteinase K digestion followed by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation. RAPD-PCR analysis was performed using the kit "Ready To Go" Amersham Pharmacia Biotech-Uppsala, Sweden with seven different primers and SSR-PCR with $(CA)_8RY$ and $CAA(CT)_6$ primers. The amplified DNA fragments were separated on 5% polyacrilamide gels, which were silver stained and photographed. RAPD-PCR and SSR-PCR profiles were used for building phenetic trees by Unweighted Pair Group Method using Arithmetic Averages (UPGMA). Phenograms built with computer software TREECONW showed great similarity in the topology of the trees. Both phenograms presented two major clusters that grouped *T. gondii* strains according to their murine virulence. The strains AS28, BV and N, which are highly virulent for BALB/c mice, were clustered with RH reference strain, the most commonly studied highly virulent strain of *T. gondii*. The other group showed that the strains which presented a level of virulence more similar to that of ME49 reference strain (avirulent) also presented a closer genetic relationship. The genetic variation within each lineage was significantly lower ($P<0,05$) than that between the lineages. Regarding out-groups, *Besnoitia* sp presented the closest relationship to *T. gondii* while *Plasmodium falciparum* the most distant. The results presented here demonstrate that intra-specific genetic variability separate Brazilian *T. gondii* strains in two groups that correlate with murine virulence phenotype, as showed for genus *Toxoplasma*.

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BM43 - LACK OF TECHNICAL SPECIFICITY IN THE MOLECULAR DIAGNOSIS OF TOXOPLASMOSIS

Kompalic-Cristo, A.[1], Nogueira, S. A.[2], Guedes, A. L.[2], Frota, C.[3], González L. F.[4], Brandão A.[1], Amendoeira M. R. [1], Britto C.[1], Fernandes O.[1]

[1]Instituto Oswaldo Cruz, Departamentos de Medicina Tropical, Protozoologia e Bioquímica e Biologia Molecular; Universidad Centroccidental Lisandro Alvarado, Departamento de Medicina Preventiva y Social; [2]Universidade Federal do Rio de Janeiro; [3]Núcleo Técnico Operacional, Diagnósticos da América; [4]Laboratório de Virologia Molecular Animal, Universidade Federal do Rio de Janeiro.

The accurate diagnosis of toxoplasmosis in immunocompetent patients or pregnant women with possible risk of *Toxoplasma gondii* transmission via placenta, is extremely important and need to be investigated. Routine serological diagnosis provides high sensitivity, but not high specificity. In this scenario, methods involving parasite detection should be implemented. Several approaches based on polymerase chain reaction (PCR) directed to distinct protozoan genomic targets have been developed and the most used is the B1 gene. Herein, we report the molecular detection standardization of *T. gondii* using PCR targeted to the B1 gene, coupled to a non-isotopic hybridization step with a probe derived from a PCR positive control performed with purified parasite DNA. Using this procedure, we were able to detect 93 fg of protozoan DNA (one parasite corresponds to 100 fg of DNA). Thirty-four individuals with suspicion of toxoplasmosis by serological diagnosis were submitted to our PCR-hybridization assay. Eight seronegative individuals were also included in the study. The presence of circulating protozoan was detected, in: (i) 50% of 10 acute phase patients presenting positive IgM anti-*T.gondii* independently of the IgG results; (ii) 12.5% of 24 chronically infected patients with positive IgG and negative IgM; (iii) 0% of 8 seronegative individuals. The data showed that in some cases, PCR products with the expected molecular size of the target sequence (194 bp) could be evidenced in the visual inspection on agarose gels, but did not hybridize with the B1 gene probe. Those amplified fragments were direct sequenced and correspond to scattered human sequences from chromosomes 2 and 10, co-amplified under the same experimental conditions, showing an homology of 88 and 89%, respectively. The PCR positive control fragment used as our molecular probe, revealed 96% of similarity with the B1 gene sequence. The results demonstrate that PCR amplification of these gene of *T. gondii*, with primers previously described in the literature, promotes the co-amplification of human sequences. These spurious products were revealed with the use of molecular approaches other than the mere visualization of the amplified products.

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BM44 - THE IDENTIFICATION OF MINICIRCLES IN *TRYPANOSOMA VIVAX*

Guerreiro, L.T.A., Souza, S.S., Traub-Cseko, Y.M., Dávila, A.M.R.

DBBM, Instituto Oswaldo Cruz, Fiocruz. Av. Brasil 4365, Rio de Janeiro, RJ, CEP 21045-900. Email: davila@fiocruz.br

Kinetoplast DNA (kDNA) minicircles are in most cases heterogeneous within a given parasite and almost completely different among trypanosome species. They are organized into one to four conserved regions (CR) representing approximately 10% of the molecule and an equal number of variable regions, depending on the species. Each CR contains three conserved sequence blocks (CSB) identified in different trypanosomatid species. The minicircles of *Trypanosoma vivax* were previously investigated by Borst et al (1985), however their sequences were not determined and are not available in the databases. Our main objective was the identification of new molecular markers for *T. vivax,* then, a semi-normalized genomic library was constructed and 400 GSS sequenced and analyzed "in silico". From 501 reads, 8 different minicircles were identified using the standalone version of Blast with the KINETO and NR databases of EBI and NCBI, respectively. Usually, best hits presented e-values worth than 0.01, nevertheless similarities were always with the conserved region of trypanosomatid minicircles. When multiple aligments were done using the ClustalX software, only 1 CR was found, containing the CSB-1, CSB-2 and CSB-3. When *T. congolense*, *T. evansi*, *T. brucei* and *T. cruzi* minicircles were included in the alignment, we confirmed that CSB-3 (GGGGTTGGTGTAA) is highly conserved to their homologs described in other trypanosomatids, and is thought to be origin of replication of minicircles. CSB-2 is less conserved among *T. vivax* minicircles and of the eight nucleotides that compose this sequence, the last five are identical. We also observed that CSB-1 sequence is approximately 85 nucleotides upstream of the CSB-3 sequence, while CSB-2 is approximately 48 nucleotides upstream of CSB-3. This organization was observed in the 8 minicircles analyzed. The size of the minicircle is ~480 bp, which would be in accordance with the 465 bp reported by Borst et al (1985). The exact size of minicircles will be determined in further experiments. Our next steps are the identification of more minicircles with a probe specific to CSB-3, and the use of those minicircles for the design of species-specific PCR-based diagnosis and typing tools. The putative gRNAs genes encoded by the minicircles will be also studied.

Supported by: IOC/ Fiocruz, CIRAD.

BM45 - *PLASMODIUM* SPP. INFECTION IN WILD BIRDS FROM MATA ATLÂNTICA, MINAS GERAIS STATE: EVALUATION BY THIN BLOOD SMEARS AND PCR

Ribeiro, SF, Sebaio, F, Branquinho, F, Marini, MA, Vago, AR & Braga, EM

The standard method for avian malaria diagnosis is the microscopic examination of thin blood smear stained with Giemsa. However, this method presents some limitations and low sensitivity. As a result, the thin blood smears-based prevalence and distribution of avian plasmodia might be underestimated. Alternative diagnostic methods based on parasite DNA detection, such as the Polymerase Chain Reaction (PCR), have been used for identification of malaria species Our present goal is evaluate the *Plasmodium* infection in 137 wild birds from Mata Atlântica (Reserva Particular do Patrimônio Natural Mata do Sossego), Minas Gerais State. The microscopic observation allowed to detect 7.3% of positive birds while a PCR protocol of 18 SSUrRNA gene showed a rate of 34.3% of *Plasmodium* infection. Correlation between both methods employed was not observed. Seventy three birds were captured in a fragmented area and 64 in non-fragmented area of Mata do Sossego. None correlation was verified between parasitism frequency and fragmentation, considering the two techniques used. Birds biological characteristics such gender, age, diet, nest and others were evaluated according to the presence of *Plasmodium* sp. Interestingly, only the diet was correlated with the parasite infection. The birds which main diet was based on insects were statistically more infected than others ones. This result indicate that the alimentary habits could be involved in efficacy of avian malaria transmission, reinforcing the importance of parasite-host interaction in ecological studies.

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BM46 - COMPARISON OF DNA EXTRACTION PROCEDURES FOR *LEISHMANIA* PCR FROM STORED SANDFLIES.

Paiva, B.R[1]; Savani E. S. M. M[2],Galati E.A.B[2], Andrade Jr, H.F[1].

1-Instituto de Medicina Tropical de São Paulo& Instituto de Ciências Biomédicas/ USP, 2-Faculdade de Saúde Pública/USP - Laboratório de Entomologia

Leishmaniases are endemic South American diseases transmitted by sand flies. In these insects, after they bite an infected mammal reservoir, the flagellated parasite access and grows in the sand fly gut, until its regurgitation in the next insect meal, closing the transmission cycle. The prevalence of natural infection by flagellates in sand fly populations could be an index of the environmental risk for the transmission of that diseases. The natural infection by flagellates rate is usually very low and its identification by taxonomic and microscopic exams demands a lot of work. The detection of infection in large numbers of insects, with determination of flagellate species would be attaining with PCR reactions. These reactions had been standardized for biopsy or scraping material, aside to flagellate culture, usually to repeated genes sequences, as mini-exon, kDNA or ribossomal genes, but most of them were tested only against mammalian DNA. In this work, we tested several ways to obtain the intestinal content of phlebotomine and their DNA, looking for interference in the PCR for leishmania detection using primers directed to ribossomal (SSUrRNA) genes. Several approaches were tested in order to obtain phlebotomine DNA, as crude total homogenate, centrifugation against pinhole using insect chitin as sieve or the classical phenol chloroform extraction. Usually, for each reaction ten laboratory breed insects (*Lutzomyia longipalpis)* and leishmania (free or that were seed with known numbers of promastigotes of *Leishmania (L.) amazonesis*, strain IFLA/BR/67/PH8) were added to individual tubes, aside of phlebotomine DNA mass extracted, to introduce a competitive DNA confounding factor.

The Leishmania PCR reaction was completely functional in the presence of added total phlebotomine DNA at 1 mg/tube. Leishmania PCR was capable to detect until 1 promastigote/sand fly. Any attempt to separate intestinal contents by centrifugation fail to maintain stable material for PCR detection, probably due extensive DNA destruction. PCR also fail in phenol-chloroform extraction of intact whole parasite, which results in sequestration of the DNA by the chitin exoskeleton.

In order to assure that the intestinal promastigote DNA was present in the material after the extraction procedure, usually by homogenizing the whole insect with destruction of chitin exoskeleton, the extraction of phlebotomine DNA must be cautiously performed.

The use of PCR, especially with Poisson distribution and extreme care in extraction, could be a powerful tool for measurement of the natural infection rate by leishmania in sand flies from endemic areas.

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BM47 - *LEISHMANIA* SPECIES-SPECIFIC PCR IN PARAFFIN EMBEDDED SKIN BIOPSIES FROM CUTANEOUS LEISHMANIASIS FROM THE ESPÍRITO SANTO STATE, AS COMPARED TO OTHER MOLECULAR BIOLOGY APPROACHES.

Paiva, B.R[1]; Passos, L.N[2]; Falqueto, A[2]., Volpini A.C.[3], dos Santos S.L.[3], Pagliari C[4], Romanha, A.J.[3] & Andrade Jr, H.F[1].

1-Instituto de Medicina Tropical de São Paulo& Instituto de Ciências Biomédicas/ USP, 2-Ambulatório de Doenças Infecciosas e Parasitárias do Hospital Universitário Cassiano Antônio de Moraes-Vitoria/ES, 3-Laboratório de Parasitologia Celular e Molecular do Centro de Pesquisas René Rachou-FIOCRUZ, Belo Horizonte/MG; 4-Laboratório de Patologia de Moléstias Infecciosas e Parasitárias/FMUSP.

The diagnosis of the infecting species in cutaneous leishmaniasis is a crucial step for adequate therapy, as the prognosis and mucosal involvement is dependent of the infecting species. Usually, the diagnosis of suspected lesions is performed by stained imprints morphology, which precludes the identification of the species of the agent. Moreover, the skin biopsies are often paraffin embedded for histology. Recently, molecular approaches had allowed the diagnosis based on PCR amplification of repeated genes present in parasite genomes, that are usually performed in fresh or culture amplified samples. We devised a new set of primers looking for amplification of mini-exon genes, and tested in 58 paraffin embedded samples from cutaneous leishmaniasis patients collected at Espírito Santo, Brazil. Consecutive sections of 5 mm from the blocks were obtained and stored, aside to process for Leishmania immunohistochemistry. Isolated sections were placed in microfuge tubes, de-waxed by xylene, and their DNA extracted with phenol chloroform proteinase K treatment. The devised sequences were performed using Primer 3 software, with a 177bp product specific to L. (V.) braziliensis, using DNA from standard promastigotes from the South American leishmanias. When isolated section were tested with the same primers, 62.1%(36/58) were clearly positive. There was no correlation between the PCR and the semi-quantitative immunohistochemistry analysis. Formol stored tissues from patients from the same area were also tested and gave a PCR positivity of 46.1%(6/13). The paraffin tissue sections were also tested by PCR- RFLP. All 58 tested samples were positive for *Leishmania* and identified as *L. (V.) braziliensis*. We concluded that different molecular approaches for the diagnosis of species of *Leishmania* in cutaneous leishmaniasis patients could be performed in paraffin-stored material. Although the methods presented different sensitivities, they showed very clear results, suggesting their inclusion as a quick tool in the management of cutaneous leishmaniasis, especially after the histological diagnosis.

This work is supported by LIMHCFMUSP and CAPES.

BM48 - DNA MICROARRAYS: LEVELS OF RNA TRANSCRIPTS DURING GROWTH OF CL BRENER AND CLUSTERIZATION OF AMASTIGOTE ESTS

C. MARTINS[1], M. MORENO[1], C. S. BAPTISTA[1], R. Z. VENCIO[1,2] and B. ZINGALES[1]

[1]Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo. Av Prof. Lineu Prestes 748, CEP 05508-090 São Paulo, Brasil. caom@iq.usp.br; [2]BIOINFO-USP – Núcleo de Pesquisa em Bioinformática, Universidade de São Paulo.

The differential gene expression during growth of CL Brener epimastigotes (doubling time = 17.7 hours) was investigated with a microarray slide containing 710 ESTs of CL Brener and 20 previously characterized genes of various *T. cruzi* strains. These targets represent 665 unique sequences. The ESTs were derived from non-normalized and normalized cDNA libraries of CL Brener epimastigotes (Urmenyi et al., 1999) and were kindly provided by Dr. W. Degrave and Dr. A. Brandão (FIOCRUZ, Rio de Janeiro, Brazil). Search for similarity with BLASTN and BLASTX programs indicates that 75% of the ESTs have no matches in protein and DNA databases. Total RNA was isolated from epimastigotes during exponential growth (48 h) and in the stationary phase (336 h). cDNAs were labeled with Cy3 and Cy 5, mixed and hybridized with the slide. To prevent dye bias, normalization of the data was achieved by dye swap. The images were analyzed with ScanAlyze 2.44 program and a M-A plot was obtained (i.e. base-2-logarithm of swap normalized ratios (M) versus base-2-logarithm of intensities (A)). We concluded that 47 targets (6.4%) exhibited up-regulation by 1.7 to 2.6-fold in mid-log phase epimastigotes as compared to stationary phase parasites. To confirm these findings, some sequences were used as probes in Northern blots of total RNA of the two populations. Hybridization with a GAPDH sequence was used for normalization. The radioactive images were collected in phosphor screens and scanned with the Storm System (Molecular Dynamics). The hybridization signals were quantified by densitometric analysis using the ImageQuant Molecular Dynamics Program. In all the cases Northern blots gave higher hybridization ratios when compared with the microarray ratios. Aiming at further construction of a more representative microarray slide, 1,233 ESTs from CL Brener amastigotes (a kind gift of Dr. A. Gonzalez, CSIC, Granada, Spain) were clustered by CAP3 program. Initial analyses were performed by Dr. F. Agüero (San Martin, Un. San Martin, Buenos Aires, Argentina), and subsequently in our laboratory. The sequences were distributed in 285 clusters (with 2 to 20 sequences/cluster) and in 300 singletons (total of 585 unique sequences). Clustering of CL Brener amastigote and epimastigote ESTs (see above) makes-up 1,162 unique sequences. We have further added 44 ESTs from Tulahuen amastigotes (kindly provided by Dr. S. Teixeira, UFMG, Belo Horizonte, Brazil), and a total of 1187 unigenes was obtained. These targets will be deposited in the microarray slide for future investigation of differential gene expression in *T. cruzi* strains displaying particular biological characteristics.

Support: FAPESP and CNPq.

BM49 - GENE EXPRESSION PROFILE OF CARDIOMYOCITES INFECTED WITH DIFFERENT FORMS OF *T. CRUZI* TRYPOMASTIGOTES

Christian M. Probst[1], Celina O. Poersch[1], Daniela P. Pavoni[1], Andrea Ávila[1], Viviane M. Goes[1], Helene S. Barbosa[2], Maria de Nazareth Meirelles[2], Mirian Claudia de S. Pereira2, Daniela Puiu[3], Darrell Mallonee[3], João Alves[3], Luis S. Ozaki[3], Li-min Wen[3], Maria Ruth Carvalho[3], Myrna G. Serrano[3], Patricio A. Manque[3], Ping Xu[3], Tadeusz A. Zwierzynski[3], Brad Freeman[3], Gregory A. Buck[3], Samuel Goldenberg[1,2], Marco A. Krieger;[1,2]

1 IBMP (Instituto de Biologia Molecular do Paran´ a), Curitiba, Brasil; 2 FIOCRUZ-RJ, Rio de Janeiro, Brasil; 3 VCU (Virginia Commonwealth University), Richmond, VA, USA

Trypanosoma cruzi, the causative agent of Chagas' disease, exhibits complex developmental programs during life cycle transitions in the infected host and insect vector. The infective form circulating in the blood stream of the mammalian host (blood trypomastigote) is originated from the amastigotes and the metacyclic trypomastigotes are originated from the cellular differentiation of epimastigotes in the insect gut. Both trypomastigote forms can infect mammalian cells; however they are adapted to interact with different hosts reinforcing the assumption that these infective forms are indeed different. We are using microarrays to study the gene expression profiles of cardiomyocites infected with the two distinct trypomastigotes in order to evaluate comparatively the host cell response. Eighteen-day murine embryos cardiomyocites were infected with Vero cell culture derived (VERO) and *in vitro* derived metacyclic (TAU) trypomastigotes and samples were taken at 1h, 2h, 4h and 6h. They were hybridized to Genechip® arrays and images were analyzed with the RMA package (Bioconductor project).

Differential expression was assessed using an F-test with correction to multiple testing (SAM software). The host cell responds quickly to the infection, increasing and decreasing genes in both infection processes but the overall result is that infection leads to more down-regulated genes. Comparison of infected cells showed that some genes are equally regulated in TAU and VERO infection (e.g, beta actin, kinesin). There are few qualitative differences between VERO and TAU infected cells, involving specially immune response genes (chemokines, NFK-α, CSF) and most of the differences are quantitative, as the tendency is the same, but the magnitude is different. These results suggest that TAU and VERO infections have different kinetics related to differences in terms of infection capability in both trypomastigotes forms.

Financial support from PRONEX, CNPq, Fiocruz, NIH

BM50 - SEQUENCE VARIABILITY OF A *T. CRUZI* RNA BINDING PROTEIN AND ITS RECOGNITION BY HUMAN CHAGASIC SERA.

DaRocha, W.D.1, Machado-Silva, A.1, Augusto-Pinto, L.1, Machado, C.R.1, Bilate, A.M.2, Cunha-Neto, E.2 and Teixeira, S.M.R.1.

1- Depto de Bioquímica e Imunologia, ICB/UFMG, Belo Horizonte, MG-Brazil. 2- Instituto do Coração (InCor), USP, São Paulo, SP-Brazil E-mail: santuzat@icb.ufmg.br

We have described a new *T. cruzi* antigen, called TcRBP48, identified by immunoscreening an amastigote cDNA library of the Tulahuen strain with human chagasic sera. TcRBP48 is encoded by a multi-copy gene family and is expressed at similar levels in all life stages of the parasite. Sequence analyses showed that TcRBP48 is the homologue of the *T. brucei* RNA binding proteins p34 and p37, which interact with 5S rRNA. To better characterize the chagasic immune response against this antigen, we expressed and purified this protein as a recombinant GST fusion protein. The GST::TcRBP48 purified fraction tested on ELISA assays showed that it is specifically recognized by human chagasic sera (n=45), but not by non-infected individuals or patients with leishmaniosis (n= 17), toxoplasmosis (n= 10) and malaria (n= 9). Levels of reactivity against TcRBP48 were not statistically different when chagasic sera from patients with severe, moderate and mild chagasic cardiomyopathy as well as asymptomatic patients were compared. Regardless the clinical manifestations, individual patients showed different levels of reactivity with recombinant TcRBP48. In light of the studies showing two or three major lineages in the *T. cruzi* population, we proposed to investigate the existence of sequence variability in the TcRBP48 genes from different *T. cruzi* strains and to test if that may cause different levels of the humoral immune response against this antigen. A 440 bp fragment corresponding to the N-terminal region of the TcRBP48 protein was amplified from the genome of various *T. cruzi* strains. Amplified fragments from 35 strains were submitted to RFLP analyses, and the sequences corresponding to the various copies of the gene present in 10 strains were determined. Comparative sequence analyses showed that the TcRBP48 gene is variable and that the patterns of amino acid substitutions can separate *T. cruzi* strains in three groups. The corresponding nucleotide changes were also detected by RFLP analyses of PCR fragments after digestion with *Hha* I.. Fragments corresponding to the genes present in the Colombiana and 1005 strains were cloned into pGEX vectors and the recombinant GST-fusions of these two isoforms were expressed and purified from *E. coli* cultures. In spite of the amino acid changes, the humoral response against these two isoforms was not different when sera from 13 patients were tested on ELISA assays. Our results suggest that the TcRBP48 is an important target of the humoral immune response in Chagas' disease and may constitute an antigen component that can be used to improve the specificity of serological tests, in spite of its variable sequence composition. This antigen can also be used for typing *T. cruzi* strains by PCR-RFLP.

Supported by grants from WHO and CNPq.

BM51 - CLONAL-HISTOTROPIC MODEL FOR CHAGAS DISEASE: A NEW STUDY STRATEGY BASED ON GENETICALLY MODIFIED *TRYPANOSOMA CRUZI* STRAINS

Pires, S.F. [1], Macedo, A. M.[1], Teixeira, S. M.[1], Da Rocha, W. D. [1], Chiari, E.[2], Pena, S. D. J.[1]

1 Departamento de Bioquímica e Imunologia – Instituto de Ciências Biológicas – UFMG. 2 Departamento de Parasitologia – Instituto de Ciências Biológicas - UFMG

Using LSSP-PCR we have demonstrated the occurrence of differential tissue distribution of distinct of *T. cruzi* populations in mice. For instance, when BALB/c mice were co-infected with *T. cruzi* JG strain and the Col.1.7G2 clone, a clear predominance of the DNA of Col.1.7.G2 was observed in the rectum, diaphragm, esophagus and blood, while JG strain DNA was detected in the heart of these same animals (Andrade et al., 1999). The presence of specific DNA patterns were used an indicators for the presence of parasites in each analyzed tissue. In the present work we developed an alternative strategy based on genetically modified *T. cruzi* (expressing Green Fluorescent Protein – GFP- or Red Fluorescent Protein – RFP) to identify the parasites directly in the infected tissues. Initially we transfected *T. cruzi* epimastigotes with pTREXGFP and pTREXRFP transient expression vectors (Vazquez et al, 1999) and obtained JG strain and Col1.7G2 clone expressing GFP and RFP. However the number of fluorescent parasites decreased with time even under selective drug pressure. Therefore, we utilized the integrative expression vector pROCKNeo (Da Rocha and cols, submitted) that allows the integration of exogeneous genes into the parasite genome by homologous recombination in the b-tubulin locus. Parasites stably expressing GFP and RFP were obtained. The transfected parasites present no significant differences in their infectivity for VERO cells when compared with the wild type populations. Pictures obtained by confocal microscopy showed intense green fluorescence in the intracellular forms of transfected parasites within VERO cells for both JG and Col1.7G2 populations.

Financial Support: CAPES, CNPq, FAPEMIG and WHO

BM52 - GENETIC ANALYSIS OF AN ANIMAL MODEL (*HOLTZMAN* RATS) EXTENSIVELY USED IN THE STUDY OF EXPERIMENTAL CHAGAS DISEASE.

Rodrigues M. A.[1], Franco J. D.[1], Fonseca C. G.[2], Machado C. R. S.[1], Vago A. R.[1]

From the Departamento de Morfologia[1] and Departamento de Biologia Geral[2]; Instituto de Ciências Biológicas (ICB).Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627 – Pampulha, CEP-31270-910, Belo Horizonte, Minas Gerais.

Chagas disease caused by the intracellular parasite *Trypanosoma cruzi*, is an infection of endemic nature in 21 countries of Latin America, with 16 to 18 million infected people. The biological, biochemist and genetic behavior of *T. cruzi* strains have been extensively studied in the experimental model at the Laboratório de Neurobiologia, ICB, UFMG. Three distinct profiles of parasitemia (low, moderate and high), have been observed in *Holtzman* rats inoculated with 10.000 trypomastigotes of CL-Brener clone (isolated from the parental CL strain) ABC and Y strains of the parasite. A recent study, developped in our laboratory investigating the organs distribution and histopatological alterations caused by mixture (10.000 + 10.000, 10.000 + 1.000, 1.000 + 1.000) of two subpopulations of *T.cruzi* (JG and CL-Brener clone) demonstrated that after 120 days of double infection JG strain was found in the heart and organs of the majority of the analyzed animals. Only the group inoculated with 10.000 trypomastigotes of each population showed the presence of CL-Brener clone in the esophagus, solium and diaphragm. The observed histotropism could be related such to the

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genetic variability of the infectant population as to the genetic background of the host. The main aim of this work was to investigate the degree of genetic polymorphism between animals (*Holtzman* rats) which have been used as model to the experimental Chagas disease. The RAPD technique was used for analysing DNA samples isolated from the blood of 20 animals from an "out-bred" colony from the Center of Bioterism of the ICB, UFMG. Six primers of arbitrary sequence were used (OPA 1, 2, 3, 8, 9 and ëGT11), which were able to produce profiles composed by multiple amplicons (5 to 14). The analysis of the profiles obtained by RAPD demonstrated a low degree of genetic variability between the analyzed animals, suggesting that the variability previously observed in the virulence and pathogenicity of subpopulations of *T.cruzi* when inoculated in *Holtzman* rats, seems to be mainly associated with factors related to the strains of the parasite.

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BM53 - PCR DETECTION OF *TRYPANOSOMA CRUZI* KDNA DIRECTLY FROM COLON SAMPLES OBTAINED FROM PATIENTS WITH CHAGASIC MEGACOLON BY USING A SENSITIVE HOT START PROTOCOL

[1]Aguiar, A. F, [2]Adad, S. J., [1]Reis, D. d´A., [3]Oliveira, E. C., [3]Luqueti, A. O., [1]Vago, A. R.

[1]Departamento de Morfologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG; [2]Departamento de Anatomia Patológica Faculdade de Medicina do Triângulo Mineiro, Uberaba, MG; [3]Laboratório de Doença de Chagas, Universidade Federal de Goiás, Goiânia, Brazil.

The pathogenesis of Chagas disease is not completly understood. Due to the difficulty in detecting parasite in tissue samples from chronic chagasic patients the role of *T. cruzy* on the development of tissue lesions observed at chronic phase was considered controversial by many years. However, recent studies in heart and esophageal tissues obtained from chronic chagasic patients using more sensitive techniques as PCR, have shown a correlation between the detection of parasite DNA and the presence of inflamation. Chagasic megacolon is clinically characterized by symptoms as obstipation, dyskinesia, meteorism and achalasia of the internal anal sphincter. The main macroscopic alterations of megacolon are dilatation and hipertrofy of the organ. Microscopically this process is characterized by presence of inflammatory infiltrates, degeneration of myocites and nervous cells. Studies on the examination of colon samples from patients with megacolon by histologycal techniques have failed to demonstrate a close correlation between the megacolon pathogenesis and the presence of *T. cruzi*. At the present study we have analysed colon samples surgically obtained from 20 chagasic patients with megacolon belonging to two distinct groups: the first group consisted of samples obtained from 11 individuals provenient from an endemic region of Goiás state, while the second one, was formed by samples from 3 different anatomic regions (sigmoid-rectum transition, sigmoid´s media portion and descendant transition of sigmoid) of 9 individuals from the region of "Triangulo Mineiro", at Minas Gerais state. By using a hot-start protocol PCR to amplify a 330 bp fragment from the variable kDNA region of parasite we detected *T. cruzi* kDNA in samples from 13 patients (65%) of the two groups. In the second group, kDNA parasite was detected in 7 of 9 patients analysed, and interestingly, from the 7 positive cases, 5 demonstrated kDNA T. cruzi only in the sigmoid-rectum transition.

Financial suport: FAPEMIG and CNPq.

BM54 - MOLECULAR TYPING OF *TRYPANOSOMA CRUZI* DIRECTLY FROM TISSUES OF CHRONIC PATIENTS WITH CHAGASIC MEGACOLON BY USING LSSP-PCR

[1]Vago, A. R., [1]Reis, D. d´A., [2]Adad, S. J., [3]Oliveira, E. C., [3]Luqueti, A. O., [1]Aguiar, A. F.

[1]Departamento de Morfologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG; [2]Departamento de Anatomia Patológica Faculdade de Medicina do Triângulo Mineiro, Uberaba, MG; [3]Laboratório de Doença de Chagas, Universidade Federal de Goiás, Goiânia, Brazil.

Chagas'disease has a variable clinical course, ranging from symptomless infection to severe chronic disease with cardiovascular or gastrointestinal involvment. The factors influencing this clinical variability have not been elucidated, but most likely genetic variation of both the host and parasite are important. Thus, researchs into intraspecific genetic polymorphisms of *T. cruzi* exploring a variability of both nuclear and kinetoplast DNA (kDNA) have failed to demonstrate a significant correlation between the genetic of parasite and the clinical course of the disease. This fact can be in part explained, because these techniques worked with parasites isolated from vectors and from the blood of patients. To unravel the molecular epidemiology of Chagas disease at a fine level, we need to be able to study parasite variability directly from clinical tissues. In this work we used LSSP-PCR (Low-Stringency Single Specific Primer PCR) technique for analysing the kDNA region of the parasite to genetically type *T. cruzy* directly from colon samples obtained from 13 chagasic patients with chagasic megacolon, wichs were proceeding from distinct endemic areas of Ghagas'disease, Goiás and Minas Gerais states. Informative genetic signatures were obtained to each analysed sample and although we cold not demonstrate strong correlation betwen the profiles obtained.

Financial suport: FAPEMIG and CNPq.

BM55 - ANALYSIS OF DIFFERENTIALLY EXPRESSED GENES AMONG *TRYPANOSOMOMA CRUZI* TRIPOMASTIGOTES

Pavoni, D.P.[1]; Probst, C.M.[1]; Poersch, C.O.; Cardoso, J.[1]; Correa, A.[1]; Arauco, P.R.C.; Ávila, A.R.[1]; Monteiro-Góes, V.[1]; Goldenberg, S.[1,2;] Krieger, M.A.[1,2]

[1] Instituto de Biologia Molecular do Paraná, IBMP, R. Prof. Algacyr Munhoz Mader, 3775, CIC, Curitiba – PR, 81.350-010, Brasil. [2] Fundação Oswaldo Cruz, FIOCRUZ, Manguinhos, Rio de Janeiro - RJ, 21.045-900, Brasil

The differentiation of epimastigotes into metacyclic trypomastigotes (metacyclogenesis) involves the transformation of a replicative, non-infectious form of *Trypanosoma cruzi* into a non-replicative, infectious one. The functional and morphological changes occurring during this process result from important changes in the parasite gene expression program. To improve our knowledge about *T. cruzi* differentiation and particularly, of the metacyclogenesis process, we have done a systematic analysis of differentially expressed genes using microarray technology. The *T. cruzi* microarray utilized was made at our laboratory, using EST sequences (from public databases and from an internal transcriptome project). The newest version of the biochip has more than 5,000 different contigs in triplicate in a 17K spots biochip. The major technical problem we face is that some cellular forms yield tiny amounts of RNA, insufficient to perform our experiments. In order to bypass this limitation, we used a method to amplify the RNA. The amplification was made linearly, using a T7 promoter to transcribe the cDNA derived from the RNA. The promoter was linked to an oligo-dT primer; consequently the RNAs generated were complementary to the original RNA (antisense RNA – aRNA). Our results demonstrated that this RNA amplification protocol did not introduce distortions that could compromise the analysis of the

microarrays. Hence, *in vitro* transcription is an adequate way to amplify the RNA of *T. cruzi*. RNA samples, extracted from *T. cruzi* epimastigotes and metacyclic trypomastigotes, were analyzed in hybridization experiments performed on the microarray slide. With this method we were able to select several metacyclic trypomastigote expressed genes. In addition we are also starting the characterization of the gene expression programs in both trypomastigotes (Metacyclic trypomastigotes and Cell Derived trypomastigotes) in order to select common and specific sets of genes.

Financial support from PRONEX, CNPq, Fiocruz

BM56 - SELECTION AND FUNCTIONAL CARACTERIZATION OF EXPRESSION REGULATED GENES OF DIFFERENTIATING EPIMASTIGOTES DURING THE METACYCLOGENESIS PROCESS

Giese, V.L.[1]; Dallagiovanna, B.[1]; Ávila, A.R.[1]; Monteiro-Góes, VS[1]; Probst, C.M.[1]; Pavoni, D.P.[1]; Krieger, M.A.[1,2] & Goldenberg, S.[1,2].

1- Instituto de Biologia Molecular do Paraná – IBMP – Rua Professor Algacyr Munhoz Mader, número 3775, 81350-010 Curitiba –PR, Brasil; 2- Fundação Oswaldo Cruz- FIOCRUZ- Manguinhos, 21045-900 Rio de Janeiro - RJ, Brasil

The differentiation of *T. cruzi* non-infective forms (epimastigotes) into infective forms (metacyclic trypomastigotes) is known as metacyclogenesis. This process is of great interest because it involves differential gene expression associated with virulence of the parasite to the mammalian hosts. The development of reproducible axenic culture conditions has made possible the study of this process *in vitro*. Differentiating epimastigotes adhere to a substrate and are released from it upon transformation into metacyclic trypomastigotes.

In order to gain further insight into the mechanism of gene expression regulation in *T. cruzi*, we are using micro-array analysis to identify genes specifically expressed in the course of metacyclogenesis process. The regulation of gene expression Trypanosomatids has unusual mechanisms as compared to other eukaryotes. The transcription in Trypanosomatids is polycistronic and it is assumed that most of the regulation of mRNA levels is determined at the post-transcriptional level. We have used a *T.cruzi* home-made micro-array in order to compare, using a competitive hybridization assay, total and polysomal RNA fractions isolated from parasites at 24h of differentiation. Eight ESTs which are differentially mobilized to the polysomal RNA fraction at 24h of differentiation were selected to further analysis. Three strategies were used to obtain the complete sequences of these 8 ESTs. Seven sequences were homologous to sequences already deposited in the GeneBank and two of them are being studied in further details, encoding a calpain-like protein and Sec-31 protein. We are presently identifying the coding sequences of these genes in *T.cruzi* Dm28c in order to express the recombinant proteins and to raise specific polyclonal antibodies. The purified recombinant proteins and the antibodies will be use in biochemical and microscopy analysis to investigate the function of these genes during *T. cruzi* metacyclogenesis.

Financial support from PADCT, PRONEX and CNPq.

BM57 - DIFFERENTIAL GENE EXPRESSION DURING THE ONSET OF *TRYPANOSOMA CRUZI* METACYCLOGENESIS.

Sotomaior, VS[1]; Dallagiovanna, B[1]; Monteiro-Góes, VS[1]; Giese, VL[1]; Nardelli, SC[1]; Stange, F[1]; Santos, LBR[1]; Pavoni, DP[1]; Picchi, G[1]; Probst, CM[1]; Goldenberg, S[12]; Krieger, MA[12].

[1] Instituto de Biologia Molecular do Paraná – IBMP – Rua Professor Algacyr Munhoz Mader, número 3775, 81350-010 Curitiba - PR, Brasil; [2] Fundação Oswaldo Cruz – FIOCRUZ – Manguinhos, 21.045-900 Rio de Janeiro - RJ, Brasil

The mechanisms involved in triggering metacyclogenesis which is driven by remarkable changes in gene expression remain unclear. *T. cruzi* metacyclogenesis can be mimicked in vitro by using chemically defined conditions that allows the selection of parasite samples at distinct time-points during the differentiation process. To address this issue mRNA differential display and *T. cruzi* DNA microarray were used to select genes differentially expressed during the onset of the metacyclogenesis process, which consists of a 2hrs nutritional stress prior to incubation in the differentiating medium (TAU3AAG). The mRNA differential display experiments resulted, after cluster analysis in 5 contigs and 8 singletons whereas the competitive hybridization experiments unraveled at least a hundred differentially expressed sequences. Thirty of these genes were selected for further characterization and were used as probes to screen a shotgun library. Newly derived sequences were compared with gene bank databases searching for similarities. It was possible to indicate putative function to 18 of these 30 selected genes. For instance, one of the sequences selected by mRNA differential display as a stress-specific gene is similar to a SWI/SNF related, matrix associated, actin dependent regulator of chromatin (gb|AAK53826.1). Another example is a gene whit expression increased during nutritional stress and is similar to a probable ubiquitin-conjugating enzyme variant from *Leishmania major* (emb|CAC14533.1). The expression patterns of the selected genes are being confirmed by Northern-blot and Real Time RT-PCR. The approach here reported will be useful in unraveling the genes that are differentially expressed during the onset of metacyclogenisis.

Financial support from PRONEX, CNPq.FIOCRUZ

BM58 - ANALYSIS OF GENE EXPRESSION CHANGES IN RESPONSE TO DIFFERENT STRESSES: SELECTION OF GENES INVOLVED IN TRIGGERING *TRYPANOSOMA CRUZI* METACYCLOGENESIS.

Monteiro-Góes, V.[1]; Sotomaior, V.S.[1]; Correa, A.D.; Santos, L.B.R.[1]; Probst, C.M.[1]; Pavoni, D.P.[1]; Goldenberg, S.[1,2]; Krieger, M.A.[1,2]

[1] Instituto de Biologia Molecular do Paraná, IBMP, R. Prof. Algacyr Munhoz Mader, 3775, CIC, Curitiba – PR, 81.350-010, Brasil; [2] Fundação Oswaldo Cruz, FIOCRUZ, Manguinhos, Rio de Janeiro - RJ, 21.045-900, Brasil

The differentiation of epimastigotes into metacyclic trypomastigotes (metacyclogenesis) involves the transformation of a replicative, non-infectious form of *Trypanosoma cruzi* into a non-replicative, infectious stage. An essential event in metacyclogenesis is the nutritional stress at which the parasites are naturally exposed in the mid-gut of the insect host. Important alterations in the gene expression program occur during this process and they might play an important role in the physiological and morphological changes observed during the metacyclogenesis. However, little is known about the ability of other types of stresses in triggering metacyclogenesis and about the genes involved in the regulation of these responses. Our approach consists in the comparative analysis of different stress conditions in triggering metacyclogenesis *in vitro* and the analysis of the expressed genes using microarray technology. We are now selecting sets of genes specifically expressed for each kind of stress. The different stress modalities were temperature increase, pH decrease and cell population density increase. Preliminary results of the comparison among the different stresses showed that temperature and pH stress present a greater efficiency than the nutritional stress in promoting parasite differentiation.

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BM59 - CHARACTERIZATION AND GENE PROFILING OF *TRYPANOSOMA CRUZI* AMASTIGOTES OBTAINED *IN VITRO*.

Correa, A. [1]; Ávila, A.R.1; Manhães, L. [1]; Motta, M.C.M. [1,2*]; Pavoni, D. [1]; Probst, C.1; Cardoso, J. [1]; Claure Arauco, P.R. [1]; Corrêa-da-Silva, M.S. [2]; Contreras, V.T. Krieger, M.A. [1,4]; Goldenberg, S[1,2,4]

[1] Instituto de Biologia Molecular do Paraná, Curitiba, Paraná, Brasil; [2] Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, UFRJ, Brasil; [3] Laboratorio de Protozoología, Centro BioMolP, Facultad de Ciencias de la Salud, Universidade de Carabobo, Bárbula, Valencia, Venezuela; [4] Fundação Oswaldo Cruz, FIOCRUZ, Manguinhos, Rio de Janeiro - RJ, 21.045-900, Brasil

Trypanosoma cruzi life cycle involves at least three distinct developmental stages: epimastigotes, trypomastigotes and amastigotes. The epimastigote forms replicate in the midgut of the insect host and develop into non-replicative metacyclic trypomastigote forms by the process of metacyclogenesis. Metacyclic trypomastigote differentiates, within the host cells, into the replicative amastigote form (amastigogenesis), which in turn differentiates into the infective bloodstream trypomastigotes. Amastigogenesis remains poorly understood; however, the development of culture media and appropriate growth conditions has allowed mimicking most of the life cycle *in vitro* paving the way to a better understanding of this process. Nowadays, *in vitro* culturing conditions render possible obtaining metacyclic trypomastigotes from epimastigotes and amastigotes from metacyclic trypomastigotes.

This work focuses on determining: first, if the amastigotes obtained *in vitro* are comparable to amastigotes obtained *in vivo* or from infected cell culture, and second, which genes are specifically expressed by amastigotes.

To accomplish the first goal, amastigotes obtained *in vitro* have been examined by electron microscopy. The ultrastructural analysis of amastigogenesis in axenic conditions, after 72 h of induction, showed that most of the parasites presented the amastigote form and many cells were actively dividing. Transmission electron microscopy showed that most parasites presented a barrel-shaped kinetoplast, which is characteristic of amastigotes and the nucleus presented a less compact chromatin displaying an evident nucleolus. We are currently characterizing this form by molecular methods, including western blot analyses using the antiserum against the amastigote specific antigen Ssp4.

To address the second goal, homemade DNA microarrays containing approximately 5000 ORFs are being used to identify amastigote either specific or non-specific genes. Firstly, competitive hybridization will be performed to compare the two replicative forms of the parasite life cycle, namely amastigotes and epimastigotes. Secondly, an *in vitro* time course experiment will be carried out starting from epimastigotes up to amastigotes, passing through the metacyclic trypomastigote form. This approach will allow us to characterize each developmental stage with a catalogue of stage-specific expressed genes and, to look for developmental regulators that could be putative targets for developing better ways to control *T. cruzi* and Chagas disease.

Supported by: PRONEX, CNPq, FIOCRUZ, FAPERJ and FUJB.

BM60 - A MEMBER OF THE WD40 GENE FAMILY IS DIFFERENTIALLY EXPRESSED IN *T. CRUZI* METACYCLIC TRYPOMASTIGOTES.

Nardelli, S.C.[1], Reis, A.[1], Ávila, A.[1], Dallagiovanna, B.[1], [1,2] Krieger, M.A. [1,2] Goldenberg,S. [1,2]

1- Instituto de Biologia Molecular do Paraná – IBMP – Rua Professor Algacyr Munhoz Mader, número 3775, 81350-010 Curitiba – PR, Brasil; 2- Fundação Oswaldo Cruz- FIOCRUZ- Manguinhos, 21045-900 Rio de Janeiro - RJ, Brasil

Metacyclic trypomastigotes are the infective forms of *Trypanosoma cruzi*. These forms are found in the posterior intestine of the invertebrate host and are released through the insect excreta during feeding in the vertebrate host. Once in the blood stream of the mammalian host they can infect different cells. The study of genes specifically expressed during this stage is important in order to identify genes involved in the infection process and that might act as virulence factors. In addition, the studies might lead to the identification of targets to be used in future attempts of rational drug design against Chagas' disease. Using the technique of Differential Display we have isolated a fragment of 901 nucleotides, which was named C20. Northern blot analysis showed that this gene is specifically expressed by metacyclic forms. The sequence of C20 showed significant homology to sequences present in gene data bases, encoding proteins that display the conserved domain WD40. Proteins of this family have essential roles in several cellular processes such as signal transduction, apoptosis and ribosomal RNA processing, among others. The complete sequence of this gene in *T. cruzi* Dm28c was obtained (2.247 nucleotides) with an open reading frame of 1.335 nucleotides. In order to express this protein in *E.coli* the complete coding sequence was cloned in the expression vector pQE31. The recombinant protein fused to a histidine-tag was purified by affinity chromatography and rabbits have been immunized to obtain a specific polyclonal antibody.

Financial support from PADCT, PRONEX and CNPq.

BM61 - EVALUATION USING MICROARRAY TECHNOLOGY OF POLYSOMAL MOBILIZATION IN GENE EXPRESSION REGULATION DURING THE *T. CRUZI* METACYCLOGENESIS

Avila, A. [1], Pavoni, D. P.[1,] Monteiro-Góes, V.[1], Probst, C. M.[1], Dallagiovanna, B.[1], Correa, A. C.[1], Claure Arauco, P. R.[1] Cardoso, J.[1], Picchi, G. [1], Sotomaior, V.[1], Reis, A. C. dos [1], Bitencur, L.[1], Stange, F.[1], Nardelli, S. [1], Fragoso, S. P[1], Nunes, L.[2], Oliveira, R. C.[2], Alves, J. [3], Ibachi, N. [3], Wen, L. [3], Manque, P. A.[3], Ozaki, L. S.[3], Serrano, M. G.[3], Puiu, D. [3], Mallonee, D. [3], Xu, P. [3], Zwierzynski, T.A.[3], Carvalho, M. R.[3], Buck, G. A.[3], Krieger, M.A.[1], Goldenberg, S.[1]

1. Instituto de Biologia Molecular do Parana, Curitiba-Parana, Brasil; 2. Universidade de Mogi das Cruzes, Mogi das Cruzes, Brasil; 3. Virginia Commonwealth University, Richmond, VA, USA

The regulation of gene expression in trypanosomatids is exerted mainly at the post-transcriptional level. The fact that most trypanosomatid mRNAs result from processing of polycistronic transcripts suggests that stage specific genes must have their expression regulated either through selective transport to the cytoplasm, or through specific degradation/stabilization pathways or, alternatively, through selection of the sequences to be translated in the polysomes. In the case of *Trypanosoma cruzi*, modulation of gene expression is of great importance because the parasite faces different environments (hosts) during its life-cycle, alternating replicative and non-infective stages with non-replicative and infective stages. The development of a chemically defined medium that supports *T. cruzi* metacyclogenesis has rendered possible obtaining parasites at various stages of the differentiation process. In order to gain further insight into the mechanisms involved in *T. cruzi* gene expression regulation, we have constructed a *T. cruzi* biochip and compared, total cytoplasmic and polysomal RNAs from parasites at various time points of the differentiation process. The results indicate that specific sets of mRNAs are mobilized to the polysomes whereas other mRNA sequences remain in the cytoplasm in a translation repressed form. These results were further corroborated by northern blot and quantitative RT-PCR analysis.

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BM62 - MOLECULAR CHARACTERIZATION OF GENES INVOLVED IN CAMP SIGNAL TRANSDUCTION PATHWAY IN *TRYPANOSOMA CRUZI*

Marchini, F.K. [1*]; Yamada-Ogatta, S.F.Y. [2]; Goldenberg, S.[1,3]; Krieger, M.A. [1,3]

[1]Instituto de Biologia Molecular do Paraná. Rua Algacyr Munhoz Mader, 3775, CIC, Curitiba, Paraná, Brasil. 81.350-010. [2] Departamento de Microbiologia, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, Campus Universitário Londrina, Londrina, PR, Brasil. [3] Fundação Oswaldo Cruz, FIOCRUZ, Manguinhos, Rio de Janeiro - RJ, 21.045-900, Brasil

The cellular differentiation from *Trypanosoma cruzi* epimastigotes to metacyclic trypomastigotes as well as the characterization of some genes and factors involved in this process, have been studied by our group on the last twenty years, including the role of cAMP as an important secondary messenger in inducing metacyclogenesis. To select and clone genes expressed at early stages of the metacyclogenesis process, we have performed the RDE (Representation of Differential Expression) technique comparing mRNA extracted from parasites at 24 h of the differentiation process with epimastigote mRNA. One gene selected using RDE was similar to a regulatory subunit of cAMP (Cyclic 3'-5' Adenosine Monophosphate) dependent protein kinase (PKA). The holoenzyme occurs naturally as a 4-membered quaternary structure, with two regulatory (R) and two catalytic (C) subunits. We are presently searching genes that encode the other subunits of PKA to be able to perform the molecular characterization of this important component of cAMP signal transduction pathway. The involvement of this pathway in metacyclogenesis has been studied by expression analysis of the different genes using microarray and real time RT-PCR methods. In addition we are making specific antibodies against recombinant proteins to corroborate the expression analysis including the characterization of the cellular localization of its components.

Financial support: PRONEX, PADCT/CNPq, Fiocruz.

BM63 - DIFFERENTIAL GENE EXPRESSION IN *TRYPANOSOMA CRUZI* STRAINS

Dost[1], C.K.; Saraiva[2], J.; Monesi[2], N.; Engels[1], W.; Albuquerque[2], S.; Zentgraf[1], U.;

[1]University of Tübingen, General Genetics, Germany; [2]University of Sao Paulo, FCFRP Ribeirão Preto, Brazil

Trypanosoma cruzi is not a homogeneous population but is rather composed by a pool of strains which circulate in both the domestic and sylvatic cycles involving humans, vectors and animal reservoirs of the parasite. Studies of isolated *T. cruzi* populations from different origins demonstrated the presence of a large range of strains with distinct characteristics. This intriguingly intraspecific variation has been extensively investigated by characterizing the morphology of blood forms, parasitemic curves, virulence, tissue tropism, pathogenicity and sensitivity to drugs. These phenotypic differences between strains of the same species are determined by differential gene expression. At least two distinct groups of *T. cruzi* were identified by a number of molecular markers and named group I and II. The differential expression of genes in these groups was analyzed by suppression subtractive hybridization (SSH). Two *T. cruzi* strains, TA and Bol-SB belonging to group I and II, respectively, were cultured in LIT-Medium and, after 3 passages, RNA from epimastigotes was extracted at the log phase. The cDNA was synthesized from mRNA and SSH Analysis was performed using the cDNA subtraction kit (BD Clontech Laboratories). Two reactions, were performed, the forward reaction with TA strain as tester and Bol-SB as driver, and the reverse reaction. Subtracted cDNA fragments from both reactions were directly cloned and 40 clones were sequenced. Sequence alignments and homology searches identified several cDNA clones that should be differentially expressed in these two *T. cruzi* strains. A couple of clones have been identified which did not reveal homology with any known *T. cruzi* gene but shared similarity with either *Leishmania, Plasmodium* or *T. brucei* genes. According to the *T. cruzi* genome project, so far the gene products of several genes are still unknown and may lead to the characterization of new proteins with interesting functions. Experiments are currently under way in order to analyze whether the genes identified from SSH analysis are also differentially expressed in other *T. cruzi* strains, and whether there is a correlation of the expression pattern with parasitemic curves, pathogenicity, morphology and virulence.

BM64 - PRELIMINARY CHARACTERIZATION OF TRANSCRIPTS ABUNDANT IN AMASTIGOTE FORMS OF *LEISHMANIA (L.) MAJOR*

Rama, E.M.; Ruiz, J.C.; Tosi, L.R. and Cruz, A.K.

Faculdade de Medicina de Ribeirão Preto – USP. Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos. Av. Bandeirantes 3900, Monte Alegre, Ribeirão Preto, SP, Brasil, CEP: 14049-900. akcruz@fmrp.usp.br

Previous work conducted in our laboratory (Iribar, PhD thesis, 2001) to characterize a *Leishmania (L.) major* gene with uncommon features led to a preliminary study of its genomic region. This analysis revealed two transcripts present in higher levels in amastigotes when compared to promastigotes. Our aim is the characterization of both transcripts, named EB4.4 and EE3.4, present in a 4.4 kb *Eco*RI-*Bgl*II and in a 3.4 kb *Eco*RI-*Eco*RI genomic fragments, respectively. A subclone from EB4.4 has been generated (pUCEB4.4) and sequenced and it bears two putative genes; a class I Fructose-biphosphate aldolase and a RNA-dependent helicase (Rev.Inst.Med.Trop.S.Paulo 44-suppl.12, 2002). We are currently characterizing the second clone, pUCEE3.4. It has been fully sequenced using a transposon-based strategy (Tosi & Beverley, NAR, 28:784, 2000). The transposon GFPKAN-mariner was used for primer-island sequencing. This process was successful and the entire fragment was sequenced to completion. The assembly and generation of the consensus sequence was accomplished with Phred/Phrap/Consed (Wilson et al, Nature, 368: 32, 1994). The 'in silico' analysis of the sequence data revealed that EE3.4 is 3441bp long, its AT content is 40.5%; and two Open Reading Frames (ORFs) were predicted to be present and coded by the same strand. The similarity search in public databank (http://www.ncbi.nlm.nih.gov) indicates that one of the ORFs from EE3.4 did not match any previously deposited known gene and the second ORF seems to be a ribosomal protein coding gene. The levels of transcripts present in pUCEB4.4 and pUCEE3.4 are being analysed in Northern experiments to determine which of them are expressed preferentially in amastigotes. These experiments will lead to further functional analyses that will involve overexpression of individual genes in the parasite and the use of insertion mutagenesis. The insertion of a selectable marker for *Leishmania* within one ORF from pUCEB4.4 or pUCEE3.4 will allow the overexpression of their second putative gene in the parasite. The generation of mutagenized versions of both recombinants is underway using a transposition system (NeoKO-mariner).

Supported by FAPESP.

BM65 - IDENTIFICATION AND ANALYSIS OF *LEISHMANIA* EXPRESSED SEQUENCE TAGS CHARACTERISTIC OF NON-CODING RNAS

Márcia A. S. Graminha, Karina Nogueira, Simone Antoniazi, Angela Kaysel Cruz

Faculdade de Medicina de Ribeirão Preto – USP, Ribeirão Preto, SP, BR. Address correspondence to: akcruz@fmrp.usp.br

Sequencing of the *Leishmania* genome has led to the identification of genes based on the predicted proteins they encode. However, sequences that lack open reading frames and encode RNAs as their final product might be overlooked during the annotation process. Accumulating evidence indicates that such non-coding RNAs (ncRNAs) can play critical roles in gene expression regulation as microRNAs (miRNAs) described in many organisms from worm to man. We are currently investigating five putative *Leishmania* ncRNAs genes whose secondary structure presents hairpin-like motif resembling those found in miRNAs. For instance, *ODD1* has a conserved hairpin in its 3´ region that presents homology to an *Arabidopsis thaliana* precursor miRNA.

Northern analyses were carried out to investigate whether these transcripts have a regulatory function. Total RNA extracted from several *Leishmania* species, in different developmental stages, was blotted on to nylon membranes and probed with antisense oligonucleotides based on the hairpin structures found in genes *ODD2* and *ODD3*. *ODD2* showed a hybridization pattern that could be related to the detection of its precursor and the processed transcript. *ODD3* presented a more complex pattern of hybridization that varied according to the species tested. Parasite transfectants carrying cosmids that contain either *ODD2* or *ODD3* were subjected to increasing levels of Hygromicin B (up to 160mg/ml) in order to overexpress these genes. Northern analyses of these transfectants suggested that at least one of the transcripts detected by *ODD2* could be a RNA target. Also, the transfectant overexpressing *ODD3* presented a cytostatic phenotype and morphological differences when compared to the parental strain. These are preliminar data and work on progress is focused in understanding the functional role of these genes.

BM66 - COHESINS AND CONDENSINS: STUDIES ON THE STRUCTURAL MAINTENANCE OF THE CHROMOSOME COMPLEXES OF *TRYPANOSOMA CRUZI*

Shimada, M. K.[1], Picchi, G. F. A.[1], Lourenço, E. E.[1], Araújo, F. R.[3], Krieger, M. A.[1,2], Fragoso, S. P.[1,2] & Goldenberg, S.[1,2]

(1) Instituto de Biologia Molecular do Paraná – IBMP – Curitiba, Paraná, Brasil. E-mail: shimada@tecpar.br. (2) Instituto Oswaldo Cruz – FIOCRUZ – Rio de Janeiro, Rio de Janeiro, Brasil. (3) Empresa Brasileira de Pesquisa Agropecuária/ CNPGC – Campo Grande, Mato Grosso do Sul, Brasil.

The factors involved in the chromosome organization, spacial distribution and condensation during the *T. cruzi* life cycle are unknown. Distinctly from the most eukaryotic cells, the chromosomes of *T. cruzi* do not condense at mitosis and are difficult to be distinguished. Moreover, little is known about the factors that play a role in the maintenance of the different levels of chromatin condensation observed along the life cycle of *T. cruzi*. Chromatin condensation may be an important mechanism for the regulation of gene expression in this parasite, since the chromatin in a highly condensed state may reduce the levels of RNA transcription, as observed in the metacyclic and bloodstream trypomastigote stages. In order to shed light into these issues we have initiated the characterization of *T. cruzi* genes encoding for cohesin and condensin complexes, that are involved in the structural maintenance of the chromosomes (SMC) in other eukaryotic cells. Cohesin is a complex necessary to maintain sister chromatid cohesion from chromosome duplication until the onset of anaphase. This protein complex contains four core subunits, two SMC subunits (SMC1 and 3) and two non-SMC proteins (Scc1/Mcd1/RAD21 and Scc3/SA). By the other hand, the condensin complex is required for the establishment and maintenance of chromosome condensation. The holocomplex of condensin is composed of two SMC subunits (SMC2/CAP-E and SMC4/CAP-C) and three non-SMC subunits (CAPD2, CAP-G and CAP-H). We have characterized nine genes of *T. cruzi* encoding for putative cohesins and condensins, which were collectively named *T. cruzi* SMC genes.The search for homology showed that the deduced amino acid sequences from *T. cruzi* SMC genes share about 45% of similarity with their eukariotic counterparts. Antisera were raised in mice either against peptides synthesized from the deduced amino acid sequences of *T. cruzi* SMC proteins or recombinant *T. cruzi* SMC proteins expressed in *E. coli*, in order to immunolocalize and study the expression pattern of these proteins through *T. cruzi* life cycle. In addition, we have launched the analysis of the expression of these genes by Northern blot and the study of their organization by PFGE and Southern blot.

Financial support: PRONEX, CNPq

BM67 - TELOMERE AND SUBTELOMERE OF *TRYPANOSOMA CRUZI* CHROMOSOME ARE ENRICHED IN PSEUDOGENES AND RETROTRANSPOSON-LIKE ELEMENTS ASSOCIATED TO HOT-SPOTS OF RECOMBINATION

1. Kim, D.H.[1], Chiurillo, M.A.[2,3], Santos, M.R.M.[1], Cerda, P.P.[1], Franco da Silveira, J.[1], Ramírez, J.L[2,3].

1. Departamento de Microbiologia, Imunologia e Parasitologia, Escola Paulista de Medicina UNIFESP, Rua Botucatu, 862, CEP 04023-062, S. Paulo, Brazil. (Email: dong@ecb.epm.br); 2. Laboratorio de Genetica Molecular, Instituto de Biologia Experimental Universidad Central de Venezuela; 3. Centro de Biotecnologia, Instituto de Estudios Avanzados (IDEA), Caracas, Venezuela

The sequencing of a ~30 kb telomeric recombinant cosmid (C6) (Chiurillo et al., *Mol. Biochem. Parasitol*. 100:173, 1999) from the pathogen protozoan *Trypanosoma cruzi* provided us with useful landmarks to establish the general features of telomeres and subtelomeres of this parasite. Our findings can be summarized as follows: we confirmed the presence of hexameric repeats and a 189-bp species specific junction at the chromosomal end; subtelomeric region appeared to be enriched in retrotransposon-like sequences and pseudogenes of the TS (Trans-sialidase)-like family; and an open reading frame corresponding to putative surface protein DGF-1. *T. cruzi* subtelomeric region also contains sequences related to the RHS (Retrotransposon Hot Spot) multigene family of *T. brucei* which present a hot spot for non-LTR retrotransposon insertion. Analysis of sequences related to Ts-like family encoding surface proteins suggests that the chromosomal ends could have been the site of generation of new GP85 variants, an important adhesin molecule involved in the invasion of mammalian cells by trypomastigote forms.

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BM68 - CHARACTERIZATION OF A NOVEL SUBTELOMERIC REPETITIVE ELEMENT FROM *LEISHMANIA*.

Silva, A.M.; Pedrosa A.L.; Ruiz, J.C. and Cruz, A.K.

Faculdade de Medicina de Ribeirão Preto – USP, Departamento de Biologia Celular e Molecular, Ribeirão Preto, São Paulo, Brasil. amdsilva@rbp.fmrp.usp.br

Telomeres are the extreme regions of chromosome ends and are crucial for cell stability and viability. The telomeric and subtelomeric regions contain repetitive elements potentially involved in events of recombination between non-homologous chromosomes. Sequencing of one end of *Leishmania major* chromosome 20 revealed the presence of a novel repetitive element, named LST-R378 (Pedrosa et al, MBP 114: 71, 2001). PCR and Southern blot analyses were performed and revealed the presence of LST-R378 in the genomic DNA from different *Leishmania* species from Old and New World. Curiously, a region of 81 nucleotides from LST-R378 is 83% identical to a fragment of a P-type

ATPase gene from *L. (L.) donovani*. Amplification reactions revealed a potential polymorphism of LST-R378 in different species; in a *L. (L.) mexicana* strain a single band of about 400bp is amplified, in *L. (L.) amazonensis* two fragments were observed and in different strains of *L. (V.) braziliensis* size-polymorphic fragments were amplified. The amplification reaction conducted on *L. (V.) braziliensis* 2904 genomic DNA revealed three fragments ranging from 300 to 600 nucleotides, all of them contain the same ATPase fragment as shown by their complete sequences. *In silico* search for the intact ATPase coding gene found it on chromosome 16. Its genomic region is currently being recovered to study its flanking sequences and to investigate the presence of a repetitive element similar to LST-R378.

Supported by FAPESP.

BM69 - HOMOLOGUES OF RPA-1 AND RBP38 ARE THE PROTEIN COMPONENTS OF TWO *LEISHMANIA (LEISHMANIA) AMAZONENSIS* G-RICH TELOMERIC (LATG) COMPLEXES

Fernández, M.F.[1], Castellari, R.R. [2], Novello, J.C. [2], Pinheiro, Hildete [3] & Cano, M.I.N. [1]

Departamento de Genética e Evolução[1], Laboratório de Química de Proteínas (LAQUIP), Departamento de Bioquímica[2], Instituto de Biologia, Departamento de Estatística, Instituto de Matemática e Estatística[3], Universidade Estadual de Campinas (UNICAMP), Campinas, SP, Brazil.

Until the present report there were no descriptions of the components of telomeric chromatin in *Leishmania* spp. Species of this genus are the ethiological agents of leishmaniasis, a worldwide spread disease of significant medical and economic importance. The chromosomal ends of the Brazilian pathogen *Leishmania* (*L.*) *amazonensis* contain conserved TTAGGG telomeric repeated sequences. Protein com-plexes that associate to these sequences (LaTG1, LaTG2 and LaTG3) were identified and characterized by gel shift assay and UV cross-linking using anion exchange purified fractions from S100 and nuclear extracts. Different physical-chemical properties of the complexes were determined. All complexes are formed at 4 ^{o}C, although LaTG2 and LaTG3 are also formed at temperatures up to 20 ^{o}C. All three complexes are stable at high salt concentrations. Size-estimation of the protein-forming complexes was achieved by in situ UV cross-linking. These proteins do not interact with double-stranded DNA and the C-rich telomeric strand, but they can associate with an RNA oligonucleotide cognate to the G-rich telomeric sequence. All three complexes were not formed i) in competition assays using specific telomeric oligonucleotides and ii) when the extracts were pre-treated with proteinase K, indicating that they are protein-forming complexes. The protein components of LaTG2 and LaTG3 were purified by affinity chromatography using a 5´-biotinilated G-rich telomeric oligonucleotide and were eluted by KCl step gradient. The column fractions were separated by SDS-PAGE and Coomassie stained to determine their protein contents. The estimated dissociation constants (K_d) for LaTG2 and LaTG3 complexed to DNA and RNA sequences are in nM range. After purification and renaturation experiments they were identified as the ~35 kDa and the ~52 kDa protein bands, respectively. These purified protein bands were in gel digested with trypsin and the resulting peptides were subjected to MALDI-TOF MS fingerprinting mass spectrometry. The peptide fingerprint analysis showed that the ~52 kDa component of LaTG2 is similar to the subunit 1 of the conserved single-stranded binding protein, replication protein A (RPA-1) and the ~35 kDa protein component of LaTG3 is a homologue of the conserved trypanosomatid RBP38 RNA binding protein.

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BM70 - GENOMIC ORGANIZATION OF TELOMERIC AND SUBTELOMERIC SEQUENCES (TAS – "TELOMERE ASSOCIATED SEQUENCES") OF *LEISHMANIA (LEISHMANIA) AMAZONENSIS*

Conte, F. F. & Cano, M. I. N.

Instituto de Biologia - Universidade Estadual de Campinas (UNICAMP), São Paulo, Brazil. micano@unicamp.br

Telomeres are protein-DNA complexes that protect linear chromosomes from degradation and end fusions ensuring genomic stability. The telomeric repetitive sequence is guanine rich and protrudes towards the end of the chromosome as a 3'-G overhang structure.

Sequences adjacent to telomeres constitute the subtelomeric region, which, in *Leishmania* spp., was characterized as "*Leishmania* Conserved Telomere Associated Sequences" (LCTAS) arranged in blocks of 100 bp flanqued by telomeric sequences. Different from *Trypanosoma brucei, Trypanosoma cruzi* and *Plasmodium falciparum*, LCTAS do not present genes encoding surface antigens. In contrast, they contain two conserved sequence blocks (CSB 1 and CSB 2) that show different organization and copy number in almost all species of the genus *Leishmania* studied so far. Due to the high degree of sequence conservation, it is suggested that LCTAS play an important role in chromosome segregation and that they could harbor binding sites for telomeric proteins.

The aim of this work is to study the genomic organization of the *Leishmania (L.) amazonensis* telomeric/subtelomeric sequences and identify proteins that specifically recognize them. In this report, we hybridized digested genomic DNA from *L. (L.) amazonensis* promastigotes with a G-rich telomeric sequence and with CSB 1 and CSB 2 sequences as probes. Kinetic experiments using *Bal*31 exonuclease were used to differentiate the telomeric fragments from the interstitial internal ones and to estimate the size of *L. (L.) amazonensis* telomeres. In addition, we used Pulsed Field Gel Electrophoresis (PFGE) in first and second dimensions to map and study the organization of telomeres and LCTAS in the parasite chromosome context.

The results of Southern blotting and *Bal*31 helped us to estimate that the Terminal Restriction Fragment (TRF) of *L. (L.) amazonensis* is approximately 7 Kb long and that the mean size of parasite telomeres is ~3 Kb. CSB 1 and CSB 2 appeared as blocks of 0.3-0.4 Kb, flanqued by *Hae*III restriction sites. They are present in lower copy number when compared to the telomeric sequences, although the hybridization signal with CSB 2 was stronger than with CSB 1, suggesting that *L. (L.) amazonensis* LCTAS are mainly composed by CSB 2 blocks. To confirm the above results, we hybridized *L. (L.) amazonensis* chromosomes separated by PFGE. Under our running conditions, all chromosomes of *L. (L.) amazonensis* were fractionated in a unique gel. They appeared as 25 bands, with molecular sizes ranging from 0.35 - $\geq$ 3.0 Mb, which were stained by ethidium bromide and hybridize with the telomeric sequence, confirming that they are linear chromosomes. Using second dimension PFGE, we demonstrated that *L. (L.) amazonensis* chromosomes present telomeres with different sizes, suggesting that as the same as in other eukaryotes, telomere replication in *Leishmania* is regulated at the chromosome level. We are currently analyzing telomeric/subtelomeric sequences cloned from purified chromosomes in order to study these regions in detail.

Financial support: FAPESP, WHO/TDR – UNDP Bank.

BM71 - GENOMIC ORGANIZATION AND TRANSCRIPTION OF A *TRYPANOSOMA CRUZI* REPEATED ELEMENT

Renata T Souza, Márcia RM Santos, José Franco da Silveira.

Depto Micro, Imuno e Parasitologia, Universidade Federal de São Paulo-UNIFESP/EPM. Rua Botucatu, 862 –cep 04023-062 São Paulo-SP Brasil (E-mail:torres@ecb.epm.br)

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We describe here further characterization of a new *T. cruzi* repeated element of ~1.5 kb named Z25. Most of Z25 element are flanked by *Eco*RI sites, and they are composed by a ~65 bp region similar to the mucin-like pseudogene followed by a ~591 bp region that shares similarity with sequences previously described in the intergenic spacer of the genes for pyrimidine biosynthesis. The 3' end (~844 bp region) shows several repetitions of 54 bp and 55 bp arranged like beads-on-a-string. In spite of the variation in the number of these small repeats (54- and 55 bp) the whole element is highly conserved. Partial digestion of *T. cruzi* genomic DNA with *Eco*RI and hybridization with selected probes indicated that most of Z25 elements are arranged in tandem arrays which are located in six chromosomal bands. To further confirm this hypothesis we did Southern blot analysis of *T. cruzi* chromosomes separated by two dimensional pulsed field gel electrophoresis after partial digestion with *Eco*RI. There is an average of 4-6 *EcoRI* fragments per chromosomal band, indicating that Z25 are arranged in tandem arrays. Northern blot analysis showed that sequences found in Z25 share homology with 1.5 kb and 0.24 kb transcripts are expressed in epimastigotes. The 1.5-kb transcript was only found in the poly A+ RNA fraction, whereas the 0.24-kb transcript was also detected in the poly A- fraction. Nucleotide sequence identity search of the GenBank database revealed a high percentage identity of Z25 with 15 ESTs (Expressed Tag Sequences), confirming that this repeated element is transcribed. Taken together, our results indicate that Z25 is organized in tandem arrays located at specific chromosomal regions, and it is expressed as oligo(A)-terminated transcripts. Further characterization of structural and functional features of Z25 repeated elements is underway in our laboratory.

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BM72 - FISHING *LEISHMANIA (L.) AMAZONENSIS* PROTEINS USING THE TELOMERIC DNA AS A BAIT

Lira, C.B.B.[1], Siqueira Neto, J.L.[1], Camillo, L.R.[2], Ramos, C.H[2]. and Cano, M.I.N.[1]

[1] Departamento de Genética e Evolução, IB, UNICAMP, Campinas, SP; [2] Laboratório Nacional de Luz Síncrotron, Campinas, SP, Brazil. liracbb@yahoo.com.br

Telomeres are specialized nucleoprotein complexes localized at the ends of linear chromosomes. They function as a cap at the chromosome end terminus and protect them from exonucleolytic degradation and end-to-end fusions, conferring genome stability and normal cellular proliferation. Telomeric DNA is composed of tandem short repeats of G-rich sequences. The G-rich strand extends beyond the complementary C-rich strand forming a single-stranded protrusion or a 3'G-overhang, which is the substrate for telomerase replication. Proteins found at telomeres can associate to the double strand or single-strand telomeric DNA forming a high order complex at the end of the chromosome. They function as positive or negative regulators of telomere maintenance and mutation or deletions of any of these protein factors can alter telomere function leading to gross effects in cell life maintenance.

Leishmania spp. telomeres are composed by the conserved TTAGGG sequence, which is maintained by telomerase. Proteins that associate to the G-rich telomeric strand were identified in affinity purified extracts of *L. (L.) amazonensis* (Fernández et al., submitted). However, there are no descriptions of proteins that associate with the double-stranded *Leishmania* spp. telomeres. Yeast one-hybrid system was chosen to screen *L. (L.) amazonensis* telomeric proteins. To conduct the assay, two integrative vectors containing the telomeric bait cloned upstream of a reporter gene were constructed: i) the pLacZi-LaTel vector, containing *lacZ* reporter gene and ii) the pHISi-1-LaTel vector containing *HIS3* reporter gene. These two vectors were used to built three different reporter yeast strains. HisTel and LacTel containing respectively pHISi-1-LaTel and pLacZi-LaTel vectors integrated in the genome. And LacHisTel, that bears both integrative vectors. *L. (L.) amazonensis* logarithmic phase promastigotes mRNA was reverse transcribed using a mixture of random and oligo dT primers generating cDNA molecules ranging from 0.4-2.0 Kb. To construct a non-normalized library the cDNA was cloned in pGAD424 expression vector, which allows the proteins to be expressed in fusion with GAL4p activation domain. The expression of *L. (L.) amazonensis* telomeric proteins activate the reporter gene transcription providing the genetic selection of positive clones using selective medium or β-galactosidase assay.

The first attempt to identify *L. (L.) amazonensis* telomeric proteins by one-hybrid system was performed with the double reporter yeast strain LacHisTel, which resulted in the selection of 13 clones from 600 possible candidates. The clones already sequenced showed any similarity with known telomeric proteins, suggesting the existence of novel *Leishmania* telomeric proteins. To confirm the above results we are currently sequencing other clones and performing the screening of new libraries.

Supported by FAPESP and WHO/TDR – UNDP Bank.

BM73 - SPECIFICITY OF A TRANSPOSON-BASED TOOL FOR GENE IDENTIFICATION IN *LEISHMANIA*

Augusto, M.J., Dias, F.C. and Tosi, L.R.O.

Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. Ribeirão Preto, SP – Brasil

The completion of genome sequencing projects stimulates the definition of new strategies aimed at the systematic analysis of the information that is being accumulated. The transposon technology has proven to be a powerful tool in tagging and analyzing genes of many organisms in a systematic fashion. In many cases transposons can be modified, controlled and used in the characterization of gene expression.

The in vitro mobilization of the Mos1 transposon, isolated from the *Drosophila mauritiana* genome, led to the development of protocols tailored for the characterization of genes of the protozoan parasite *Leishmania*. In a previous report we showed that fusion proteins generated between parasite genes and reporter genes carried in transposons can be efficiently detected within the parasite cell. The detection of reporter expression was based on the use of an immunofluorescence microscopy protocol. Here we report the study of the efficiency of the element /NEO*SAT to trap *Leishmania* genes. The specificity of the trapping system was confirmed by the fact that the Neomycin Phosphotransferase (NPT) gene is expressed within the parasite only if inserted into the open reading frame of a given gene. Random insertions of the /NEO*SAT transposon into genomic libraries were characterized by *primer island* sequencing and insertions of interest were identified. Selected events bearing the reporter gene inserted in-frame into annotated genes were transfected into the parasite. Out-of-frame insertions and intergenic events were also selected and used as negative controls. Since all constructs were cloned into the shuttle vector pELHYG, transfectants were selected in hygromycin B. Transfectants bearing in-frame insertions were able to grow in the presence of G418. Those transfectants carrying intergenic or out-of-frame insertions could not be selected for the expression of NPT. This was the first indication of the specificity of the trapping tool. Immunodetection of NPT in transfectant cells confirmed that the reporter gene introduced by the transposon insertion was expressed only as part of a fusion protein.

Two other specialized transposons, carrying the b-glucuronidase (GUS) and the Green Fluorescent Protein (GFP) genes as reporters, were also constructed and tested. In the elements /GUS*SAT and /GFP*NEO the reporter genes do not contain an ATG start codon and will be expressed only if part of a fusion protein. The expression of GUS and/or GFP is screened rather than selected for. This characteristic, and the possibility of detection of reporter expression in live

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cells, is a major advantage of these elements as gene trapping tools. Work in progress is focused on defining strategies for genome-wide implementation of the established protocols.

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BM74 - SYSTEMATIC DISRUPTION OF GENES AT A *LEISHMANIA* CHROMOSOME END.

Squina, F.M.; Pedrosa, A. L.; Cruz, A. K. Tosi, L. R. O.

Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. Ribeirão Preto, SP – Brasil. Email:luiztosi@fmrp.usp.br

The development of transposon technology and its ability to destroy endogenous phenotypes allow a global perspective on genome expression in a systematic manner. Such strategy is especially appealing when dealing with the protozoan parasite *Leishmania,* whose genome-sequencing project is approaching completion. However, the presence of repetitive elements at chromosomal extremities hampers the assembly and annotation of sequence while restricting the implementation of strategies aimed at the study of genes located therein.

Here we report the use of a specialized mariner transposon adequate not only as the source of reagents for precise gene disruption protocols, but also for the sequencing of repetitive elements. The extremity of the 580 Kb chromosome of *L. (L.) major* LV39 was assembled through mapping of two cosmid clones named 008B2 and 056G8, isolated from a genomic library. Southern analysis of PFGE-separated chromosomes associated these clones to chromosome 7. Southern hybridization revealed the presence of repetitive sequences common to both clones, but only clone 008B2 presented the typical telomere-associated hexameric repeats. The restriction map of these clones revealed an overlapping region of approximately 12 Kb. Clones 008B2 and 056G8 cover 60 Kb of the chromosome end.

Clone 056G8 was restricted with *Bam*HI/*Bgl*II and further subcloned into the shuttle vector pELHYG. Two of the recombinant plasmids, carrying 15 Kb and 10 Kb, were subjected random insertions of the mariner element ELNEOKO. This specialized transposon contains a selectable marker preceded by the protozoan splicing acceptor sites and a prokaryotic promoter sequence. *Primer-island* sequencing of these insertion events and *in silico* analysis of the data generated revealed the presence of two putative ABC-transporters genes. Clones carrying the element ELNEOKO inserted within a 5.2 Kb ABC transporter-like gene were identified and isolated. The plasmid DNA of a chosen clone was digested to produce a linear fragment to be used in a gene disruption protocol. The fragment was transfected into the parasite and integration events were selected in media containing G418. The homologous recombination of the selectable marker carried by the transposon, and flanked by the target sequences, was confirmed by Southern analyses of PFGE-separated chromosomes and digested genomic DNA. The clone 008B2 was used as target for transposition of the element /GFP*KAN. This transposon contains the Green Fluorescent Protein (GFP) as a reporter for translational fusion events. *Primer-island* sequencing of transposition events into 008B2 enabled not only the characterization of telomeric and sub-telomeric repetitive elements, but also the establishment of a higher resolution map of this peculiar genome structure. The strategy presented here is a valuable tool for systematic disruption and/or discovery of genes, and an important source of reagents for comparative and functional studies of *Leishmania* genome.

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BM75 - FUNCTIONAL STUDIES OF THE TCRHO1 GTPASE OF *TRYPANOSOMA CRUZI*: CONSTRUCTION OF MUTANTS (POSITIVE AND NEGATIVE DOMINANTS) AND RNA INTERFERENCE ASSAYS.

De Melo, L.D.B.[1], Nepomuceno-Silva, JL.[1], Eisele, N.[1], De Souza, W.[2], Cunha-e-Silva, N.L.[2], Lopes, U.G.[1]

1Laboratório de Parasitologia Molecular, Instituto de Biofísica Carlos Chagas Filho, CCS, UFRJ, Rio de Janeiro. 2Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, CCS, UFRJ, Rio de Janeiro. E-mail: dione@biof.ufrj.br

Rho family proteins are members of the Ras superfamily of small GTPases. In higher eukaryotes, these proteins play critical roles in the cell mobility, phagocytosis, intracellular transport, cell adhesion and maintenance of cell morphology. Other cellular processes under Rho family control are the regulation of activity of the NADPH oxidase complex, progression of the G1 phase of cell cycle, transcription regulation and metastasis induced by different oncogenes. The TcRho1 gene identified in our previous work is the only Rho family member in *Trypanosoma cruzi.* This GTPase presents a C-terminus motif (CQLF), which is targeted to farnesylation by a *T. cruzi* farnesyltransferase. A *T. cruzi* cell line expressing a mutant TcRho1-ÄCaaX (a protein unable to be farnesylated) demonstrated poor metacyclogenesis levels in TAU-3AAG medium. To proceed with the functional characterization, we carried out site-directed mutagenesis to obtain the G15V and Q76L positive dominants (constitutive active form) and the T20D negative dominant (constitutive inactive form), which were transfected in *T. cruzi* and selected on agarose plates. Growth curve revealed a significative decrease on proliferation of the TcRho1-T20D cell line, with poor adhesion ability during metacyclogenesis process. Currently, all cell lines are under ultrastructure investigation by electron microscopy. Other functional approach to study TcRho1 function is the RNA interference. A fragment sense-loop-antisense of TcRho1 was subcloned in the pTEX and pRIBOTEX vectors to produce a dsRNA in vivo and parasites transfected with these constructions are presently under selection for posterior phenotypics studies.

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BM76 - DIFFERENTIAL EXPRESSION OF GENES IN *TRYPANOSOMA CRUZI* TREATED WITH THE BENZNIDAZOLE DRUG

Triana-Chávez O[1], Escolar F[2], Zapata-Madrigal C[1], del Mazo J[2]

1. Grupo de Chagas, Instituto de Biología, Universidad de Antioquia, AA 1226, Medellín – Colombia. otriana@matematicas.udea.edu.co. 2. Grupo de Gametogenesis, Consejo Superior de Investigaciones Científicas, Madrid - España

The Chagas disease is one of the parasitic diseases more broadly distributed in America and it affects among 16-18 millions of people from different regions of the continent. In Colombia, it is calculated that 1.3 million people are infected and 3.6 millions are in risk of contracting the infection.

In the last years, numerous studies have been carried out about the biochemistry and physiology of the *T. cruzi*. However, the treatment of the infection taken place by the parasite is considered as one of the more unsatisfactory and the advances in the chemotherapy in order to control the Chagas disease have been very few. The treatment is based on two old and unspecific medications, nifurtimox and benznidazole. The necessity exists of looking for new chemotherapeutic targets and effective drugs against the different forms of *T. cruzi*, and with less deleterious effects than those of classic medications.

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In this work, as an approach, an analysis is shown of expression of genes when a Colombian stock of *T. cruzi* is treated with benznidazole, in comparison to the genes expressed in the stock control without treatment. Epimastigotes of *T. cruzi* were treated with the drug from 25 ug/ml to 500 ug/ml with the purpose of determining the DL_{50}. The RNA total of treated parasites and the controls were isolated, and the RNA messenger was purified with a primer poly T. The cDNAs of the control stock were cloned in the vector l ZAP and evaluated by the technique of hybridisation subtractive, with cDNA of the treated parasites and non treated ones, with the purpose of identifying genes that are expressed differentially when the parasite is exposed to the drug. The differentially expressed clones were isolated, amplified by PCR and subjected to a second screening. The doubly positive clones were sequenced and compared in the database. A great number of genes has been isolated, some of them corresponded to well-known genes, as it is the case of the histone H1, and other genes to be studied. In a future, knowing the sensibility of the stocks of Colombia to the antichagas drugs, is interesting to the identification of genes that are expressed differentially in stocks with sensitive and resistant phenotype.

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BM77 - ISOLATION OF CDNA GENES OF AMASTIGOTE SURFACE PROTEIN-2 EXPRESSED IN THE SYLVIO X10 CLONE 4 STRAIN OF *TRYPANOSOMA CRUZI*.

Claser C, Boscardin SB and Rodrigues MM

Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo-Escola Paulista de Medicina, Rua Botucatu 862 6A, São Paulo, SP, Brazil, 04023-062. E-mail: carlaclaser@ecb.epm.br

Recently, independent groups studied the immunogenic properties of plasmids containing genes encoding the Amastigote Surface Protein-2 (ASP-2). Genetic immunization with *asp-2* genes generated immune responses mediated by antibodies, $CD4^+$ and $CD8^+$ T cells. Most relevant, DNA-vaccinated mice displayed remarkable protective immunity, surviving lethal infection with *T. cruzi* (Boscardin *et al*., 2003, Infect. Immun. 71:2744, Fralish & Tarleton, 2003, Vaccine 21:3070). To gain further information on the sequence polymorphism of ASP-2 genes, we isolated and characterized genes encoding members of this protein family expressed in the Sylvio X10 clone 4 strain of *T. cruzi*. RNA purified from intra-cellular amastigotes was used as template for reverse transcriptase reaction in the presence of oligo-dt primers. The cDNA was used as target for PCR in the presence of primers specific for the previously described ASP-2 gene. PCR products of ~2.1 Kb were obtained from cDNA of amastigotes and cloned into the pMOS vector. Several clones containing the ~2.1 kb inserts were analyzed by enzymatic restriction. We found that the amastigote cDNA contained at least 4 groups of genes. When compared to the previously described genes of ASP-2, their restriction patterns were completely distinct indicating a clear polymorphism. We are currently sequencing theses genes to determine their predicted amino acid sequences.

Supported by FAPESP.

BM78 - *LEISHMANIA (L.) AMAZONENSIS*: MOLECULAR AND IMMUNOLOGICAL ANALYSIS OF A CDNA LIBRARY FROM AMASTIGOTES AND MAPPING OF EXPRESSED SEQUENCE TAGS (ESTS) IN CHROMOSOMAL BANDS.

Girotto, L.G., Lasakosvitsch, F., Silveira, J.F., Santos, M.R.M. & Barbiéri, C.L.

Disciplina de Parasitologia, Escola Paulista de Medicina – Universidade Federal de São Paulo, São Paulo, SP, Brasil.

The present work describes the construction of a cDNA expression library from *Leishmania (L.) amazonensis* amastigotes, as well as the generation and characterization of expressed sequence tags (ESTs).

The randomly analysis of 322 clones showed that all of them reacted with rabbit immune serum against *L. (L.) amazonensis* amastigotes, demonstrating the reliability of the *L. (L.) amazonensis* cDNA library. Among the isolated clones, 100 were sequenced generating new *L. (L.) amazonensis* ESTs from which 53% are not related to any other sequences in databases, whereas 47% presented significant similarities to known genes: 3.2% showed similarity to *L. (L.) amazonensis* genes, 23.4% to *L. (L.) major* chromosomal sequences, and 19% to genes from other trypanosomatids. The chromosome mapping of *L. (L.) amazonensis* resolved by pulsed field gel electrophoresis (PFGE) of 9 ESTs and 2 cysteine proteinase genes was used to study the chromosomal polymorphism between *L. (L.) amazonensis* and *L. (L.) major*. Results from these experiments indicate a possible rearrangement in chromosome 19 of *L. (L.) major*, since two clones from *L. (L.) amazonensis* which present similarity to cosmid L4766 mapped in two different chromosomes of *L. (L.) amazonensis*, one of 1250 kb (*LlaAmEST*0087) and another of 720 kb (*Llacys1* gene). On the other hand, the ESTs hybridizing in a single band may be used as a chromosome-specific marker in the determination of the molecular karyotype of the *L. (L.) amazonensis* LEM690 strain.

The ESTs characterization is useful for discovery of new genes and for the physical mapping of genomes.

Supported by CNPq and FAPESP.

BM79 - GENE SURVEY OF *EIMERIA* SPP. OF DOMESTIC FOWL USING OPEN READING FRAME ESTS (ORESTES)

Solai, C.[1]; Fernandez, S.[1]; Melo, J.C.M.[1]; Nagao, L.T.[1]; Sobreira, T.J.P.[1]; Ahagon, P.H.[1]; Durham, A.M.[2]; Gruber A.[1] & Madeira, A.M.B.N.[1]

[1]Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo. Av. Prof. Orlando Marques de Paiva 87, São Paulo SP, 05508-000, Brazil. [2]Instituto de Matemática e Estatística, USP. E-mail: albackx@usp.br

Coccidiosis of the domestic fowl is an important enteric disease caused by seven different protozoan species of the genus *Eimeria*. The most studied species, *E. tenella*, presents a genome of circa 60 million base pairs distributed in 14 chromosomes. Sequencing initiatives are being carried out, including an EST project (Washington University - USA) and a 5-fold genome coverage (Institute for Animal Health and Sanger Institute, UK). As a member of the *Eimeria* Genome Consortium (http://www.sanger.ac.uk/Projects/E_tenella/consortium.shtml), our laboratory has initiated an alternative EST sequencing project using the open reading frame expressed sequences tags (ORESTES) approach. ORESTES reads are produced by low-stringency RT-PCR of mRNAs and are biased towards the central region of the transcripts (Dias-Neto *et al.*, PNAS 97: 3491-3496, 2000). The primary aim of our study was the generation of at least 10,000 reads of each one of the three most important *Eimeria* species: *E. tenella*, *E. acervulina* and *E. maxima*. We have obtained so far a total of 7,741 high-quality reads from sporozoites and second-generation merozoites of *E. tenella* H, 10,175 reads from *E. acervulina* H sporozoites and sporulated oocysts, and 1,851 reads from *E. maxima* H sporulated oocysts. Clustering analyses resulted in 2,382, 2,233 and 549 distinct events, respectively. When the *E. tenella* reads were clustered together with 26,955 conventional 5' ESTs already deposited on NCBI, a total of 5,926 clusters were obtained. Comparing to clustering data restricted to the NCBI subset (4,571 clusters), this data suggests that our set of reads contributed with the discovery of 1,355 new putative transcripts (61% of our clusters represented novel sequences). This result is in agreement with the assumption that ORESTES-generated data is complementary to the information obtained by conventional ESTs. Preliminary annotation revealed that sporozoites and merozoites express very distinct transcript profiles. Future perspectives include

ORESTES generation and sequencing for other developmental stages of each species, plus a comprehensive annotation aiming at identifying new potential candidates for vaccine development.

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BM80 - *TRYPANOSOMA CRUZI:* USE OF PERMEABLE CELLS AS MODEL OF STUDY THE ACTION OF DRUGS IN THE PROCESSING OF THE MESSENGER RNAS"

BARBOSA,C.F., CICARELLI,R.M.B., CHUNG, M.C.

Faculdade de Ciências Farmacêuticas, Departamento de Ciências Biológicas, Universidade Estadual Paulista, Rodovia Araraquara-Jaú Km 01, Araraquara 14801-902, SP, Brasil

Pre-mRNA maturation in Trypanosomatids occurs through a processs called trans-splicing, which involves excision of introns and union of exons in two independent transcripts; a short transcript spliced leader (SL RNA) is trans-spliced to the acceptor pre-mRNA, originating the mature mRNA. In this work we have used permeable cells from epimastigote forms of *Trypanosoma cruzi* (Y, NCS and Bolivia strains) as a model for study of drug interference in trans-splicing reaction. The cells were treated with lysolecithin and hydroxymethyl-nitrofuran, a nitrofuran-derived drug which can inhibit trypanotione reductase, an important enzyme in the metabolism of *T. cruzi*. The reciprocal drug nitrofural had its activity observed in different concentrations, showing itself a sensible reduction in the RNA processing concentration of 8mM. After newly-formed RNA extraction, they were hybridized with the SL RNA antisense sequence as a probe followed by RNAse treatment to localize SL exon and intron formation to demonstrate that trans-splicing reaction occurred.

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BM81 - IDENTIFICATION OF CANDITATE GENES WITH HEMOLITIC FUNCTION IN *TRICHOMONAS VAGINALIS*

Carvalho, S.E.; Freitas, D.M.; Feijó, G.C.; Silva, J.X.; Simões-Barbosa, A.*

Catholic University of Brasilia, Post Graduation Program in Genomic Sciences and Biotechnology, SGAN 916, 70910-160, Brasília DF, Brazil. E-mail: sbarbosa@ucb.br

Trichomonas vaginalis, a flagellate parasitic protozoan, is the etiologic agent of trichomoniasis. The pathogenesis includes events such as hemolysis, cytoadhesion and secretion of proteases, causing epithelial injuries with some pathological consequences in the geniturinary tract of its host. Hemolysis is also an important source of nutrients for the protozoan. Some molecules are not synthesized *de novo* by the parasite. In particular, the microelement iron controls the metabolic activities of the parasite as well as regulation of some genes related to virulence. The hemolytic process must therefore be characterized. The objective of this study was to identify candidate genes with hemolytic function in a cDNA library of *T. vaginalis*. Messenger RNA was purified from fresh clinical isolates, grown in Diamond growth media. A cDNA library was developed from transcripts orientated for transient expression in *Escherichia coli* under control of the *lac* promoter. Additionally, functional screenings were performed in total of 5 x 10^4 clones of the cDNA library. This functional screening was based on the formation of bright halos surrounding the bacterial colonies after induction with a layer of IPTG/blood/agar. Six distinct hemolytic clones were selected based upon this screening procedure. Their sequences show that the parasite has different genes with hemolytic activity, with transcript length varying from 0.4kb to 1.5kb. Bioinformatic analysis based on homologies, domains and possible functions showed that the parasite may use different pathways to induce hemolysis in the host.

BM82 - MAPPING AND CHARACTERISATION OF THE *LEISHMANIA (L.) AMAZONENSIS* GENOMIC REGION CONTAINING THE META 1 GENE: COMPARATIVE STUDY WITH THE *L. (L.) MAJOR* GENOME

Franco FAL, Ramos CS and Uliana SRB

Instituto de Ciências Biomédicas, Depto. de Parasitologia, Universidade de São Paulo. Av. Prof. Lineu Prestes, 1374, São Paulo, SP, Brazil, CEP 05508-900. srbulian@icb.usp.br

The metacyclic upregulated meta 1 gene is conserved in both Old and New Wold *Leishmania* species. Overexpression of the meta 1 protein in *L. (L.) amazonensis* generates parasites that are more virulent *in vivo,* suggesting an important function for this protein during infection (Uliana et al, Exp. Parasitol. 92:183-191,1999). Previous efforts to characterise other transcripts derived from the region surrounding the meta 1 gene in *L. (L.) amazonensis* allowed the description of a meta 1-related, metacyclic upregulated gene named meta 2 (see Ramos and Uliana, this meeting).

Three other fragments of the same genomic region identified as complementary to cDNAs prepared from metacyclic stage parasites were characterized. The first fragment, 5 kb in length, was derived from the 5' region of the meta 2 gene and hybridized to 2 transcripts: a 1,5 kb transcript upregulated in metacyclic promastigotes and an unregulated 1 kb RNA. The analysis of the nucleotide sequence of this clone enabled the identification of two ORFs and a possible coding strand switch region. The translated sequence of one of this ORFs is similar to the "VsrD" protein of *Pseudomonas solanacearum,* a virulence related, transcription regulator protein.

The second fragment, located between meta 1 and meta 2 genes, identified a 2,2 kb transcript predominantly expressed in promastigotes and encoded a putative protein without significant similarities to proteins previously described.

The last clone, obtained from a fragment located 3' to the meta 1 gene, identified a 2 kb transcript upregulated in amastigotes. The nucleotide sequence of this fragment indicates the presence of an ORF similar to the NOD3 human protein, involved in immune reactions.

The contig composed by all these *L. amazonensis* gene fragments was aligned with a *L. (L.) major* contig (*L. (L.) major* Genome Project) revealing a high degree of similarity. Regions containing ORFs, as expected, are more conserved, with sequence similarities ranging from 87 to 92%, while intergenic/untranslated regions are more heterogeneous, with sequence similarities ranging from 58,66 to 87,02%.

Supported by FAPESP.

BM83 - CHARACTERIZATION OF THE ABC TRANSPORTER GENE *PRP1* RELATED TO PENTAMIDINE RESISTANCE IN *LEISHMANIA (L.) MAJOR*

Coelho, A. C. [1], Beverley, S. M. [2], Cotrim, P. C. [1]

[1] Instituto de Medicina Tropical, USP, Av. Dr. Enéas de Carvalho Aguiar, 470, 4º andar, São Paulo – SP, 05403-900, Brasil. [2] Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110.

Pentamidine (PEN) is a second-line agent in the treatment of leishmaniasis whose mode of action and resistance is not well understood. Here we used a genetic strategy to search for genes able to mediate PEN resistance when overexpressed in *Leishmania (L.) major*. A shuttle cosmid library containing

genomic DNA inserts was transfected into wild type promastigotes and screened for PEN-resistant transfectants. Two different cosmids identifying the same locus were found, which differed from other known *Leishmania* drug resistance genes. The PEN resistance gene was mapped by deletion and transposons mutagenesis to a protein belonging to the P-glycoprotein/MRP ABC transporter superfamily that we named PRP1 (Pentamidine Resistance Protein 1). The predicted PRP1 protein encodes 1,807 amino acids with the typical dimeric structure involving 10 transmembrane domains and two nucleotide-binding domains. PRP1 mediated PEN resistance could be reversed by verapamil and PRP1 overexpressors showed cross-resistance to trivalent antimony but not to pentavalent antimony (glucantime). Although PEN resistance was modest (1.7-3.7 fold), this may be potentially significant in clinical drug resistance given the marginal efficacy of PEN against *Leishmania*.

This research is sponsored by: CNPq, FAPESP, LIM-48 and TDR-WHO.

BM84 - MULTILOCUS GENOTYPE DATA PROVIDE EVIDENCES THAT HYBRID *TRYPANOSOMA CRUZI* STRAINS CONSTITUTE A THIRD MAJOR LINEAGE

Luiz Augusto-Pinto, Juliana R. Pimenta, Santuza M. R. Teixeira, Octavio Fernandes, Égler Chiari, Sérgio D. J. Pena, Andréa Mara Macedo and Carlos Renato Machado

Universidade Federal de Minas Gerais,Rua Antônio Carlos 6627, Departamento de Bioquímica ICB, Pampulha, Belo Horizonte, Minas Gerais, Brasil, CEP:31270-901. augusto@icb.ufmg.br

Several molecular markers corroborate the existence of two major lineages for *Trypanosoma cruzi*, denominated *T. cruzi* I and *T. cruzi* II, related to zymodeme 1 and 2, respectively. However, a number of strains presenting hybrid characteristics cannot be classified into any of these two lineages. The major goal of this work was to ascertain the evolutionary relationship of these hybrid strains, by performing a multilocus analysis in 98 *T. cruzi* isolates. Two nuclear genes, one mitochondrial gene and five microsatellite repeats were chosen for this analysis. Maximum parsimony phylogenetic inferences based on microsatellite genotypes showed a good correlation with the 24Sa rRNA dimorphism, but revealed the existence of a third major cluster that included all hybrid strains studied. Likewise, single-nucleotide polymorphism of *TcMSH2* gene and RFLP analyses of the mitochondrial *COII* gene support the existence of three major lineages (haplotypes A, B and C) with all strains displaying hybrid characteristics being classified into *MSH2* haplogroups B and C or the mitochondrial clade B. The hybrid nature of these strains were confirmed by median-joining network analyses for the microsatellites loci. In the light of these results, we discuss new insights into the evolutionary origin of hybrid strains of *T. cruzi* and propose that these strains may constitute a third major lineage in the population.

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BM85 - CHARACTERIZATION OF A ZINC FINGER PROTEIN IN *TRYPANOSOMA CRUZI*

Ericsson, A.R.O.; Martins de Sá, C. & Lima, B.D.

Departamento de Biologia Celular, Universidade de Brasília, Brasília, DF, 70910-900. E-mail: ericsson@unb.br

Zinc fingers are compact protein domains composed of an a-helix and a b-sheet held together by a zinc ion. Tandem arrays of zinc fingers are commonly used to recognize nucleic acids. Among other activities, they are involved in the processes of replication, transcription, and DNA repair. The nucleocapsid protein of HIV-1 contains a zinc finger motif $CX_2CX_4HX_4C$ that contributes to multiple steps of the viral life cycle, including the proper encapsidation of HIV RNA. In trypanosomatids, only a few of the proteins that contain such fingers are studied. In *Leishmania chagasi*, a protein containing nine zinc finger motifs $CX_2CX_4HX_4C$ was identified. This protein, called HEXBP, binds to the 5'unstranlated region of the most abundant membrane glycoprotein of this protozoan and is likely to be involved in DNA replication, structure and repair. In *Trypanosoma cruzi*, a protein containing five zinc finger motifs $CX_2CX_4HX_4C$ was identified. This protein, called TcZinc1 or PDZ5, is homologous to the *Crithidia fasciculata* UMSBP protein that is probably involved in minicircle replication. Here, we report the identification and characterization of a protein in *Trypanosoma cruzi* containing eight zinc finger motifs $CX_2CX_4HX_4C$, denominated TcZinc2. Molecular cloning of the *Tczinc2* gene and heterologous expression of TcZinc2 as a fusion with an His-tag was performed in *E. coli*. The recombinant protein TcZinc2 was purified by immobilization in metal affinity chromatography Ni-NTA (Nickel-nitrilotriacetic acid) and used for antibody production in rabbits. Western blot analysis using total protein extracts of the three forms of the parasite has shown that TcZinc2 is expressed equally in all of them. Western blot experiments using cytoplasmic and nuclear/mitochondrial fractions from epimastigote forms have shown that TcZinc2 is present at the nuclear/mitochondrial fraction. Since the TcZinc2 protein does not have peptide signal for mitochondrial localization, it is probably a nuclear protein. SELEX (Systematic Evolution of Ligands by Exponential enrichment) experiment will be performed in order to determine the nucleic acid nature, as well as the consensus sequence of the target molecule to which this specific protein binds. For that purpose, molecular cloning of the *Tczinc2* gene and heterologous expression of TcZinc 2 as a fusion with GST (Glutathione-S-Transferase) were performed in *E. coli*. The recombinant protein TcZinc2 was purified by affinity chromatography to glutathione. This study will provide valuable information regarding the function of this protein in *T. cruzi*.

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BM86 - TCZFP1: A *TRYPANOSOMA CRUZI* PROTEIN THAT BINDS TO C-RICH SEQUENCES

Mörking P A(1), Foti L(1), Umaki A C(1), Dallagiovanna B(1) (2), Garat B(2), Fragoso S P(1) and Goldenberg S(1)

(1)Instituto de Biologia Molecular do Paraná, Brasil. (2)Laboratorio de Interacciones Moleculares. Universidad de la Republica, Uruguay

The control of mRNA stability plays a fundamental role in the regulation of eukaryotic gene expression. This is particularly relevant in trypanosomes where the organization of the genome into polycistronic transcription units implies that most genes must be regulated at the post transcriptional level. This control can be influenced by the basal mRNA decay machinery, regulatory factors that respond to various stimuli and sequence-specific decay components.

We have cloned a *T. cruzi* gene encoding a zinc finger protein named TcZFP1. TcZFP1 is homologous to the TbZFP1 (67.6%) which was implicated in the regulation of the morphogenesis and differentiation in *T. brucei*. These proteins share the unusual zinc finger motif (CCCH) found in a diverse range of RNA-binding proteins involved in various aspects of the control of cell homeostasis and differentiation. Other proteins bearing this motif are intimately associated with RNA stability, transport or translation and operate at different steps of gene expression regulation. Electrophoretic mobility shift assay (EMSA) showed that TcZFP1 binds specifically to some synthetic oligoribonucleotides containing C-rich sequences. This kind of sequences are present in the untranslated region of several mRNAs of trypanosomatids, raising the possibility that TcZFP1 might interact with RNAs or ribonucleoprotein complexes in the cell via the CCCH domain, regulating the mRNA stability or translation.

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XXX ANNUAL MEETING ON BASIC RESEARCH IN CHAGAS DISEASE - XIX MEETING OF THE BRAZILIAN SOCIETY OF PROTOZOOLOGY - HOTEL GLÓRIA, CAXAMBU, MG, BRASIL - 10-12 NOVEMBER 2003. **Rev. Inst. Med. trop. S. Paulo, 45**(Suppl. 13), November, 2003.

BM87 - THE *META 2* GENE: GENOMIC COMPARISON IN TRYPANOSOMATIDS AND CHARACTERIZATION OF THE META 2 PROTEIN OF *LEISHMANIA (L.) AMAZONENSIS*

Ramos CS and Uliana SRB

Instituto de Ciências Biomédicas II (Depto. Parasitologia) - Universidade de São Paulo. Av. Prof. Lineu Prestes 1374, Cid. Universitária, São Paulo, SP, Brazil, CEP: 05508-900. camila@icb.usp.br

We had previously identified the *meta 2* gene in the region flanking the *L. (L.) amazonensis meta 1* gene. Both are single copy genes upregulated in metacyclic promastigotes and conserved in all *Leishmania* species analyzed to date.

The region of the *L. (L.) amazonensis* genome containing the *meta 1* and *meta 2* genes was compared with orthologous *L. (L.) major*, *Trypanosoma brucei* and *T. cruzi* contigs provided by Genome Projects. Conservation of synteny and linkage between these species was observed in a segment comprising at least five genes. Interestingly, a cluster of eight meta 1 related genes was found in *T. brucei* with five highly conserved copies of the *meta 1* homologue and three copies of the *meta 2* gene. On the other hand, in *T. cruzi* three copies of *meta 1* gene were identified while the *meta 2* gene was not present.

The *meta 2* gene had been structurally characterized and an open reading frame (ORF) encoding a 444 amino acid protein was identified. The transcribed amino acid sequence contains three copies of the META domain, which is defined as a conserved domain present in secreted proteins of bacteria and in the meta 1 protein of *Leishmania*, followed by a sequence similar to calpain-like proteins. The recombinant meta 2 protein, expressed in fusion to the maltose binding protein (MBP), lacks the C-terminal end. We immunized Swiss mice with this protein in order to obtain anti meta-2 polyclonal sera. Immunofluorescence experiments suggest that the meta 2 protein is localized in vacuoles dispersed in the cytoplasm of the cell. These sera will be used in immunobloting experiments against *L. (L.) amazonensis* protein extracts prepared from log and stationary phase promastigotes and amastigotas to investigate the pattern of expression at the protein level.

Functional analysis of the meta 2 protein has been initiated. We are currently preparing constructs to be employed in obtaining meta 2 and meta 2-GFP ovexpressing mutants. These mutants will be characterized by *in vitro* and *in vivo* infection experiments.

Supported by FAPESP.

BM88 - SEQUENCING AND CHARACTERIZATION OF GENES WHICH CODE FOR ANTIGENS FROM *L. (L.) CHAGASI* COMPOSED OF MULTIPLE REPETITIVE DOMAINS.

*Bedor C.N.G, #Pontes-de-Carvalho, L. C., #Oliveira, G. G. S; #Teixeira M. C. A. T; and de *Melo Neto, O. P.

*Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Av. Moraes Rego s/n, Recife PE 50670-420. # Laboratório de Imunologia Celular e Molecular, Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121, Brotas, Salvador BA 40295-001 *e-mail*: chebedor@cpqam.fiocruz.br

The visceral leishmaniasis (VL) is a potentially fatal disease caused by *Leishmania chagasi* in Brazil, responsible for about 3.500 new cases each year. It is present in almost all the Brazilian territory, but it occurs mainly in areas where the health services are scarce. Due to the difficulties in the diagnosis of VL in low income areas and the lack of non toxic treatment, many alternatives are being investigated for the development of simple, economical and non-invasive tests for the early diagnosis of VL or the production of a vaccine against this disease. The identification of new antigens from *L. (L.) chagasi* which could be used as part of such tests/vaccines is a major goal in the *Leishmania* research.

In this report we describe the sequencing and characterization of genes that were previously selected from a *L. (L.) chagasi* amastigote cDNA library in a screening with sera from dogs infected with this parasite. Our results show that of the 6 analyzed clones (LC 9, LC 12, LC 14, LC 16, LC 18 and LC 30), 5 codify for proteins composed of regions that contain multiple repetitive domains, varying in size from 14 to 74 aminoacids. The 6th clone (LC 30) lacks the small repeats, but is nevertheless composed by two identical sequences *in tandem* (over 1000 aminoacids in lenght). Clones LC 9 and LC 12 code for the same protein differing only in the number of repetitive units. The same is observed for clones LC 14 and LC 16. The individual repeats from the LC 9/LC 12, LC 14/LC 16 and LC 18 proteins are unrelated and of different sizes, suggesting that, at least in *L. (L.) chagasi*, proteins with repetitive regions are good inducers of humoral immunity irrespective of their sequences. These antigens therefore could be important components of kits for the diagnostic of visceral leishmaniasis.

This work was supported by BioManguinhos, FINEP and FIOCRUZ.

BM89 - CHARACTERIZATION OF A HIGHLY REPETITIVE ANTIGEN FROM *L. (L.) CHAGASI*

Albuquerque, A. L., Pontes de Carvalho, L. C., * Oliveira G. G. S.* and de Melo Neto, O. P.

Centro de Pesquisas Aggeu Magalhães, Fundação Oswaldo Cruz, Av. Moraes Rego s/n, Recife PE 50670-420. * Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão 121, Brotas, Salvador BA 40295-001. +*e-mail*: ale@cpqam.fiocruz.br

The visceral leishmaniasis (VL) is an endemic parasitic infection which occurs in both tropical and sub-tropical countries. In Brazil it is caused by *Leishmania (L.) chagasi* and it is considered an important cause of morbidity and mortality. This disease still presents great difficulty in its diagnosis and treatment, with the current chemotherapy being highly toxic. Control of LV therefore requires the development of better diagnostic methods as well as alternatives to reduce the number of affected individuals, such as development of a vaccine which could prevent its occurrence in both humans and dogs, its natural reservoir.

In earlier work we identified by immunoscreening of a *L. (L.) chagasi* amastigote cDNA library several clones coding for proteins containing long stretches of tandem repeats. One of these proteins, named LC 14, codes for 22 copies of a 14 amino-acids (aas) long repeat followed by a unique 230 aas C-terminus. A homologue from *L. (L.) major* has also been identified containing over 100 copies of the 14 aas repeat plus a very similar C-terminus. Here we start the functional characterization of this protein in *L. chagasi* by subcloning its cDNA in a prokaryotic expression vector (pRSET), expression of the recombinant protein in *Escherichia coli* fused to a N-terminus His-tag and production of specific rabbit polyclonal antiserum. The recombinant protein transcribed and translated *in vitro* as well as the bacterially expressed protein behaves abnormally in SDS-PAGE gels, migrating with an apparent molecular weight much higher than expected. We believe this may be a consequence of the highly repetitive region. Western-blotting of total *L. (L.) chagasi* extract with the specific antibody produced multiple bands, several of which with molecular weights higher than 170kDa. A different profile is observed in *L. major* extract, with a reduced number of bands, although several bands with high molecular weights can also be observed. In both *L. (V.) braziliensis* and *Trypanosoma cruzi* the recognition by the antibody is impaired, and in the latter only a minor band is detected indicating that these antigens are not very conserved within the order Kinetoplastida and even within the *Leishmania* genus. Further work will be required to understand the reason for the multiple protein bands recognized by the antibody as well as the protein's function and intracellular localization in *L. (L.) chagasi*.

This work was supported by BioManguinhos, FINEP and FIOCRUZ.

XXX ANNUAL MEETING ON BASIC RESEARCH IN CHAGAS DISEASE - XIX MEETING OF THE BRAZILIAN SOCIETY OF PROTOZOOLOGY - HOTEL GLÓRIA, CAXAMBU, MG, BRASIL - 10-12 NOVEMBER 2003. **Rev. Inst. Med. trop. S. Paulo, 45**(Suppl. 13), November, 2003.

BM90 - CHARACTERIZATION OF THE PTERIDINE REDUCTASE 1 GENE (PTR1) OF *LEISHMANIA (VIANNIA) BRAZILIENSIS*

Renzi, A. and Tosi, L.R.O.

Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos. Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo. Ribeirão Preto, SP – Brasil.

A critical step in the infectious cycle of *Leishmania* is the differentiation of parasites from the non-infective stage to the highly infective metacyclic promastigote stage. The gene Pteridine reductase 1 (*PTR1*) of *Leishmania major* was shown to play an important role in differentiation process, the metacyclogenesis. PTR1 acts reducing biopterin into its active form, tetrahydrobiopterin (H_4B). A decreased level of intracellular H4B is an important factor controlling the extent of metacyclogenesis. The disruption of *L. (L.) major PTR1* gene resulted in transfectants with an increased virulence phenotype, as judged by the rate of lesion formation in mice. The increased virulence observed in these transfectants is connected with an increased number of metacyclic forms. In *L. (L.) major* the *PTR1* gene is located in the H region, a 45 Kb locus of chromosome 23. The H locus can be easily amplified and codes for genes involved in drug resistance, such as *PTR1* (methotrexate resistance), *PGPA* (antimonials resistance) and *HTBF* (terbinafine resistance). Therefore, the H_4B levels, being so important in the differentiation of infective forms of the parasite, could be affected or controlled by the amplification of *PTR1*. The aim of this work is to characterize the expression of *PTR1* in *L. (V.) braziliensis*, as well as the effect of its inactivation. The availability of *PTR1* mutants of *L. (V.) braziliensis* will permit the correlation between levels of gene expression and the number of metacyclics forms found in stationary-phase cultures.

The *L. (V.) braziliensis PTR1* (*LbPTR1*) was isolated from a partial genomic library using the *L. (L.) major* gene as a probe in Southern analysis. The disruption of the gene will be done by homologous recombination of a disruption fragment generated by in vitro transposition into the cloned *LbPTR1*. The isolated clone was subjected to transposition of two specialized transposable elements ELNEOKO and ELSATKO. Both transposons carry a shuttle selectable marker preceded by the protozoan splicing acceptor sites and a prokaryotic promoter sequence. The sequence of the *LbPTR1* is being determined by *Primer-island* sequencing of these insertion events. The availability of the gene interrupted by two different selectable markers permit the production of two different reagents for homologous recombination into the genome. Therefore, it is possible that the strategy employed will generate *LbPTR1* null mutants. Although the overexpression of PTR1 had no effect on the *L. (L.) major* virulence, *LbPTR1* was also cloned into the expression vector pXG and will be transfected into the parasite. The effect of *LbPTR1* inactivation and/or overexpression will be accessed by Northern analysis and by the determination of number of metacyclic forms in stationary-phase cultures of the parasite.

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BM91 - CLONING AND CHARACTERIZATION OF *LEISHMANIA (VIANNIA) BRAZILIENSIS* H-REGION ASSOCIATED GENES

Dias, F.C. and Tosi, L.R.O.

Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos. *Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo.* Ribeirão Preto, SP – Brasil. E-mail: luiztosi@fmrp.usp.br

Gene amplification is a mechanism of cellular self-preservation observed in the protozoan *Leishmania* cell lines subjected to drug pressure. The R and H region of *Leishmania (L.) major* genome are the best studied loci that undergo amplification in response to unrelated drugs. The H region is 45 Kb present in *L. (L.) major* chromosome 23. Three genes associated to drug resistance were identified within this locus. The genes *PTR1* (Pteridine reductase-1), *PGPA* (P-glycoprotein A) and *HTBF* (H region associated terbinafine resistance gene) are involved in resistance to methotrexate, antimonials and terbinafine respectively. In spite of the importance of the phenomenon, and its relevance to the definition of therapeutic strategies, the H region has not been studied in the parasite of the *Viannia* subgenus, *L. (V.) braziliensis*.

We have used the genes mentioned above and two other loci as probes in Southern analysis in order to draw a comparison between the H locus of *L. (L.) major* and that of *L. (V.) braziliensis*. The presence of the four loci in the same chromosome of 800 Kb was confirmed by Southern analyzes of PFGE-separated chromosomes. Although all loci analyzed were present in the same chromosome, the genomic DNA Southern data revealed important differences in their restriction pattern. The mapping of the different loci in the *L. (V.) braziliensis* genome was the basis for the construction of partial genomic libraries. We have used these libraries to isolate three *L. (V.) braziliensis* H region genes. Clones bearing the genes *PTR1*, *TTRS* (Tryptophanil-tRNA synthetase) and *V-ATPase* were isolated and are being characterized. The isolated clones were subjected to the in vitro transposition of the specialized transposable element /GFP*NEO. This transposon carries a shuttle selectable marker (NEO) preceded by the protozoan splicing acceptor sites and a prokaryotic promoter sequence. /GFP*NEO also contain the reporter gene GFP (Green Fluorescent Protein), which will be expressed only if part of a fusion protein. The use of the in vitro transposition system will allow not only the determination of the sequence of these *L. (V.) braziliensis* genes, through *primer-island* sequencing, but also the characterization of their expression. The functional characterization of these genes can be furthered due to the fact that the insertion of the /GFP*NEO transposon generates the reagents for protocols of gene disruption.

We are also investigating the possible amplification of the H locus through the characterization of a terbinafine-resistant *L. (V.) braziliensis* cell line selected in 9mg/ml of terbinafine. This inhibitor of esqualene-epoxidase mediates the amplification of the H region as circular amplicons in *Leishmania* species of the *Leishmania* subgenus.

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BM92 - HOMOLOGS OF THE YEAST LONGEVITY-ASSURANCE GENE (*LAG-1*) IN *TRYPANOSOMA CRUZI*: UNRAVELING THE MECHANISMS OF CERAMIDE BIOSYNTHESIS

Sampaio, T.R., Figueiredo, J.M., Previato, J.O., Mendonça-Previato, L. and Heise, N.

Laboratório de Glicobiologia, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro

Ceramide, originally identified as mere lipid moiety of membrane sphingolipids such as sphingomyelin and surface glycoconjugates, is now emerging as a vital lipid mediator that influence key aspects of cell growth, regulation, differentiation and death in eukaryotes. Ceramides are synthesized *de novo* or by recycling pathways after the *N*-acylation of dihydrosphingosine (DHS), reaction catalysed by the enzyme ceramide synthase. Recently, two highly homologous ER membrane proteins associated to longevity in *Saccharomyces cerevisae* (Lag1p and Lac1p) were shown to be essential for the acyl-CoA-dependent ceramide synthase reaction. Recently, *LAG1* homologous sequences have been identified in several eukaryotes, including human. In yeast, the *LAG1Sc* and *LAC1Sc* genes are essential for viability since in haploids, deletion of both genes result in a nonviable strain.

In the present work, we assayed microsomal membranes from epimastigotes with [^{3}H]-DHS, and substrates such acyl-CoA derivatives, free fatty acids or an *in situ* acyl-CoA generation system to identify and characterize the ceramide synthase activities of *Trypanosoma cruzi*. The radiolabelled products were extracted, separated on TLC using $CHCl_3$: MeOH: 2N NH_3OH (40:10:1, v/v) and visualized after autoradiography. In order to obtain further molecular evidences about the existence of the ceramide synthase in the parasites, the *T. cruzi* genomic database in the web was searched for putative *LAG1* homologues using BLAST and the yeast Lag1p-motif "RKDYKELVFHHIVTLLLIWSSYVFHFTKMGLAIYITMDVSDFFLSLSKTLNY". Underlined amino acids in this sequence are conserved in all Lag1-proteins described to date.

Using the cell-free assay system described above, we have observed that the *T. cruzi* ceramide synthase was able to use only acyl-CoA derivatives as substrates, but not free fatty acids. The activity was completely blocked by Fumonisin B1 (FB1), a mycotoxin able to inhibit the acyl-CoA-dependent ceramide synthases from fungi, plants and mammals. However, contrary to previous observations with yeasts and mammals, FB1 was not toxic to the parasite. From the total Lag1p-motif related individual reads found in the *T. cruzi* whole genomic sequence database at TIGR (http://www.tigr.org), 20 sequences (between 600 and 1000 bases each) producing high-scoring segment pairs (E = 3.9e-172) and presenting variable degrees of overlap to each other were used for the construction of two mini-contigs. The first one contained a putative open reading frame (ORF) of 1212 (*LAG1TcA*) bases and the second contig contained another putative ORF of 1218 (*LAG1TcB*) bases. As observed for *LAG1*Sc and *LAC1*Sc, the predicted amino acid sequence of *LAG1TcA* is 74% identical to the *LAG1TcB* protein sequence. Therefore, in analogy to yeast, the first sequence was named *LAG1Tc* and the second *LAC1Tc*. Both sequences presented homology to *LAG1* genes from other eukaryotes. Further evidence of homology came from hydrophobicity plots and suggested that the *T. cruzi* putative *LAG1* members encode membrane proteins with a predicted 45kDa molecular mass.

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BM93 - CHARACTERIZATION OF TIF34 AND PRT1 HOMOLOGS IN *TRYPANOSOMA CRUZI*: TWO SUBUNITS OF THE EUKARYOTIC INITIATION FACTOR EIF3

Lourenço, E. E.[1] Picchi G. F. A.[1], Araújo, F.R.[2], Krieger, M. A.[1,3], Goldenberg, S.[1,3] Fragoso, S.P.[1,3]

1) Instituto de Biologia Molecular do Paraná – IBMP, Curitiba, PR, Brasil; 2) Embrapa Gado de Corte – Campo Grande – MS – Brasil; 3) Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brasil
edio_eligio@yahoo.com.br

Translation is an important step in gene expression, since the genetic information encoded in mRNAs has to be, ultimately, translated into proteins. Initiation of protein synthesis is promoted by at least 10 initiation factors. The largest eukaryotic initiation factor is eIF3, a multisubunit complex that stabilizes the ternary complex eIF2-GTP-$tRNA_i^{Met}$ and promotes mRNA binding to the 40S ribosomal subunit. eIF3 also functions as a ribosome subunit anti-association factor. In yeast, eIF3 is composed by five subunits, including TIF34, TIF35 and PRT1.

Through the analysis of sequences from a normalized *T.cruzi* epimastigote cDNA library, we have identified a gene that encodes a TIF34 homolog protein (56% of similarity), which was named *TcTIF34*. *TcTIF34* is a sigle-copy gene, displaying an open reading frame of 1,0 kb and encodes a polypeptide of 37 kDa, that presents several WD repeats, which are suposed to facilitate the interactions among subunits of multiprotein complexes. It has also been shown that in yeast eIF3, TIF34 interacts directly with TIF35 and PRT1. The presence of these two other genes in the *T.cruzi* genome was investigated from the analysis of the sequences that were deposited from the *T.cruzi* genome sequencing project (www.tigr.org). We have identified only a *T.cruzi* PRT1 homolog gene 2.0 Kb when the yeast PRT1 and TIF35 were used as query sequences in the BLAST search. *TcPRT1* is a single copy gene that encodes a polypeptide of 80 kDa, which shares 39% homology with yeast PRT1.

Polyclonal antisera were raised against the recombinant TcTIF34 and TcPRT1 in order to analyse the expression pattern of theses proteins along *T.cruzi* cell cycle and differentiation. Furthermore, we are currently using a bacterial two-hybrid system to investigate whether TcTIF34 and TcPRT1 can physically interact with each other.

Financial support CNPq, PRONEX, Fiocruz

BM94 - STRUCTURE OF PDZ5 CODING GENE OF *TRYPANOSOMA CRUZI*: A ZINC FINGER PROTEIN WHICH BINDS TO UNIVERSAL MINICIRCLE SEQUENCE

dos Santos, G.V.; Coelho, E.R.; Urmenyi, T.P.; Rondinelli, E. e Silva, R.

Instituto de Biofísica Carlos Chagas Filho – CCS – UFRJ – RJ – Brasil

PDZ5 is a protein of *Trypanosoma cruzi* that is homologous to the *Crithidia fasciculata* UMSBP (Universal Minicircle Sequence Binding Protein). We have previously characterized the PDZ5 as a five zinc-finger domain protein that is recognized by the antibody generated against the protein UMSBP from *C. fasciculata*, and also binds to UMS – the Universal Minicircle Sequence, (Coelho, E.R. International Journal for Parasitology 33: 853-858, 2003). The UMS is a dodecamer found in all minicircles of the trypanosomatids and it is involved with the minicircle DNA replication. The UMSBP binds to UMS just before replication of the minicircles and was first characterized in *C. fasciculata*. It is believed that UMSBP recruits the protein complex responsible for the replication itself.

The gene pdz5 is localized in the chromosomal band XX of *T. cruzi* genome (da Silveira, Gene 308: 53-65, 2003), downstream to the proteasome beta 5 subunit gene. Preliminary results indicate that the pdz5 gene is present as a single copy. We have mapped the trans-splicing and polyadenylation sites. Reverse transcription assays suggested that this gene is transcribed as a polycistronic unit. We have found a DNA polymorphism represented by a 72 base pair deletion in the proteasome-pdz5 intergenic region. Analysis of other genera or strains of Trypanosomes indicates that this DNA polymorphism is present in some strains and absent in others. We are investigating the role of this polymorphism in mRNA transcription.

Supported by FAPERJ and CNPq

BM95 - GENE CHARACTERIZATION OF THE MOLECULAR CHAPERONE HSP10 OF *TRYPANOSOMA CRUZI*

Fernandes, M., Silva, R., Rondinelli, E., Ürményi, T. P.

Instituto de Biofísica Carlos Chagas Filho – IBCCF/UFRJ

The HSP10 it's a component of a proteic multimeric apparatus composed of HSP60 and HSP10 proteins. This chaperone machine is responsible for helping cellular proteins to reach their stable tridimensional conformation and also reduces protein denaturation in stressing conditions and prevents the formation of protein aggregates. Both HSPs are inducible proteins and must be coordinately regulated, and are, therefore, good models for studying kinetoplastid gene regulation. The *Trypanosoma cruzi* HSP60 gene has been previously isolated and characterized in our laboratory. As a first step to characterize the coordinated regulation, we begun by studying the other component of the *T.cruzi* chaperone machine, HSP10.

The complete coding sequence was obtained from the EST sequencing effort of the *T. cruzi* genome project (Verdun *et al*, 1998 - Infect. Immun. 66(11):5393-

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5398). The comparison of the predicted amino acid sequence of *T. cruzi* HSP10 with other HSP10 sequences shows small conserved regions spread over the entire length of the protein. These regions matches with the hydrophobic and hydrophilic peaks of the peptidic chain. In this alignment we also detected a 5 amino acid deletion conserved only in tripanosomatids. When molecular modeling methods were performed in silico, this deletion provokes a two fold increase in the size of the orifice located on the upper surface of the HSP10 structure, upon comparison with the E.coli analog protein (Gro ES). Moreover, the tripanosomatids maintain a negatively charged circle around the orifice, putting there an aspartate since they lost the glutamate that performs this role in E.coli. This information could help to elucidate the still obscure role of this orifice on the chaperone machine function.

A phylogenetic tree built from the sequences mentioned above point to a vertical evolution of the HSP10 protein. Southern blot experiments suggest that HSP10 is present as a multicopy gene arranged in tandem, estimated to be between 5 and 10 copies based on gene equivalent experiments. The genes are located at chromosomal band XVIII, which was verified by PFGE. Comparison of the genomic pattern of different strains of *T.cruzi* revealed a restriction fragment length polymorphism (RFLP). The level of HSP10 mRNA, of about 0.5 Kb, does not increase upon heat shock at 37°C and 40°C. However, a smaller mRNA has an expression increase in higher temperatures. The complete coding sequence was cloned in an expression vector and transformed into bacteria (E. coli BL21 strain) in order to produce recombinant HSP10 as a GST fusion protein. The fusion protein generated inclusion bodies, which were solubilized using urea. The isolated recombinant protein was then tested against chagasic human serum, and the HSP10 protein shown to be immunogenic. We are currently amplifying by RT-PCR the 5´ and 3´ UTRs in order to identify RNA processing sites.

BM96 - IDENTIFICATION OF THE HEAT SHOCK ELEMENT(S) IN THE POST-TRANSCRIPTIONAL REGULATION OF HSP70 GENES OF *TRYPANOSOMA CRUZI*.

De Carvalho, DR. Silva, R. Rondinelli, E & Ürményi, TP.

Instituto de Biofísica Carlos Chagas Filho, CCS, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ 21949-900, Brasil.

Kinetoplastid protozoa show unusual mechanisms of RNA processing, such as trans-splicing, polycistronic transcription and RNA editing, and a predominance of regulation of gene expression at the post-transcriptional level. Post-transcriptional control of gene expression have been shown in kinetoplastids, as in other eukaryotes, to be mediated by sequence elements present in untranslated regions (UTRs) of mRNAs and/or intergenic regions.

The HSP70 genes of *Trypanosoma cruzi* are organized as 7-10 copies arranged in tandem, and the protein is synthesized at normal temperatures. Upon heat shock, both HSP70 synthesis and mRNA levels are increased in a transcription-independent manner. As a first step to identify the heat shock responsive elements, the HSP70 trans-splicing acceptor and polyadenylation sites were identified. We found a major and a minor trans-splicing acceptor site, and three distinct cleavage/polyadenylation sites. In addition,analysis of several 3'UTR sequences cloned by RT-PCR shows polymorphism of the length of a central TTA repeat region. Plasmids containing the chloranfenicol acetyltransferase (CAT) reporter gene are being constructed. In these constructs the CAT gene is flanked by segments of the HSP70 intergenic region containing either the 5' UTR or 3' UTR and their respective regulatory sequences. Rab7 UTRs containing sequences are being used as control plasmids. The reporter genes will be under the control of the 18S ribosomal RNA promoter. The promoter containing sequence is currently being validated in transient transfection essays. CAT enzymatic activity and mRNA levels resulting from transfection of the plasmid constructs will be determined.

Supported by CNPq, FAPERJ and FUJB.

BM97 - CHARACTERIZATION OF THE FIRST *TRYPANOSOMA CRUZI* RAB-LIKE ENCODING GENE

Ramos, F. P.[1**], Araripe, J. R.[1], Cunha e Silva, N. L.[3], Urmenyi, T. P.[1], Silva, R.[1], Rondinelli, E.[1,2]

[1]Laboratório de Metabolismo Macromolecular Firmino Torres de Castro, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, CCS, Rio de Janeiro, Brazil. [2]Departamento de Clínica Médica, Faculdade de Medicina, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil. [3]Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, CCS, Rio de Janeiro, Brazil.

Rab GTPases are small GTP-binding proteins that are involved in several steps along the exocytic and endocytic pathways. Here we report the characterization of the 552 bp-complete coding sequence of *TcRABT*, the first Rab-Like gene of *Trypanosoma cruzi*. *TcRABT* is present in a discrete number of copies and the chromosomal location of its locus is being performed. *TcRABT* is transcribed as a single 1.2 kb mRNA in epimastigotes and its presence is being investigated in the other parasite forms. The *TcRABT* open reading frame predicts a 183 amino acid polypeptide and a 20.5 kDa protein that shares the highest values of similarity and identity with human and rat Rab23, presenting all the five GTP-binding domains. However, the cystein residues at the C terminus, which is carried by all members of Ras superfamily, essential to isoprenylation, an absolute prerequisite for the membrane association of those proteins, are not present in *TcRABT*. This finding may indicate that *TcRABT* may represent a novel member of a small GTPase family as it does not seem to be a typical Rab, neither an obvious member of another family. Polyclonal antibodies raised against a polypeptide of the *TcRABT* C-terminus were able to recognize a single 20 kDa band in DM28c and CL Brener *T. cruzi* strains as well as a single 49 kDa band corresponding to the GST fusion protein expressed in *Escherichia coli* BL21 strain. Functional and immunolocalization assays are being performed in order to better understanding *TcRABT* function.

This work was supported by CNPq and FAPERJ.

BM98 - CLONING AND CHARACTERIZATION OF TOPOISOMERASE IV GENE IN THE ENDOSYMBIONT OF *CRITHIDIA DEANEI*

Maria Cristina Machado Motta[1,2], Edio Eligio Lourenço[2], Leonardo Foti[2], Samuel Goldenberg[2,3], Stenio Perdigão Fragoso[2,3]

(1) Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, UFRJ, Rio de Janeiro, RJ, Brazil. (2) Instituto de Biologia Molecular do Paraná, Rua Professor Algacyr Munhoz Mader 3775, Curitiba Paraná Brasil, 81350-010. (3) Fiocruz, IOC, Av. Brasil 4365, Rio de Janeiro, RJ, Brasil

In eubacteria there are four topoisomerases which present distinct roles in DNA topology. Topo I and III are type I topoisomerases, that make single-stranded breaks in DNA, whereas the type II topoisomerases, DNA gyrase and Topo IV promote double-stranded breaks in DNA. Topo I and IV counter gyrase activity in order to maintain DNA supercoiling and play essential roles for cell viability, participating in processes as DNA replication, transcription and recombination. Regarding type II topoisomerases, gyrase introduces negative supercoils, which are required for the initiation of replication and removes positive supercoils to allow fork progression. The main role of Topo IV in replication is to unlink the catenated DNA generated during the replication or recombination, allowing chromosome segregation.

Some trypanosomatids present a obligate symbiotic bacterium in the cytoplasm, which divides in synchronicity with the host cell. How the symbiont

co-evolves with the host protozoon is a question of great interest, since it is related to the origin of organelles as the mitochondrion and the chloroplast. In the present study, we investigate the presence of type II topoisomerases in the endosymbiont of *Crithidia deanei*. For this purpose, the DNA used in molecular assays were obtained from isolated symbionts after cell fractioning. Primers were constructed from conserved domains of several prokaryotic Topo IV in order to amplify such gene in the symbiotic bacterium. PCR amplifications produced fragments sharing high similarity with those described in bacteria from *Pseudomonas* genus. A polyclonal antiserum raised against the recombinant Topo IV was produced to detect this protein by Western blotting. A band of approximately 70 kDa was detected in extracts of *Escherichia coli* which super-expressed the protein and in *C. deanei*, but not in the aposymbiotic strain of this protozoon. Immunocytochemical approaches were performed using the same antiserum, in order to determine the localization of this protein. A spefic labeling was observed over the symbiotic bacterium, but not in other structures of *C. deanei*. Further studies are necessary to better characterize other topoisomerases and to elucidate how the expression pattern of these enzymes affect the division process of this endosymbiotic bacterium.

Supported by: CNPq, FAPERJ and FUJB

BM99 - CHARACTERIZATION OF A *TRYPANOSOMA CRUZI* GENE ENCODING A PROTEIN BEARING REPETITIVE EPITOPES

Souza, F.S.P.[1], Picchi, G.F.A.[1], Lourenço, E.E.[1], Araújo, F.R.[2], Goldenberg, S.[1], Fragoso, S.P.[1]

1.Instituto de Biologia Molecular do Paraná – IBMP – Curitiba – PR – Brasil.
2.Embrapa Gado de Corte – Campo Grande - MS. butter_flaybr@yahoo.com.br

Trypanosoma cruzi, the etiological agent of Chagas disease has a complex life cycle that involves at least three distinct developmental stages: epimastigote, trypomastigote and amastigote. The differentiation of *T.cruzi* epimastigote into metacyclic trypomastigote involves the transformation of a replicative, non-infectious form into a non-replicative, infectious stage, by a process named metacyclogenesis. The identification of genes that play a role in cell differentiation of this unicellular organism is essential to determine how the parasite survives in different hosts and which molecules are involved in its infectiveness. Among the genes identified from the sequencing of ESTs from a *T.cruzi* metacyclic trypomastigote normalized cDNA library, there is one, named Tc445, that encodes a protein bearing repetitive epitopes. The Tc445 epitopes are highly conserved each other and contains 149 amino acid residues. Data base searching revealed that Tc445 protein shares a 48% of similarity with NUP-1, a protein of *Trypanosoma brucei*, which is associated with the nuclear lamina. NUP-1 is believed to be an orthologue of metazoan lamins. Since *T.cruzi* metacyclic trypomastigote is a non-replicative form, Tc445 protein might have a distinct role from the metazoan lamins which are involved in the reorganization of the nuclear envelope after the mitosis. Instead, Tc445 protein might play a role in the organization and maintenance of the highly condensed chromatin level that is observed in the trypomastigote stage. Antiserum was raised against peptides corresponding to either the sequence of Tc445 repetitive epitope or a hydrophilic non-repetitive region of the protein and it is currently being tested in imunolocalization assays.

Financial support: PRONEX, CNPq, Fiocruz

BM100 - CLONING AND CHARACTERIZATION OF THE PHOSPHOGLUCOMUTASE (PGM) GENE FROM *TRYPANOSOMA CRUZI*.

Penha, L.L., Mendonça-Previato, L., Heise, N., Previato, J.O. and Lima, A.P.C.A.

Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ.

The surface of *Trypanosoma cruzi* is covered by a dense coat of highly O-glycosylated sialoglycoproteins, which are thought to play a key role in host cell adhesion and invasion by trypomastigotes. There is evidence that the degree of surface sialylation of galactopyranose residues (Galp) is critical to parasite virulence. In order to further study the requirement of O- sialylglycans for *T. cruzi* survival and virulence, we aim to reduce the amount of Galp residues in the parasite by down regulating the activity of the enzyme phosphoglucomutase (PGM). PGM catalyses the reversible conversion of Glc-6-PO4 to Glc-1-PO4. The latter phosphorylated product is a key compound for the formation of UDP-Glc that is transformed into UDP-Galp by UDP-Galp-4-epimerase. In *T. cruzi* this is the only pathway for generation of UDP-Galp. In order to identify the *PGM* gene by PCR, we used a combination of degenerated primers based on sequence similarity with the *PGM* gene from others organisms and specific primers based on the 3' end of the *T. cruzi* PGM sequence (GenBankAI066127). The full lenght gene was isolated by inverted PCR of Dm28c genomic DNA, cloned and sequenced. The *PGM* gene of T. cruzi shows approximately 60% identity with that of Leishmania major and approximately 50% sequence identity with the *PGM*1genes from human, mouse and *A. thaliana*. Southern blot analysis indicates the presence of one *PGM* gene copy and Northern blot analysis revealed similar PGM RNA levels in epimastigotes and tissue culture trypomastigotes. The heterologous expression of the full lenght gene is underway.

BM101 - STRUCTURAL AND FUNCTIONAL ANALYSES OF *TRYPANOSOMA CRUZI MSH2*, A GENE INVOLVED IN THE MISMATCH REPAIR PATHWAY

Alice Machado-Silva, Sérgio D. J. Pena, Santuza M. R. Teixeira, Carlos Renato Machado.

Universidade Federal de Minas Gerais,Rua Antônio Carlos 6627, Departamento de Bioquímica ICB, Pampulha, Belo Horizonte, Minas Gerais, Brasil, CEP: 31270-901. alicems@icb.ufmg.br

Recent studies have demonstrated that the species *T. cruzi* can be divided into three distinct haplogroups, named A, B and C. This division was based upon polymorphisms found in the *MSH2* of 32 *T. cruzi* strains and is supported by another study with a *T. cruzi* antigen, *TcAg48*. The *MSH2* codifies for a protein involved in the mismatch repair pathway (MMR), which is responsible for the correction of mismatched bases in DNA. It was recently verified that strains belonging to haplogroups B and C (JG and Cl Brener, respectively) present a less efficient MMR when compared to Colombiana, a haplogroup A strain, after treatment with cisplatin and hydrogen peroxide. Since each of the haplogroups is characterized by a distinct *MSH2* isoform and as this protein has a central role in MMR, we decided to investigate whether *MSH2* could account for the difference in MMR found between the haplogroups. The *MSH2* was amplified from genomic DNA of two *T. cruzi* strains, Colombiana (haplogroup A) and Cl Brener (haplogroup C) and its whole coding region was sequenced. A high percentage of the SNPs found between the *MSH2* from these strains have resulted in amino acid substitutions, some of them in regions described as important for the protein's structural or functional maintenance. Analysis *in silico* through the program SIFT and modeling by Swiss model have indicated that the differences found between the *MSH2* of Colombiana and Cl Brener do not lead to significant variations in this protein's function or structure. Aiming to verify the protein's activity i*n vitro*, we have expressed the Colombiana's MSH2 and the purified protein was used in an ATPase assay. Through this assay it was possible to verify the ATPase activity of the Colombiana MSH2 protein

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BM102 - CLONING AND CHARACTERIZATION OF *TRYPANOSOMA CRUZI* GENES ENCODING PUTATIVE NUCLEOLAR PROTEINS

Vancini, T.R.G.[1], Picchi, G.F.A.[1], Araújo, F.R.[3], Goldenberg, S.[1,2] & Fragoso, S. P.[1,2]

3) Instituto de Biologia Molecular do Paraná – IBMP, Curitiba, PR, Brasil; 4) Fundação Oswaldo Cruz, Rio de Janeiro, RJ,Brasil; 5) Embrapa Gado de Corte – Campo Grande – MS - Brasil

Trypanosoma cruzi, the causative agent of Chagas disease, shows several peculiarities related to the mechanisms of gene expression regulation. This parasite regulates its gene expression predominantly by a post-transcriptional process, involving either the processing of long polycistronic transcripts by trans-splicing and poly-A tail addition or mechanisms based on stage-specific changes in mRNA stability and translation. Less attention has been payed, however, to the role of the ribosomal RNA processing as a stage-specific gene expression control mechanism.

Most of the steps of ribosome biogenesis in eukaryotic cells, like transcription and processing of ribosomal RNAs (rRNAs) take place primarily in the nucleolus and are performed by small nucleolar ribonucleoproteins (snoRNPs). We have shown that a gene named *TcImp4*, encoding for a protein putatively associated with U3 small nucleolar ribonucleoprotein (U3snoRNP), is present in *T.cruzi* epimastigote forms but not in metacyclic trypomastigote forms. This observation associated to the fact that there is an extensive reorganization of the nucleolus of trypomastigotes of *T.cruzi*, leaded us to hypothesize that components of some snoRNPs, might be absent in this stage and consequently might contribute to shut down the translation of mRNAs, by blocking the synthesis of new ribosome particles. Recently, we have identified two other genes encoding for homolog nucleolar proteins Nop10p and Nop58p, which are involved in the rRNA processing in yeast. *T.cruzi* homolog proteins, named TcNop10 and TcNop58, were expressed in *E.coli* and antisera were raised against either synthetic peptides or the recombinant proteins, in order to study the expression of these proteins during *T.cruzi* metacyclogenesis process and give support to our hypothesis of differential gene regulation through the modulation of nucleolar protein synthesis.

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BM103 - CLONING AND CHARACTERIZATION OF A *TRYPANOSOMA CRUZI* GENE ENCODING FOR A PROTEIN WITH A STRUCTURAL SIMILARITY WITH THE HEAVY CHAIN OF DYNEIN

Manhães, L. [1]; Carreira, M.A.C.[2]; Goldenberg, S.[1] & Fragoso, S.P. [1];

[1]Instituto de Biologia Molecular do Paraná. Rua Algacyr Munhoz Mader, 3775, CIC, Curitiba, Paraná, Brasil. 81.350-010. sfragoso@tecpar.br. [2]Instituto Oswaldo Cruz. Av. Brasil, 4365, Rio de Janeiro, RJ, Brasil. 21.045-900

Trypanosoma cruzi is the protozoan parasite causative of Chagas disease, which afflicts millions of people in Central and South America. Its life cycle involves at least three distinct developmental stages: epimastigotes, trypomastigotes and amastigotes. The epimastigote forms replicate in the midgut of the insect host and develop into non-replicative metacyclic trypomastigote forms by the process of metacyclogenesis. Metacyclic trypomastigotes are released in the excreta of insects of the *Reduviidae* family (triatomine insects) during feeding and invade the cells of the mammalian host. Within the host cells, the parasite differentiates into the replicative amastigote form, which in turn differentiates into bloodstream trypomastigotes, which infect new cells. The analysis of sequences of ESTs from a *T.cruzi* cDNA library has identified a gene that was named *Tc22*. The larger *Tc22* ORF is 530 bp long and encodes a 20kDa polypeptide. Southern blot analysis indicated that *Tc22* is a single copy gene in the *T.cruzi* Dm28c genome. The search for homology using the BLAST algorithm (www.ncbi.nlm.nih.gov) showed that Tc22 shares considerable structural similarity with an extensive region of the heavy chain of dynein. In eukaryotic cells, dynein, in association with microtubules, is required for vesicular and organelles transport, as well as flagellar motility. To investigate whether Tc22 is a novel *T.cruzi* microtubule-associated protein, we have expressed this gene in *E. coli* in order to raise an antiserum against the recombinant protein, which will be used for *in situ* localization approaches by electron and imunofluoresce microscopy. Furthermore, the expression pattern of Tc22 protein will be analysed by western blot to determine if the protein is present in all stages of *T.cruzi* life cycle.

Financial support: PRONEX, PADCT/CNPq, FIOCRUZ.

BM104 - MOLECULAR CLONING AND EXPRESSION OF THE *TRYPANOSOMA CRUZI* CATHEPSIN B

Cadavid, G., Lacerda, D.P., Teixeira, A.R.L., and Santana, J.M.

Laboratório Multidisciplinar de Pesquisa em Doença de Chagas, Universidade de Brasília, Brasília-DF; corresponding author: jsantana@unb.br

Cysteine proteases are involved in several aspects of host-parasite interactions and are the most abundant proteinases in most parasitic protozoa. Cathepsin B was recently identified in all forms of the *Trypanosoma cruzi*. In order to characterize this enzyme both molecularly and functionally, the procathepsin B gene was inserted into the pET15b expression vector to generate an N-terminal His-tagged recombinant protein. rTCCB was successfully expressed in *E. coli* and showed approximately a 36 kDa protein in SDS-PAGE under reducing conditions. Recombinant protein was then purified from the inclusion bodies fraction utilizing a column charged with nickel. rTCCB was solubilized in denaturants and was refolded. After extended incubation in acidic conditions, rTCCB showed activity on the fluorogenic substrate Phe-Arg-AMC. The antibodies against the recombinant protein were developed in mice and rabbit. The expression of an active recombinant allows a better inhibitor screening technique and the production of TCCB crystals to determine the three-dimensional structure. The complete *T. cruzi* TCCB characterization can elucidate its relevance to the metabolism of the parasite as well as the evaluation of its potential as a drug target.

This research is supported by CNPq.

BM105 - GENETIC, ENZYMATIC AND STRUCTURAL PROPERTIES OF PROLYL OLIGOPEPTIDASE FROM *TRYPANOSOMA CRUZI*

Bastos, I.M.D.[1], Grellier, P.[2], Cadavid, G.[1], Ault, M.R.[1], Joyeau, R.[3], Teixeira, A.R.L.[1], Schrével, J.[2], Martins, N. [4], Maigret, B., Silveira J. F.[4], Santana, J.M[1].

[1]Laboratório Multidisciplinar de Pesquisa em Doença de Chagas, Universidade de Brasília, Brasília-DF; corresponding author: jsantana@unb.br, [2] USM 0504, [3]USM0503, Département Régulations, Développement, Diversité Moléculaire, Muséum National d'Histoire Naturelle, Paris, [4]Embrapa Recursos Genéticos e Biotecnologia, [5]Escola Paulista de Medicina Unifesp, Sao Paulo.

We have demonstrated that *Trypanosoma cruzi* produces an 80 kDa enzyme (Tc80 proteinase) that is secreted and hydrolyzes purified and native collagens. Lesser activity is observed on fibronectin, but none on BSA, laminin and immunoglobin G. We have suggested that this proteinase could be involved in

the infection process by facilitating parasite migration through extracellular matrix (ECM). Previous data have indicated that this *T. cruzi* protease is a prolyl oligopeptidase (POP Tc80). POP Tc80 selective irreversible inhibitors have blocked parasite entry into host cells, reinforcing its role in infection. Here, we report the identification of the POP Tc80 gene located on a 3.5 Mb chromosome as a single copy. Recombinant POP Tc80 expressed in *E. coli* presents biochemical and kinetic properties similar to those of the native enzyme, and is strongly enhanced by reducing agents. POP Tc80 expression was found to be higher in infective than in non-infective parasitic forms. Three-dimensional modeling based on crystalized porcine POP structure indicates that POP Tc80 is composed of the a/b-hydrolase domain containing the catalytic triad Ser548-Asp631-His667 and of the seven-bladed b-propeller non-catalytic domain.

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BM106 - MOLECULAR STUDIES OF *TRYPANOSOMA BRUCEI* ADENOSINE DEAMINASES TAD2P AND TAD3P.

Pimenta Jr., A. A. [1]; Rubio, M. A.[2]; Alfonzo, J. D. [2] and Thiemann, O. H. [1]

[1] Departamento de Física e Informática, Instituto de Física, Universidade de São Paulo, Caixa Postal 369, São Carlos, 13560–970, SP, Brasil. [2] Department of Microbiology, The Ohio State University, 376 Biological Sciences Bldg., Room 440 484 W. 12th Ave.Columbus, OH 43210, USA.

Inosine is found at position 34, the wobble (first) nucleotide of the anticodon of several tRNAs. Inosine at this position (I_{34}) is believed to play an important role in protein synthesis by allowing the base pairing with U, C or A in the third position of the codons. Biochemical studies have revealed that I is in tRNAs is a result of deamination of a genomically encoded A and suggest the involvement in this process of a tRNA-dependent deaminase. Such A34I tRNA deaminase has been cloned from yeast *Saccharomyces cerevisiae* and recently from *Trypanosoma brucei*.

In *Trypanosoma brucei* it has been identified a mitochondria specific editing process that changes the C34 residue of tryptophane tRNA to an I residue. This process results in a $tRNA^{trp}$ that can incorporate a tryptophane at the in frame stop codon AUG. The TAD2p and TAD3p genes have been cloned from *T. brucei* cells for molecular characterization.

These genes consist of 675bp (TAD2p) and 1011bp (TAD3p) sequences encoding proteins of 225 and 337kDa, respectively. Both open reading frames have been cloned into the expression vector pTrcHis2-TOPO (Invitrogen) and introduced into BL21(DE3) *Escherichia coli* cells for overexpression experiments. Several pilot expression experiments have been done in an attempt to obtain the two proteins in the soluble fraction of *E. coli* lysates. Here we report the initial results of this project aim at the structural and functional characterization of *T. brucei* TAD2p and TAD3p proteins and their involvement in the kinetoplast $tRNA^{trp}$ editing.

Financial Support: FAPESP and PRONEX.

BM107 - DEVELOPMENT OF RECOMBINANT REPLICATION DEFICIENT ADENOVIRUSES EXPRESSING *TRANS*-SIALIDASE OR AMASTIGOTE SURFACE PROTEIN-2 OF *TRYPANOSOMA CRUZI*.

VIEIRA MACHADO**, A. M., CLASER C. **, BOSCARDIN, S. B. **, GAZZINELLI, R. T*., RODRIGUES, M. M. **, BRUÑA-ROMERO, O*.

*Centro de Pesquisas René Rachou - FIOCRUZ, Belo Horizonte, Minas Gerais, Brazil; **Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal de São Paulo-Escola Paulista de Medicina, São Paulo, São Paulo Brazil

Chagas's disease is caused by the intracellular parasite *Trypanosoma cruzi*. This disease is still a major health problem for many countries in Latin America, where it affects more than 15 million of people and is the cause of thousand of deaths every year. Presently, there is no vaccine candidate able to prevent the infection by this parasite. Moreover, the effectiveness of conventional chemoterapy, specially during the chronic phase of the infection, is very poor. On the other hand, results of various experimental studies have suggested that CD8 T cells could play an important protective role against *T. cruzi* infection. Epitopes recognized by *T. cruzi* specific CD8 T lymphocytes were recently found in the *trans*-sialidase (TS) and amastigote surface protein 2 (ASP-2) of *T. cruzi*. In recent years, one of the most successful approaches for induction of potent CD8 cellular immune responses has been the use of recombinant viral vectors as vehicles to deliver antigens. Among the most studied viral vectors, replication-defective adenoviruses possess several attractive features with regard to the development of sub-unit vaccines. They generate self-limited infections, have the ability to infect antigen-presenting cells, possess strong protein expression capabilities, and can be purified to high titers.

In the present work, we describe the construction of genetically stable replication-defective recombinant adenoviruses that express either TS or ASP-2 antigens. The *in vitro* characterization of these recombinant viruses was performed following infection of 293 A cells. By immunoblot, we detected high levels of expression of both *T. cruzi* antigens. We are currently evaluating whether the recombinant adenoviruses expressing TS or ASP-2 can induce an effective immune response against *T. cruzi* in mice.

Financial support: Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP); Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG); PDTIS and PAPES III - Research programs from Fundação Oswaldo Cruz (FIOCRUZ)

BM108 - AUTO-REGULATED EXPRESSION OF ALPHA AND BETA TUBULIN GENES OF *TRYPANOSOMA CRUZI*

Rosiane A. da Silva[1]; Daniella C. Bartholomeu[2] e Santuza M.R. Teixeira[1]

1- Departamento de Bioquímica e Imunologia - Instituto de Ciências Biológicas – UFMG. 2- The Institute for Genomic Research - TIGR - USA

We have been studying the molecular mechanisms responsible for the expression of alpha and beta tubulin genes in *T. cruzi* as a model to understand the control of stage-specific gene expression in trypanosomatids. In higher eukaryotes, tubulin mRNA levels are subjected to auto-regulatory mechanisms affecting mRNA stability: when the pool of free tubulin increases in the cytoplasm, polysomal tubulin mRNAs are destabilized by binding of protein factors to nascent N-terminal tubulin tetrapeptide. It is hypothesized that this binding results in recruitment of RNAses that degrade polysomal tubulin mRNAs. Using vinblastine and taxol, drugs that disrupt tubulin dynamics by opposite mechanisms, we found evidences indicating similar regulatory process operates in *T. cruzi*. Vinblastine causes significant morphological alterations in *T. cruzi* epimastigotes whereas taxol does not alter the shape of these parasites. This result parallels with the effects of these same treatments on the levels of microtubule-associated tubulin: vinblastine causes significant despolimerization of microtubule-associated tubulin whereas taxol maintains the microtubule structure unchanged. In accordance with these effects, only vinblastine treatment was found to alter the levels of alpha and beta tubulin mRNAs. Two hours after the addition of 50 mM vinblastine, treated parasites present a significant reduction in the levels of both tubulin mRNAs. Experiments using actinomycin D showed that this reduction is due to a decrease in the half-life of alpha and beta tubulin mRNAs. Western blot analyses are in agreement with the auto-regulatory model: epimastigotes, which have higher levels of tubulin mRNA, contain less amount of free tubulin subunits compared to amastigotes and trypomastigotes. To investigate the involvement of the sequences within tubulin mRNAs, particularly

the regions encoding the N-terminal tetrapeptide which have been shown to be a target for the regulatory process in other eukaryotes, plasmids containing the luciferase reporter gene associated with these sequences have been constructed. These plasmids are presently being used in transient and stable transfections assays of epimastigotes.

Supported by CNPq.

BM109 - PHOSPHOGLYCERATE KINASE GENES FROM *LEISHMANIA (L.) MAJOR* AS TOOLS FOR THE STUDY OF GENE EXPRESSION.

Azevedo, A.; Pedrosa, A.L.; Toledo, J.S.; & Cruz, A.K.

Faculdade de Medicina de Ribeirão Preto – USP. Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Ribeirão Preto, São Paulo, Brasil. aazevedo@rbp.fmrp.usp.br

All members of the order Kinetoplastida contain microbodies harboring glycolytic enzymes. Some enzymes are exclusive to the glycosome, whereas others are present in both glycosome and cytosol. *Leishmania* has two *PGK* genes. One, *PGKC,* encodes a glycosomal variant and the second gene, *PGKB*, encodes the cytosolic enzyme. We have previously localized those genes in one end of chromosome 20 of *Leishmania (L.) major* (Pedrosa *et al.* MBP 114:71, 2001). At the nucleotide level these genes are 99.5% identical. Our aim is to study some aspects of the control of expression of each PGK isoform. Fragments containing PGKB and PGKC genes were subcloned in pX63Neo and transfected in *Leishmania*. Both transfectants were submitted to high levels of drug pressure (G418) to specifically overexpress the corresponding gene in the parasite. We observed that cells bearing the exogenous *PGKB* would respond to the drug pressure and increase the episome copy number. On the other hand, the PGKC overexpressor could never reach similar number of copies of the recombinant, the estimated difference between them is about 18 times. Furthermore, a similar difference was observed in the transcript levels of *PGKC* and *B* in northern experiments, even if drug concentration is 10 times higher. To extend this analysis we are currently investigating the PGKC and PGKB protein levels. Our data supports the speculation that an element present either in the 3' or 5' UTR of *PGKC* controls the episome replication. This is also affecting the level of the *PGKC* transcript. Therefore, we decided to investigate such regions and the strategy chosen involved the construction of chimeras of both *PGK* recombinants for further transfection and phenotype analyses. *PGKC* and *PGKB* recombinants were digested and the fragments were used to build four chimeras in which 5' of one of the PGKs was ligated to the 3' of the other gene and vice versa. Transfectants were recovered from plates and are currently under analysis.

Supported by FAPESP

BM110 - MOLECULAR CHARACTERIZATION AND FUNCTIONAL COMPLEMENTATION OF *LEISHMANIA (L.) AMAZONENSIS* AP-ENDONUCLEASE (*LAMAP*) HOMOLOGOUS GENE

Verissimo-Villela, E*., Reis, A.B.B*.; Albano, R.M**.; Bello, A.R*.

*Laboratório de Biologia Molecular. Disciplina de Parasitologia, FCM, UERJ - Rio de Janeiro. RJ; ** Laboratório de Biologia Molecular, Departamento de Bioquímica, IBRAG, UERJ. E-mail: bello@uerj.br

Introduction: *Leishmania* spp. parasites are able to thrive inside fagolisosomes despite of the constant generation of oxygen and nitrogen reactive species, resulting in the emergence of abasic (apurinic and apyrimidinic - AP) sites, considered cytotoxic and mutagenic to prokaryotic or eukaryotic cells. It is believed that these parasites might possess efficient DNA repair mechanisms, extremely important for its intracellular survival. Objectives: Our goal was the isolation, molecular characterization and functional complementation studies in mutant *E. coli* strains of *Leishmania (L.) amazonensis* homologous genes involved in the repair of oxidative DNA lesions. Methodology: Based on the *L. major* AP-endonuclease (APEX) sequence *L. (L.) amazonensis* PCR primers were designed, cloned into TOPO TA cloning vector and subjected to automated DNA sequencing. These amplicons within the expected size (1.34 kb), were used in functional complementation assays of Exonuclease III (*xth*$^-$), Endonuclease IV (*nfo*$^-$), Endonuclease III (*nth*$^-$) *E. coli* strains challenged with various concentrations of H_2O_2 and methylmethanesulphonate (MMS). This product was cloned into *Leishmania* sp. shuttle vector (pXG-GFP$^+$) at both orientantions. The transfected parasites expressing the *L. (L.) amazonensis* homologous *apex* gene, named *Lamap*, and consequently sense and antisense mRNAs will be used in *in vitro* assays with culture and peritoneal macrophages as well as in *in vivo* assays with BALB/c mice. Results: The *L. (L.) amazonensis Lamap* gene displayed a 95% overall homology with the *L. (L.) major* one. We have observed a positive correlation for the *apex* gene and its ability to repair oxidative and alkylating lesions, conferring higher resistance to MMS and a less significant one to H_2O_2. Conclusions: We have isolated an AP-endonuclease homologous that allowed us to conclude that the activities for Endonuclease III and Exonuclease III enzymes are the most significant ones detected at this parasite, being the former more relevant when alkylating agents are involved and more effective when coupled to the second one. The Endonuclease IV activity is irrelevant when compared to the other ones. These data point towards a divergence on what concerns the activities previously shown in *L. (L.) major* and might be further exploited for the characterization of oxidative responses displayed by this protozoan parasite.

Financial support: WHO/TDR, FAPERJ and UERJ

BM111 - EIF4A HOMOLOGUES FROM *LEISHMANIA (L.) MAJOR*: QUANTIFICATION AND FUNCTIONAL PROPERTIES OF TWO DIFFERENT HOMOLOGUES

Reis, C.R.S., Rocha, P. O., 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

In the kinetoplastid protozoans the presence of a modified cap, cap4, plus the spliced leader sequence on the 5'end of the mRNAs suggests the existence of differences in the way the mRNA is recruited for translation. However very little is known about the translation machinery in these pathogens. In plants, yeast and animals, initiation of protein synthesis starts with the binding of the translation initiation complex eIF4F - eIF4A, eIF4E and eIF4G – to the monomethylated cap present on the 5'end of the mRNAs. This complex allows the recognition of the mRNAs by the small ribosomal subunit and the initiation of translation. eIF4A is a very conserved protein within the eukaryotes and possibly even in prokaryotes. It has a helicase activity and is a member of the DEAD box family of RNA helicases. In translation eIF4A binds eIF4G and, with the help of another translation initiation factor – eIF4B, is responsible for melting secondary structures along the mRNA 5'UTR allowing the small ribosomal subunit to scan the mRNA and find the translation initiation codon. We have previously reported the initial characterisation of one *L. (L.) major* eIF4A sequence (LmeIF4A1). Here we describe a second eIF4A homologue from this parasite (LmeIF4A2) identified within the *L. (L.) major* partial genome sequence available online at the Sanger Center Home-PAGE. In order to characterise it functionally the LmeIF4A2 sequence was amplified the gene,

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cloned and expressed as a recombinant protein in *Escherichia coli* fused to a tag of 6 Histidines on its N-terminus. His-tagged recombinant LmeIF4A2 was then used for the production of polyclonal sera in rabbit and its expression compared with that of LmeIF4A1 through Western-Blots. So far we have not been able to detect LmeIF4A2 in *L. (L.) major* promastigotes, in contrast with LmeIF4A1 which is expressed as a very abundant protein with over 10^6 molecules per cell. Also, in pull down assays to investigate interactions between the *Leishmania* His-eIF4A homologues and a candidate eIF4G protein from the same organism, labelled with 35S, LmeIF4A2 differs from LmeIF4A1 homologue in that it doesn't seem to bind the eIF4G protein. Further experiments will be required in order to define LmeIF4A2's function in the *Leishmania*'s protein synthesis and during its complex life cycle.

This work was supported by CNPq, CAPES, FIOCRUZ, FACEPE and The Wellcome Trust.

BM112 - IDENTIFICATION AND PRELIMINARY CHARACTERIZATION OF PUTATIVE HOMOLOGUES OF THE TRANSLATIONAL INITIATION FACTOR EIF4G FROM *LEISHMANIA (L.) MAJOR*.

Katz, R., Reis, C.R.S., 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

Our research has as its major objective the study of protein synthesis in protozoan parasites of the order Kinetoplastida, which includes major human pathogens responsible for diseases such as Chagas' disease, sleeping sickness and the leishmaniasis. With the sequencing of the *L. (L.) major* and *T. brucei* genomes and the availability of the sequences in the internet (Sanger Center and NCBI databases), we opted to investigate translation initiation and mRNA recognition in these pathogens. Our approach was the identification within these databases of homologues of translation initiation factors, through homology analysis with known vertebrate factors. We concentrated on the translation initiation complex eIF4F (eIF4E, eIF4G and eIF4A) since in other eukaryotes it binds the mRNA at its capped 5' end and recruits it for translation. eIF4G is a very large polypeptide (approx. 200 kDa), which provides a scaffold for the other eIF4F subunits, and has binding sites for other proteins such as the eIF3 initiation factor (which binds the 40S ribosomal subunit) and the poly(A) binding protein. In metazoans eIF4G is the subject of multiple regulation events required to control protein synthesis and is able to bind RNA on its own. Here we describe the preliminary characterization using bioinformatic and biochemical tools of four putative eIF4G homologues identified within the *L. (L.) major* genome database (LmeIF4G1-4). They all share the central conserved eIF4A binding domain which allowed their identification. However they lack any significant similarities outside this domain and no binding sites for the other eIF4G partners could be identified. Likewise, homology to described eIF4G proteins from other organisms is restricted to the eIF4A binding domain. The four sequences differ significantly in the size and sequence content of their N and C-terminus, but homologues for the 4 proteins can be clearly identified in *T. brucei* genome sequences indicating their conservation within the order Kinetoplastida. To begin the functional characterization of the LmeIF4G1-3 their sequences were amplified, cloned, expressed and used for antibody production. So far we have been able to confirm expression of LmeIF4G3 in *L. (L.) major* promastigotes but LmeIF4G1-2 are either expressed in very low amounts or are expressed in others stages of the *Leishmania* life cycle.

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BM113 - CLONING AND EXPRESSION OF RECOMBINANT *LEISHMANIA* SP. TRYPANOTHIONE REDUCTASE

Castro-Pinto DB.[(1)], Mendonça-Lima L. [(2)] and Leon LL. [(1)]

(1)Departamento de Imunologia, (2) Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Fiocruz.

Leishmania parasites have elaborated a variety of strategies to evade the host defense mechanisms, among them a redox system involving trypanothione (T)/trypanothione reductase (TR), equivalent to the glutathione (G)/glutathione reductase (GR) system in mammals. Trypanothione is a tripeptide (glutathione) associated to spermidine, and it is maintained intracellularly as a dithiol $T(SH)_2$ through a unique enzyme, the trypanothione reductase (TR, EC1.6.4.8). Based in previous data from our group demonstrating this system in *Leishmania amazonensis* promastigotes and axenic amastigotes (Castro-Pinto et al., 2003), we decided to better characterize this protein through recombinant DNA technology. In a preliminary comparative analysis of coding sequences for TR in different trypanosomatids, a high degree of conservation was observed among the amino acid sequences from the species studied up to now, allowing the design of oligonucleotides in order to amplify the TR gene of *L. (L.) amazonensis* and *L. (V.) braziliensis*. The PCR fragments obtained were subcloned in a sequencing DNA vector and expressed in *E. coli* (pBAD/Thio - TOPO). The nucleotide sequences from both genes were determined through automatic sequencing of double stranded DNA (ABI Prism 377). *E. coli* strains over-expressing recombinant TR of *L. (L.) amazonensis* and *L. (V.) braziliensis* were obtained and, after purification, these proteins will be used to obtain polyclonal sera in mice and for functional and structural studies.

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BM114 - MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF *LEISHMANIA (L.) MAJOR* ADENYLOSUCCINATE LYASE (ADSL).

Mantovani, M.[1]; Rojas, A.[1]; Polikarpov, I.[1]; Pedrosa, A.L.[2]; Cruz, A.K.[2]; Aguilar, C.F.[3] & Thiemann, O.H.[1]

1- Laboratory of Protein Crystallography and Structural Biology, IFSC-USP, Av. Trabalhador Sãocarlense 400, PO Box: 369, 13566-590, São Carlos/SP, Brazil. 2- Department of Cellular and Molecular Biology and Patogenic Bioagents, FMRP-USP, Av. Bandeirantes, 3900, 14040-904, Ribeirão Preto/SP, Brazil. 3-Laboratory of Structural Molecular Biology, Department of Chemistry, Biochemistry and Pharmacy, National University of San Luis, Av Ej. de los Andes 950 (5700) San Luis, Argentina.

Many species of *Leishmania* are responsible for serious visceral or skin diseases that show high incidence in tropical and subtropical regions. These Kinetoplastid protozoa parasites are auxotrophs to purine nucleotides and require those compounds from the medium or host cell. As a consequence, they have developed a specific set of enzymes involved in the purine nucleotide salvage pathway, such as adenylosuccinate lyase (ADSL). The characterization of purine salvage enzymes would contribute to our knowledge of this central biochemical pathway in the kinetoplastid protozoa. In this context, the present work aims to characterize the *adsl* gene and the recombinant ADSL enzyme from *Leishmania (L.) major* Friedlin. The *adsl* gene was already cloned into an expression vector and a purification protocol of the recombinant enzyme was established. The mature mRNA transcript, containing 2032 nucleotides, was defined by 5' and 3'RT-PCR. Restriction analysis and Pulse Field Gel Electrophoresis (PFGE) followed by Southern hybridizations showed that *adsl* is a single copy gene and is located in the chromosome 5 of this parasite. These characteristic allows to future knockout experiments. The tetrameric form of the recombinant ADSL

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enzyme was confirmed by native gel electrophoresis and Dynamic Light Scattering. ADSL has an experimental pI of 6.07 and exhibited maximum enzymatic activity at pH 8.5. The kinetic parameters were analyzed by a Lineweaver-Burk plot. K_m, V_{max} and K_{cat} values of 10.0mM, 14.3mmoles/min, and $33s^{-1}$, respectively, were obtained for adenylosuccinate substrate. A polyclonal mouse antibody raised against ADSL was produced and have been tested. In addition, X-Ray diffraction data, from a Cesium derivative crystal, were collected and are being processed.

Work supported by: FAPESP, HHMI, WHO/TDR, PRONEX and the University of São Paulo

BM115 - CLONING, EXPRESSION AND PURIFICATION OF A *LEISHMANIA (L.) MAJOR* MITOCHONDRIAL TRYPTOPHANYL-TRNA SYNTHETASE.

LEITE, N. R.[1]; Alfonzo, J.D.[2] and THIEMANN, O. H.[1]

1-Laboratory of Protein Crystallography and Structural Biology, IFSC-USP, Av. Trabalhador Sãocarlense 400, PO Box: 369, 13566-590, São Carlos/SP, Brazil. 2-Department of Microbiology, The Ohio State University, 376 Biological Sciences Bldg., Room 440 484 W. 12th Ave.Columbus, OH 43210

The fidelity of protein synthesis is dependent on the correct charging of tRNAs with their cognate amino acids. This process is catalyzed by aminoacyl tRNA synthetases (aaRSs) specific for each particular tRNA. From the comparison of sequence homologies and crystal structures, aminoacyl-tRNA synthetases could be divided into class I and II. Tryptophanyl-tRNA synthetase (WARS) belongs to class I aaRS, which share the consensus sequences "HIGH" and "KMSKS" and the Rossman fold domain. The mitochondria *L. (L.) major* tryptophanyl-tRNA synthetase, LmWARS2, plays an important role in the organelle protein synthesis were it is required to distinguish and charge the edited and the unedited $tRNA^W$. This unique feature may render LmWARS2 a potential target for the design of novel inhibitors.

Sequence analysis of this protein revealed a putative mitochondrial signal represented by the first 24 amino acids and alignment with the sequences of others mitochondrial WARS indicated this protein has insertions along of its amino acids sequence. The mature form of this protein was amplified by the PCR and cloned into expression vectors pCR-TOPO/NT and pCR-TOPO/CT, pET-28a and pET-29a and introduced into *Escherichia coli* BL21-CodonPlus (DE3)-RIL cells. The pET-28a construct resulted in the overproduction and purification of LmWARS2 by metal chelate affinity chromatography. One construct was made by the introduction of an in-frame stop codon into vector pET29a. This LmWARS2 (LmWARS2/pET29a-DH) allowed to express the protein without a His-tag. Both constructs showed expression after induction with IPTG. The expression level of LmWARS2/pET28a was higher than LmWARS2/pET29a and LmWARS2/pET29a-DH. A purification protocol has been developed to this protein, however, the resulting LmWARS2 is unstable, precipitating shortly after purification. We have obtained mouse polyclonal antibodies against LmWARS2 for further imunolocalization and imunoprecipitation experiment.

Work supported by: FAPESP, HHMI, WHO/TDR, PRONEX and University of São Paulo

BM116 - MOLECULAR CHARACTERIZATION AND COMPARATIVE ANALYSIS OF THE MITOCHONDRIAL GENOMES OF *EIMERIA* SPP. OF DOMESTIC FOWL

Romano, C.M. [1], Fernandez, S. [1], Madeira, A.M.B.N. [1], Shirley, M.W.[2] and Gruber, A.[1]

[1]Faculdade de Medicina Veterinária e Zootecnia, USP. Av. Prof. Orlando Marques de Paiva 87, São Paulo SP, 05508-000, Brazil. [2]Institute for Animal Health, Compton, Berks., United Kingdom. argruber@usp.br

Most of the protozoan parasites of the phylum Apicomplexa, including the genus *Eimeria*, present three distinct DNA genomes: nuclear, mitochondrial and plastid genomes. The nuclear genome comprises 14 chromosomes and is currently the subject of a major genome sequencing effort (http://www.sanger.ac.uk/Projects/E_tenella/). The mitochondrial genome of Apicomplexa has been characterized in *Plasmodium* spp., corresponding to a linear molecule composed by 15-150 tandemly repeated copies of a 6 kb element. In *Theileria*, however, the genome is comprised by a 7 kb monomeric element. In both organisms three cytochrome genes were found (*cy*b, *cox*I and *cox*III), as well as some stretches of rRNA genes, but no tRNA genes were present. Dunn *et al.* (1998) have reported in *E. tenella* the occurrence of 170-220 kb molecules hybridizing to a mitochondrial probe. Since the mitochondrial genome is maternally inherited (through the macrogametocyte), it represents an interesting target for monitoring cross-infections. Moreover, this genome can also be used for phylogenetic and epidemiologic analyses. In order to characterize the mitochondrial genome of *Eimeria* spp., our group has determined the complete mitochondrial sequences of the seven *Eimeria* species that infect the domestic fowl. In addition, the mitochondrial genomes of 5 distinct strains of *E. tenella* were also sequenced, thus allowing the characterization of the intra-specific variability. All the genomes sequences showed a size of circa 6 kb and contained the cytochrome genes reported for other Apicomplexa. A multiple sequence alignment, using sequences derived from the seven *Eimeria* species, revealed a similarity of around 90% and a conservation of the synteny and gene order. When the mitochondrial genomes five *E. tenella* strains were compared, a very high conservation was observed. Two haplotypes were found, with two consecutive timines being deleted in strains MC (Brazilian) and Wisconsin (North American) when compared to strains H, TA and Wey (isolated in the UK). This high level of conservation was already reported for *Plasmodium falciparum* strains isolated from different continents (Conway *et al*. 2000), and may reflect a very recent common origin of the strains. Comparing the mitochondrial sequences of several species of *Eimeria*, *Theileria* and *Plasmodium*, we observed that the gene order and orientation are conserved among different species of any genus, but not across distinct genera. This result suggests a gene "shuffling" event during the evolution of these mitochondrial genomes. A possible explanation can be found on the replication mechanism of this genome, which is primarily based on recombination events and rolling circle activity (Preiser *et al*. 1996). Further studies will be carried out to better understand this finding.

BM117 - FUNCTIONAL CHARACTERIZATION OF TRYPSIN AND CHITINASE GENES OF *LUTZOMYIA LONGIPALPIS* THROUGH POLYCLONAL ANTIBODIES.

Letícia M. Batista.& Yara M.Traub-Cseko.

Department of Biochemistry and Molecular Biology, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro leticiamoulin@hotmail.com

While transgenic mosquitoes incapable of transmitting malaria have already been created, little is known about molecules involved in feeding and infection by *Leishmania* in *Lutzomyia longipalpis.* By using differential display techniques and EST sequencing, we have already identified many candidates for a role in these processes. Among these is a gut-specific chitinase gene with high transcription levels after 72 hours of blood feeding, probably involved in the degradation of the peritrophic matrix.. We have also identified 3 trypsin genes, expressed early (6 hours) after the bloodmeal. We are interested in carrying out functional studies of these genes, and for that we have as objective the production of polyclonal antibodies. Not only it is well know that levels of RNA not always reflect presence of active protein, due to post-transcriptional gene regulation, but also, polyclonal antibodies also permit immunolocalization studies. We are using two different approaches for the production

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of these antibodies: production of recombinant antigens in the expression vector pET28a, and use of the vector pCDNA3 as a DNA vaccine. The pET28a vector is designed with enhanced features to permit easier sub-cloning, detection, and purification of target proteins. pET28a has a promoter that directs transcription of the T7 RNA polymerase gene, which is inducible by isopropyl-b-D-thiogalactopyranoside (IPTG). Addition of IPTG to a growing culture of the lysogen induces the T7 promoter, which in turn transcribes the target DNA cloned in the plasmid. pCDNA3 is a DNA vector designed for high-level stable and transient expression in eukaryotic hosts, being effective in a wide range of mammalian cells. The pCDNA3 vector is designed to be used as a DNA vaccine. These plasmids, containing specific genes, enter the cells, where the DNA is transcribed and translated resulting in the production of specific proteins. The produced proteins are processed similarly to intracellular viral antigen resulting in the activation of the immune system and production of antibodies. PCR was used to amplify both cDNAs (chitinase and trypsin) and the fragments were cloned into pCRII. The inserts were purified and ligated into either the pET28a or pCDNA3 plasmids. The positive clones were confirmed by sequencing. The pET28a constructs were successfully induced for expression by IPTG (1mM) and the recombinant protein is presently being purified for inoculation of rabbits. The pCDNA3 constructs are presently being inoculated in rabbits. It will be interesting to evaluate the efficiency of antibody production using both techniques, since it is so much easier to work with DNA vaccines. These antibodies will be used in Western blots and immunolocalization studies of the chitinase and trypsin proteins, for temporal and quantitative studies.

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BM118 - STUDIES ON FLAG, A FLAGELLAR PROTEIN OF *LEISHMANIA*

Marcelino-Nascimento, L., Traub-Cseko, Y. M.

Department of Biochemistry and Molecular Biology, Instituto Oswaldo Cruz, Fiocruz. Rio de Janeiro, RJ, Brazil, luanda_nascimento@hotmail.com

The pathogen causing leishmaniasis is a protozoan with basically two morphologies along its life cycle, an amastigote form in the mammalian host and a flagellated form called promastigote in the sand fly vector. A successful infection by the promastigote in the invertebrate depends on the capacity to adhere to the epithelial cells in the gut of the sand fly. Some molecules were implicated in this process. The main one is the parasite surface LPG (lipophosphoglican). Another *Leishmania* molecule, a protein named FLAG, recognized by anti-flagellar monoclonal antibodies (mAb), seems to be involved also in this phenomenon. Its location is exclusively flagellar and there is no function formally described for it. Evidences showed that the anti-FLAG mAb can inhibit the adhesion of *Leishmania* promastigotes to the epithelial cells of the sand fly midgut (Warburg *et al*.,1989, *J. Protozool*., 36:613). There is a lack of information about flagellar proteins of *leishmania*. Also, the precise mechanism by which proteins are directed to the flagellar pocket and flagellum remains unsolved. We have sequenced the FLAG gene in many species of *leishmania*, and found a high level of conservation. Data base searches also found similar sequences in trypanosomes. We intend to study the sorting signal of FLAG and in this way contribute to the formal characterization of this protein and to the current knowledge of flagellar targeting. For this purpose we are constructing fusion proteins of FLAG and GFP (green fluorescent protein). Two *leishmania* expression vectors with the GFP cassette were used to obtain FLAG linked to the C- or the N-terminal of the reporter protein. These constructs are being transfected into leishmania, that will be examined by fluorescence microscopy. Another objective is the production of policlonal antibody, a useful tool for many different assays. This will be done by the injection into rabbits of either recombinant FLAG or by DNA vaccines.

Supported by PAPESIII-Fiocruz.

BM119 - TRAFFICKING OF THE CYSTEINE PROTEINASE LPCYS1 IN *LEISHMANIA*: FUNCTION OF THE PRE-PRO DOMAIN

SANTOS, J. P. T.; VILLA, M. M.; TRAUB-CSEKO, Y. M.

Department of Biochemistry and Molecular Biology, Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro, RJ, Brazil. joaopaulots@hotmail.com

Leishmaniasis is a serious disease with about 400,000 news cases per year, that nowadays is also considered an opportunistic disease in AIDS. *Leishmania* is a dimorphic parasite that has its cycle of life in two hosts, a sand fly vector, and a mammal. In the invertebrate host, it presents promastigote forms (with flagellum), and in the mammalian host the amastigote form (without external flagellum). Cysteine proteinase may be linked to many pathological processes caused by parasites. Two distinct lysosomal cysteine proteinases have been identified in *Leishmania (L.) pifanoi,* Lpcys1 and Lpcys2. Studies of the targeting of Lpcys2 showed the lack of a role for glycosylation in this process, although a role for the pro domain has been determined. Assays of immunolocalization have shown the presence of both proteinases in the lysosome, but of relatively more abundant quantities of Lpcys1 in the flagellar pocket than of Lpcys2. Lpcys1 does not have the C-terminal domain characteristic of most cysteine proteinases of trypanosomatids, that is also present in Lpcys2, and have some sequence differences in the pre-pro domain. We are interested in investigating possible differences in targeting mechanisms between Lpcys 1 and 2. Constructs that express the pre-pro domain of Lpcys1 fused with GFP (green fluorescent protein) were made in a *leishmania* expression vector. To study the targeting of this protein to the lysosome, these constructs are being transfected into *Leishmania* through electroporation. In this technique the parasite, in the presence of DNA, is submitted to an electric shock, when pores are formed in its membranes and the plasmids enter the cell. The transfected parasites are incubated in medium with a concentration of selective drug determined by EC50 (dose of drug needed to reduce the growth in 50%), and the transfected cells are examined by fluorescent microscopy.

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BM120 - LYSOSOMAL TRAFFICKING IN *LEISHMANIA*: USE OF YEAST TWO-HYBRID SYSTEM AND STUDIES OF SIGNAL CONSERVATION

Morgado,G.S., Villa,M.M., Traub-Cseko,Y.M.

Department of Biochemistry and Molecular Biology, Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro grazisamor@hotmail.com

Leishmaniasis are diseases caused by protozoan haemoflagelates of the genus *Leishmania* that affect many people in various parts of the world, predominantly poor and underdeveloped countries. The lack of efficient vaccines and therapies leads to the need for the development of new targets for control measures. Molecules involved in mechanisms of cellular trafficking in *Leishmania* can be used as a potential target for disease control. Cysteine proteinases have been implicated in processes that might be important in parasite invasion, infectivity and pathogenicity, making them a good investigation target. Previous studies showed that in Lpcys2, an abundant lysosomal cysteine proteinase from *L. (L.) pifanoi*, the pro domain plays a role in targeting (Costa-Pinto et al., 2001). We are presently going deeper into these studies and searching for molecules that interact with the pro region signal utilizing the yeast two-hibrid system. The system is based on yeast co-transformation of the vectors: pBD-GAL4 Cam containing the pro domain sequence of cysteine proteinase (bait) and HybriZAP-2.1 containing an amastigote leishmania cDNA library (target). Subsequently, molecule interaction activates the b-galactosidase and Histidine gene reporter

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expression, identified by the appearance of blue colonies. Until this moment no positive colonies were identified. We are also investigating the evolution of trafficking signals by fusing the Lpcys2 prepro and pro domains to the reporter GFP (green fluorescent protein) and transfecting these constructions into yeast, *Saccharomyces cerevisiae* YRG-2. For this purpose we used two yeast specific expression vectors: pGFP-N-FUS34 and pGFP-C-FUS23. The cells transfectants were examined by fluorescence microscopy. When the signal sequence was fused to the GFP amine end, the cells showed a compartmentalized fluorescence compatible with the vacuole, the equivalent to the lysosome of *Leishmania*. This observation indicates the recognition of the *Leishmania* signal by yeast, and might explain the lack of positive results by the yeast two-hibrid system.

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BM121 - CHARACTERIZATION OF GENES ENCODING PROTEINS INVOLVED IN INTRACELLULAR TRAFFICKING IN *TRYPANOSOMA CRUZI*.

Macêdo CDS, Cardoso ER, daRocha WD, and Teixeira SMR.

Departamento de Bioquímica e Imunologia, ICB, UFMG, Belo Horizonte, MG, Brasil

In this study we propose to characterize two cDNAs isolated from a *Trypanosoma cruzi* library of amastigote forms screened with sera derived from of chagasic patients. Sequence analyses of *Tc*AG38 and *Tc*AG49 cDNAs reveal homologue sequences and indicated that they corresponds to a new class of *T. cruzi* gene, which encodes proteins presenting significant homology with VIP36 and ERGIC 53, mammalian proteins involved in cellular sorting or recycling. The complete sequence of the *Tc*AG38 cDNA was determined revealing an ORF of 550 amino acids and the *Tc*AG49 sequence is slightly bigger. Using GFP tranfections, we have previously showed that these proteins localized near to the flagellar pocket and co-localizes with kinatoplast and golgi region markers. Another strategy in the briefing of the localization of this protein is the analysis of the distribution of this pAG38 in cells of mammals. For this, already we are carrying through the construct of the vector presenting the codding region for pAG38 in fusion with GFP for transfecção of fibroblasts. We identified a microssatelite within the coding region which is translated into a poli-Glu region. PCR amplifications of this region reveled polymorfism among diferent strains of parasite. Southern blot analysis indicated that the antigen is encoded by a one to three copies of this gene in the genome of parasite. Northern blot analysis revealed that *Tc*AG38 is constitutively transcribed into a 2,5 kb mRNA, which is slightly more abundant in amastigote than epimastigote and trypomastigote forms. Transformed *E.coli* expressing the recombinant proteins as GST fusions were produced using the pGEX vector (Pharmacia). GST::pAG38 and GST::pAG49 were submited to purification protocol and are being used to generate specific antibodies.

APOIO: WHO, CNPq e CAPES

BM122 - STUDIES ON THE TYPE II DNA TOPOISOMERASE OF THE TRYPANOSOMATID *BLASTOCHRITHIDIA CULICIS*

Lourenço, E. E.[1],Cavalcanti D.P.[3], Picchi G. F. A.[1], Motta, M.C. M.[3], Araújo, F.R.[4], Krieger, M. A.[1,2], Goldenberg, S.[1,2] Fragoso, S.P.[1,2]

1) Instituto de Biologia Molecular do Paraná – IBMP, Curitiba, PR, Brasil. 2) Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brasil. 3) Instituto de Biofísica Carlos Chagas Filho – UFRJ, Rio de Janeiro, RJ. 4) Embrapa Gado de Corte – Campo Grande – MS – Brasil. edio_eligio@yahoo.com.br

Type II topoisomerases (topoII) are enzymes that catalyze changes in the topology of the DNA molecule by introducing transient double-stranded cuts. They are involved in many cellular processes, including DNA replication, transcription, recombination and chromosome segregation. Moreover, topoII are targets for many antitumoral agents and might be also important targets in the chemotherapy of diseases caused by parasites, including Chagas disease and leishmaniasis. We have cloned and characterized the gene encoding *TOP2* from the monoxenic trypanosomatid *B. culicis* (*BcTOP2*), since this enzyme might be used as a prototype for topoII enzymes from pathogenic trypanosomatids and consequently a good model for future structural and functional studies. BcTOP2 was expressed in *E. coli* and antiserum was raised against the recombinant protein. Western blot analysis revealed a polypeptide of 138 kDa, comparable to that observed in extracts of *C. fasciculata* (CfmtTopoII). In *C. fasciculata*, monoclonal antibodies against CfmtTopoII showed that the enzyme is localized in the mitochondrium. On the other hand, in *T.cruzi*, topo II was localized in the nucleus. However, in *Leishmania (L.) donovani* and *Bodo salt*ans, topo II imunolocalizes in both compartiments. In view of this conflicting results we decided to determine the cellular compartiment in which BcTopo II is localized, in order to approach the role that this enzyme could perform either in the kDNA replication or in chromosome replication and segregation.

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BM123 - *TC*RABS FUSED TO GFP - AN ALTERNATIVE STRATEGY TO STUDY THE LOCATION OF *TC*RAB7 AND *TC*RAB5A PROTEINS IN *TRYPANOSOMA CRUZI*.

da Cunha, I. M. [(1)], Cunha e Silva, N. [(2)], Silva, R.[(1)], Ürményi,T.P.[(1)], de Souza, W.[(2)] Araripe, J.R.[(1)], Rondinelli, E. [(1)].

(1)Lab. Metabolismo Macromolecular Firmino Torres de Castro, (2) Lab. Ultraestrutura Celular Hertha Meyer, IBCCF, CCS, UFRJ

Rab proteins are specifically found at different cellular compartments. They are small GTPases anchored in the organelle membranes cytoplasmatic face, with a role in the vesicular traffic control of the eukaryotic cells. The *rab7* and *rab*5A genes of *Trypanosoma cruzi* have already been sequenced and characterized in our laboratory. We have located *Tc*RAB7 protein in the Golgi Apparatus of epimastigote forms by immunoelectronmicroscopy using a specific polyclonal antibody and now we are performing the *Tc*RAB5 localization. A heterologous antibody, raised against a carboxi-terminal region of Rab5A protein of *Trypanosoma brucei*, is being used to localize Rab5A protein in *T. cruzi* and we have already produced recombinant protein in order to produce a specific antibody against *Tc*RAB5A.The use of the GFP (green fluorescent protein) fusion protein is another powerful strategy to localize proteins in live cells, distinguishing their functional sites from their synthesis or recycling pathways. To further investigate *Tc*RAB7 site and also in a first attempt to localize the *Tc*RAB5A, we have constructed and will express *TcRAB* genes fused in their N-terminal regions to GFP. Oligonucleotides were drawn to amplify, from the cloning vector pEGFP-C1, a DNA cassette (850 kb fragment) containing a truncated version of the coding region for GFP, without stop codon, suitable for expression of the protein fusion. Two vectors (pRIBOTEX-GFP and pTEX-GFP) will be used to insertion of *Tc*RAB7 and *Tc*RAB5 ORFs. The first vector will be originated from the pRIBOTEX plasmid whose expression is driven by a ribosomal RNA gene promoter. Another vector will be originated from the pTEX whose expression is controled by flanking regions of the GAPDH genes. The constructs will be sequenced, amplified and transfected in epimastigote forms. The cells, live or fixed, will be examined for GFP expression using the Confocal Laser Scanning microscope Zeiss LSM 310.

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BM124 - REGULATION OF POLYSOME ASSOCIATED POLYPETIDES AND HSP47 BIOSYNTHESIS BY ANTISENSE OLIGONUCLEOTIDES IN *HERPETOMONAS SAMUELPESSOAI*

Ferreira, L. R.[1], Barroso, A. P.[1]; Costa, K.G.[2]; Dias, P.A.[2], Fonseca, C.P.[1]

[1]PUC-MINAS, Poços de Caldas-MG, [2]HCRP-USP, Ribeirão Preto-SP.

Herpetomonas samuelpessoai corresponds to a Trypanosomatidae parasite from the *Herpetomonas* genus. These parasites can be grown in culture medium *in vitro*. Hsp47, an endoplasmic reticulum resident protein, has binding properties and has been hypothesized to function as a molecular chaperone in regulating protein folding and assembly. In this study, we investigate the interacion of Hsp47 with polysome- associated proteins following antisense oligonucleotide treatment directed to Hsp47 in *Herpetomonas samuelpessoai* parasites. For these studies, we employed phosphorothioate oligodeoxynucleotides directed to the first five codons of Hsp47 that straddle the predicted translation initiation site of Hsp47. The levels of Hsp47 were assessed by immunoprecipitation and/or Western Blot analysis. Labeled nascent polypeptide chains were isolated from polysome preparations as peptidyl-tRNA complexes using ion exchange chromatography. The eletrophoretic profiles of labeled polysomes revealed that the majority of Hsp47 was associated with nascent chains of polypeptides. Treatment of *Herpetomonas samuelpessoai* with antisense Hsp47 oligonucleotides after 48 days reduced the levels of Hsp47 and caused a "knock out" of the synthesis of nascent polysome associated polypeptide chains. This study revealed that diminished levels of Hsp47 induced by antisense oligonucleotide treatment of *Herpetomonas samuelpessoai* cells also reduce the levels of polysome associated polypeptides. The results provide further evidence that Hsp47 is associated with polysomes at a very early point during translocation of polysome associated nascent polypetide chains.

This study was sponsored by FAPEMIG and PROBIC-PUC-MINAS.

BM125 - TYPE I DNA TOPOISOMERASES OF *TRYPANOSOMA CRUZI*: CELLULAR LOCALIZATION OF TOPOISOMERASE I AND CHARACTERIZATION OF TOPOISOMERASE III

Umaki, A.C.S.[1]; Araújo, F.R.[2]; Mörking, P.A.[1]; Krieger, M. A.[1,3]; Goldenberg, S.[1,3] & Fragoso, S.P.[1,3]

[1]Instituto de Biologia Molecular do Paraná, IBMP, Curitiba, PR, Brasil. [2]Empresa Brasileira de Pesquisa Agropecuária, EMBRAPA, Campo Grande, MS, Brasil. [3]Fundação Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, RJ, Brasil. acsumaki@hotmail.com

Type I topoisomerases are classified in two subfamilies, IA and IB, based on differences in amino acid sequence and reaction mechanisms. The type IA enzymes link covalently to cleaved DNA through the 5' phosphate. They are represented by reverse gyrase, bacterial topo I and topo III and the eukaryotic topo III enzymes. Type IB topoisomerases, in contrast, become attached covalently to 3'-phosphate end of the cleaved strand of the DNA. The presence of at least one type I DNA topoisomerase in all organisms indicates that these enzymes play a key role in replication and transcription processes, and recent studies have pointed to their role in recombinational repair and in chromosome segregation. Furthermore, topo I is a target for clinically anticancer drugs, such the analogous of camptotecin. Hence, we decided to clone and characterize the genes encoding these enzymes in *T. cruzi* (*TcTOP1* and *TcTOP3*) in order to get insight into its importance during the cell cycle and differentiation of the parasite and evaluate them as potential targets for chemotherapic treatment of Chagas disease. The sequencing of *TcTOP3* gene showed that it contains an ORF of 2,5 Kb, encoding for a polypeptide of 95 kDa. The deduced amino acid sequence of *TcTOP3* shares a similarity of ~ 49% with Topo IIIb of *Xenopus laevis* and *Homo sapiens*. We have also initiated the characterization of a gene encoding a second topo III, which the sequence showed to be divergent from that first one and shares significant homology with topo III of *Arabidopsis thaliana*, instead. It indicates that *T.cruzi* might have more than one topo III with different functions. Recombinant Tctopo III was expressed in *E. coli* and an antiserum was raised for further studies of cellular localization.

Regarding to Tctopo I, which the gene was previously characterized by us, immunofluoresce analysis shows that this enzyme immunolocalizes into the nucleus of epimastigote forms of *T.cruzi*, as expected. Our preliminary data also suggest that TcTopo I enzyme might have a differential expression pattern through the cell cycle of *T.cruzi* epimastigote forms.

Financial support: PRONEX, PADCT/ CNPq.

BM126 - EVALUATION OF THE ROLE OF THE MINIEXON GENE IN THE VIRULENCE OF *LEISHMANIA (V.) BRAZILIENSIS.*

Santos, A.F.J., Toledo, J.S., Silva, A.M., Antoniaz, S.A., Cruz, A.K.

Laboratorio de Parasitologia Molecular, Departamento de Biologia Celular, Molecular e Bioagentes Patogênicos – FMRP/USP; *andrefjsantos@yahoo.com.br*

Trans-splicing is the preferred route for the generation of mature mRNA among trypanosomatids and the miniexon gene plays a central role in the splicing process of mRNAs. The splice leader is added to the 5' extremity of each mRNA supplying its 5' capped structure. In previous studies carried out in the laboratory we have shown that the over expression of the miniexon gene leads to an attenuation of the virulence of *Leishmania (L.) major* in BALB/C mice (Antoniazi et al. MBP 107: 57, 2000). To verify if the same phenomenon would be observed in different species of *Leishmania*, we have selected three distinct strains of *L. (V.) braziliensis* (2904, Ba 27b2 e Ce 3227). The EC50 for Hygromycin B (HYG) was calculated for each one of the *L. (V.) braziliensis* strains, in order to define the drug concentration for selection of transfectants. The drug concentration to be used in liquid media is 6ug/ml, 4ug/ml e 6ug/ml, for strains 2904, Ba 27b2 e Ce 3227, respectively. These strains were transfected with clone 32D05 that contains approximately 100 copies of the miniexon gene, or with the vector (cLHYG) with no insert, as a control. The transfectants of *L. (V.) braziliensis* had been selected on solid media and submitted increasing drug pressure to stimulate the overexpression of the episomal genes. Molecular characterization of the transfectants confirmed the identity of the episome and was followed by *in vivo* investigation of virulence. A suspension of 10 million promastigotes from stationary phase (Ce 3327) was injected in the hind footpad of hamsters. Evaluation of lesion development was carried out for five months by weekly measurements of the footpad with a kaliper, the contra-lateral footpad was used as a control. Each experimental group was composed of 10 animals infect with: (1) parental line, (2) cLHYG transfectant, (3 and 4) transfectant with 32D05 under low (12 ug/ml of HYG) and high selective pressure (120 ug/ml of HYG), respectively. Lesion development was observed in groups 1 and 2, but not in animals from groups 3 and 4. Present data confirms that the gene miniexon, when overexpressed, attenuates the virulence of *L. (V.) braziliensis*. We are currently evaluating another strain of the same species and also investigating the possible mechanism that leads to the observed attenuation.

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BM127 - PILOT SURVEY OF EXPRESSED SEQUENCE TAGS (ESTS) FROM THE ASEXUAL BLOOD STAGES OF *PLASMODIUM VIVAX* IN HUMAN PATIENTS

Emilio F Merino[1], Carmen Fernandez-Becerra[1], Alda MBN Madeira[2], Ariane L Machado[3], Alan Durham[3], Arthur Gruber[2], Neil Hall[4] and Hernando A del Portillo[1*]

[1]Departamento de Parasitologia, ICB, Universidade de São Paulo, São Paulo, Brazil. [2]Departamento de Patologia, FMVZ, Universidade de São Paulo, São Paulo, Brazil. [3]Departamento de Ciências da Computação, IME, Universidade de São Paulo, São Paulo, Brazil. [4]The Sanger Institute, Wellcome Trust Genome Campus, Hinxton, UK. *Correspondence (hernando@icb.usp.br)

Plasmodium vivax is the most widely distributed human malaria, responsible for 70–80 million clinical cases each year and large socio-economical burdens for countries such as Brazil where it is the most prevalent species. Unfortunately, due to the impossibility of growing this parasite in continuous *in vitro* culture, research on *P. vivax* remains largely neglected. A pilot survey of expressed sequence tags (ESTs) from the asexual blood stages of *P. vivax* was performed. To do so, 1,184 clones from a cDNA library constructed with parasites obtained from 10 different human patients in the Brazilian Amazon were sequenced. Sequences were automatedly processed to remove contaminants and low quality reads. A total of 806 sequences with an average length of 586 bp met such criteria and their clustering revealed 666 distinct events. The consensus sequence of each cluster and the unique sequences of the singlets were used in similarity searches against different databases that included *P. vivax*, *Plasmodium falciparum*, *Plasmodium yoelii*, *Plasmodium knowlesi*, Apicomplexa and the GenBank non-redundant database. An E-value of $<10^{-30}$ was used to define a significant database match. ESTs were manually assigned a gene ontology (GO) terminology. A total of 769 ESTs could be assigned a putative identity based upon sequence similarity to known proteins in GenBank. Moreover, 292 ESTs were annotated and a GO terminology was assigned to 164 of them. These are the first ESTs reported for *P. vivax* and, as such, they represent a valuable resource to assist in the annotation of the *P. vivax* genome currently being sequenced. Moreover, since the GC-content of the *P. vivax* genome is strikingly different from that of *P. falciparum*, these ESTs will help in the validation of gene predictions for *P. vivax* and to create a gene index of this malaria parasite. Most important, as these ESTs represent parasite genes expressed during the stages responsible for the pathology associated with vivax malaria, sequence comparisons with the data from the *P. vivax* genome should assist in identifying SNPs for genetic mapping and population diversity studies.

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BM128 - ROLE OF THE SPLEEN IN EXPRESSION OF VARIANT GENES FROM *PLASMODIUM CHABAUDI* IN BALB/C MICE.

Rodriguez S, Fernandez-Becerra C and del Portillo HA. (hernando@icb.usp.br)

Departamento de Parasitologia, ICB, Universidade de São Paulo, São Paulo (Brazil).

Antigenic variation has been noted in all *Plasmodium* species where it has been sought, including the simian malarias, *P. knowlesi* and *P. fragile*, the rodent malarias, *P. chabaudi* and *P. yoelii* and the human malarias, *P. vivax* and *P. falciparum*. Significantly, a key role of the spleen in the expression of variant antigens from *P. knowlesi* was initially observed in splenectomized monkeys. Thus, a schizont-infected cell agglutination test allowed demonstrating that splenectomized monkeys no longer expressed a highly variant multigene family, *pk235*, involved in antigenic variation whereas normal monkeys expressed different *pk235* genes in the course of chronic infections. Recently, a multigene family likely involved in antigenic variation in *P. chabaudi* has been identified and termed *cir* (*P. chabaudi* variant genes). *cir* genes, along with nine other multigene families whose function and subcelular localizations are unknown, are located within the subtelomeric regions of different (if not all) chromosomes. We are thus using mouse models to determine the role of the spleen in the expression of these multigene families in *P. chabaudi*. To this end, we have established chronic infections in normal and splenectomized BALB/c and CBA/J mice by using non-sterilizing treatments with chloroquine at each peak of parasitemia. This treatment allows us to study the pattern of transcription of all the subtelomeric gene families, including the *cir* genes, and to determine the extent of cytoadherence of these parasites after several passages in splenectomized animals.

Supported by CAPES, FAPESP and CNPq

BM129 - SYMPTOMATIC AND NON-SYMPTOMATIC MALARIA INFECTIONS ARE CAUSED BY THE SAME SET OF *PLASMODIUM FALCIPARUM* GENOTYPES

dalla Martha, R.C. (1), Pereira da Silva, L.H. (1), Wunderlich G. (2)

1: CEPEM, Br. 364, km 4,5 Porto Velho, Rondônia, Brazil. 2: Department of Parasitology, ICB-USP, São Paulo, Brazil

In order to elucidate if genotypes of *Plasmodium falciparum* parasites causing either symptomatic or non-symptomatic malaria infections were different from each other, *Plasmodium falciparum* parasites obtained from residents of different suburbs of Porto Velho were compared by means of microsatellite analysis.

Fourty-four samples, divided in five groups were analyzed: two groups of samples from non-symptomatic infections (Candelaria, taken at different timepoints) and three groups of samples from symptomatic patients (from Candelaria, Bate-Estaca and CEPEM, all from suburbs of Porto Velho-RO, Brazil) were analyzed using eleven different microsatellite markers. For each distinct group, the diversity index, the fixation index (F_{st}-value) and the percentage of mixed (polyclonal) infections was determined. All goups presented multiple infections to a similar degree. As expected, the group of samples from CEPEM showed the highest degree of heterozigosity, since samples were from patients of several different locations. There was no significant difference in heterozygosity between samples from asymptomatic and symptomatic infections of the same location. The largest genetic differentiation was observed between Candelária (asymptomatics) and Bate Estaca (symptomatics) samples, in spite of the geographical proximity of the two locations. This study confirms that the rather similar circulating *Plasmodium falciparum* genotypes, determined by microsatellite analysis, can either both cause symptomatic or asymptomatic infetcions and that the reason for this probably lies in the differential expression of virulence associated genes.