Contract N° 003716

TRYPADVAC2

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Specific Targeted research or innovation Project

GLOBAL ACTIVITY REPORT

Period covered: from 01/06/2005 to 31/05/2009 Date of preparation: 31/05/2009

Start date of project: 01/06/2005 Duration: 48 months

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Project coordinator organisation name: *Centre de coopération internationale en recherche agronomique pour le développement* ***(CIRAD)*** *Unité mixte de recherche IRD-CIRAD « Trypanosomes »* ***(UMR 017)***

Publishable final activity report

1. Summary description of project objectives

The aim of the project was to contribute to the improvement of livestock productivity in the developing world through limitation of trypanosome-associated pathology and accurate diagnostics of trypanosome infections.

It was proposed to develop immunization strategies against pathogenic factors of the parasites ("anti-disease" vaccine approach). In a previous INCO project under FP5, we had focused on congopain, an immunosuppressive cystein protease (CP) of *Trypanosoma congolense*. As the effects of immunization with congopain appeared limited, association with other antigens was deemed necessary. The current proposals expanded the initial work to screening and characterization of other pathogenic molecules, especially those responsible for anemia. A number of other trypanosome proteases, CPs, serine and metallo- proteases, required characterization of their biological roles in the parasite and the host. Trypanosomes also contain protease inhibitors, some of which have immunomodulatory effects. They may thus be manipulated, directly or in association with their partner enzymes, to modulate the disease. Other, non-proteolytic, pathogenic factors had been identified. The GPI (glycosyl phosphatidyl inositol) anchor of the *T. brucei* Variant Surface Glycoprotein (VSG) had been shown to be a major inducer of TNF, a cytokine that mediates anemia. Finally, development in the field of proteomics and progress in the genome mapping of trypanosomes provided tools to study new pathogenic pathways.

In order to improve diagnosis of the disease, procedures for antibody detection were to be developed and/or validated. They are based on recombinant technology, which circumvent problems associated with the current use of parasite extracts. Recombinant and synthetic peptides from CPs and heat shock proteins, both previously identified as major antigens, as well as newly described molecules, were to be assessed for their diagnostic potential.

2. Contractors involved

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4. Work performed

The work performed and results achieved during the four years of the project can be summarised under the following six themes:

1. Molecular studies on trypanosome proteases and their inhibitors (WP1, WP2, WP3, WP4, WP6)

The previous INCO-DEV Trypadvac project in FP5 centered exclusively on congopain. It was shown that congopain indeed acts as a pathogenic factor of *T. congolense* during infection, but immunization/challenge experiments remained inconclusive. The present FP6 Trypadvac2 project had proposed to expand the anti-disease vaccine concept to other pathogenic factors, while still attempting to improve the protective potential of congopain.

Congopain-like cysteine proteases (clan A enzymes).

Knowledge of the three-dimensional structure of congopain is essential for the production of an effective anti-disease vaccine based on congopain. It is all the more important that not only does congopain dimerises at physiological pH (a unique feature of this class of proteases), but also that protective epitopes may be dimmer-associated. For this endeavor, it was first shown that congopain is sufficiently stable at the high concentrations required for crystallization. As problems were encountered in the purification protocol initially developed, the methodology was improved, through the application of three phase partitioning, followed by molecular sieve and ion-exchange chromatography. This resulted in the production of large amounts of the protein, that was sent to the Institut de Biologie Structurale de Grenoble for crystallization. In the interim, the dimerisation mechanism was investigated through site-directed mutagenesis based on a theoretical model, allowing defining the regions of the molecule involved. Alternative forms of congopain, with the active site cysteine mutated to alanine as well as an inactive, full length congopain variant were also sent for crystallisation to improve chances of success. Unfortunately, as per the end of the project, no crystals were obtained, and future work shall refine the screens and also consider alternative fragments of the molecule.

An exhaustive analysis of the variability of congopain-like CPs was completed during the project. Several congopain-related enzymes were identified by various methods, including constructing and screening a subgenomic library, using PCR with degenerate primers, and by bioinformatics data-mining. Interestingly, the bulk of variation occurs at the vicinity of the catalytic site, what could have implications for host-parasite interactions. The most exciting discovery was the presence of a serine residue instead of a cysteine in the active site of the variants. More importantly, this variability was confirmed at protein level, using two-dimensional analysis of the proteome of *T. congolense* by western blotting. Some variants were expressed as recombinant proteins and characterized, showing that indeed the variant Cys / Ser are active, with cysteine proteinase characteristics, an occurrence described for the first time. The optimum activity of the variant at physiological pH raises interesting questions regarding their role in host-parasite relationships. However, as far as vaccine is concerned, the high level of sequence identity makes the variability unlikely to have any incidence.

The protective potential of congopain was further analysed by comparing the effect of different adjuvants on anti-congopain antibody production. An experiment was conducted in mice to compare the efficacy of eight conventional adjuvants using congopain as the model, following four parameters: height and sustainability of the response, booster effect of infection, and inhibition of protease activity by the resulting antibodies. As expected, no single adjuvant emerged as the “best”. For this reason, the three that gave the most adequate response were tested anew in cattle. Two adjuvants, QuilA (Saponin-based) and Adjuphos (Alum-based) gave comparable and synergistic results. Consequently both were used together in the immunization/challenge experiment with a cocktail of recombinant pathogenic factors detailed at the end of this section. Moving away from conventional adjuvants towards molecular adjuvants, an assessment of the adjuvant potential of α_2-

Macroglobulin was conducted in goats in Mozambique, to confirm the general validity of the results obtained in rabbits in the previous project. The findings confirmed the ability of this molecule to induce antibodies that inhibit the activity of congopain. Finally, we sought to investigate the potential of the BiP/hsp70 of *T. congolense* (a protein initially identified as a diagnostic candidate) as a molecular adjuvant, through the engineering of different genetic chimeras between C2 and various domains of BiP. Most constructs expressed an insoluble protein that required the development of complex protocol of solubilisation, purification under denaturing conditions, and renaturation. The constructs were ready to be tested by the end of the project.

The success of an anti-disease vaccine also goes hand in hand with establishing the role of the pathogenic factor, congopain and related CPs, in the host-parasite relationship. This was attempted using reverse genetics and to this end, recombinant RNAi transfer vectors were generated for both congopain and oligopeptidase B (see hereafter for the latter), in *T. brucei* and *T. congolense*. The technology was transferred from France to South Africa, with the establishment of cultures of *T. congolense* procyclic and trypomastigote forms, and of parasite transformation techniques. Significant progress was made and the first results of gene silencing obtained at the end of the project are encouraging.

Homologues of congopain in African *T. vivax* and in South American species of trypanosomes (*T. vivax and T.* evansi) were also studied, through cloning of their genes and recombinant expression. Two forms of evansain were cloned and expressed (glycosylated and non-glycosylated) in *Pichia pastoris*. Although they appeared inactive, thwarting any attempt to enzymatic characterization, they showed some strong potential in diagnostics. Three full-length clones of the *T. vivax* Cathepsin L protease called vivaxpain were obtained from a *T. vivax* Venezuelan isolate using primers based on the peptidase sequence from the *T. vivax* Y486 African strain. Sequence analyses and alignment showed 81% identity between African and American *T. vivax* strains. The vivapain cloned from an African strain was expressed as an active protein, and characterized. The protein is glycosylated, and displays differing substrate specificity from congopain, suggesting the possibility for species-specific diagnostics, as well as from that of mammalian cathepsin L, which may be exploited in peptide based chemotherapy. As a mirror to what was done with *T. congolense* BiP/hsp70 and congopain, a South American *T. vivax* vivapain-HSP70 peptide binding domain construct was cloned and expressed in a yeast system for testing as a vaccine.

Cathepsin B (clan A enzyme)

An exciting finding of the project has been the discovery of numerous cathepsin B-coding genes in the trypanosome genome. Whereas the T. b. brucei and T. vivax genomes only showed one or two genes, respectively, coding for cathepsin B (CB), 14 different genes were found dispersed in the T. congolense genome. Sequence analysis showed that these genes are conserved within the savannah isolates, which is a prerequisite for protection trials. The proteases show only 25% identity with congopain and related enzymes, and cluster in two major families based on amino acid sequence alignment. CBc1 to CBc6 share the classical catalytic amino acid triad (C,H,N) and CBs7 to CBs13 contain unusual catalytic amino acid triads (S,X,N), reminiscent of what was discovered in the congopain variants. Two representative isoforms from each family were characterized. Successful expression in Pichia pastoris necessitated the disruption of the glycosylation sites in the proteases by site-directed mutagenesis. Attempts at maturation of the 38-40 kDa CBc1, CBc9 and CBc12 proforms and the higher molecular weight glycosylated CBc6 proform, was only successful for CBc1 and CBc6, resulting in the mature 27 and 30 kDa forms. Following further maturation attempts, CBc9 and CBc12 were processed with pepsin, but the mature forms were not active. Whereas CBc1 showed both endo- and carboxy-dipeptidase activity, CBc6 had only exopeptidase activity. Unlike its mammalian homologue, TcoCB1 is not inhibited by the leupeptin group of inhibitors. However it is sensitive to the cysteine proteinase class specific inhibitor E-64 and the cathepsin B specific, cell-permeable inhibitor CA-074. This inhibitor was trypanostatic in in vitro cultures of T. congolense and rapidly killed trypanosomes at higher concentrations. It also increased the accumulation of undigested BSA in lysosomes, suggesting that cathepsin B possibly

plays a role in endocytosed protein degradation and in the survival of T. congolense bloodstream forms in vitro.

Antibodies were produced to study differential expression of CB in bloodstream, procyclic, epimastigote and metacyclic trypanosome forms. Processed CB was present in large amounts in bloodstream forms (in vesicles between the nucleus and flagellar pocket) and at very low levels in epimastigotes. At the messenger level, mRNA coding for CBc12 was present in all stages, while the mRNA coding for CBc1, 2, 3, 4 and 5 was present in bloodstream forms.

The sera from *T. congolense* infected trypano-susceptible Boran cattle showed the presence of anti-CBc1 and –CBc9 antibodies. While the catalytic domain of CBc1 elicits an early, pronounced humoral response to CBc1, and a lower response to CBc9, almost no response was detected against the catalytic domain of congopain. These results suggest that CBc1 is a good diagnostic candidate for *T. congolense* infection, and this will be followed up as a possible species-specific diagnostic test.

Towards assessing the functional role of the different proteases characterized in *T. congolense* using reverse genetics tools, a *T. congolense in vitro* culture system and transformation of parasites were developed in the fourth year. The greatest stumbling block has been the long-term *in vitro* culture of bloodstream forms (BSF) and stable differentiation of the BSF into the various life cycle stages *in vitro*. A new enriched adaptation medium was designed for the isolation of BSF from infected mouse blood and for their long term *in vitro* culture for drug trials and preservation through freezing and transfection assays. Furthermore the efficiency of procyclic form transfections was improved. A further break-through was setting up a tetracycline inducible system for the *T. congolense* IL3000 reference strain. Techniques to transform the parasites into metacyclics in both wild type isolates and genetically modified parasites in a significantly shortened time were also achieved. The ability to perform *in vitro* metacyclogenesis as well as transgenic procyclic form technique are essential tools to investigate the functional role of *T. congolense* genes through the life cycle as well as *in vivo* during experimental infections in mice. The development of these new tools, although they required major time and efforts, constitute one of the main achievement of this project.

Metacaspases (Clan CD enzymes).

Metacaspases are cysteine protease present in plants, fungi and protozoa, but not in mammals. As a consequence they have diagnostic and vaccine potential in the context of this project. There are five metacaspase genes in *T. b. brucei*, three of which are predicted to encode active cysteine peptidases (MCA2, MCA3 and MCA5) while two other (MCA1 and MCA4) have a substitution of the active site cysteine by a serine, as observed in congopain and cathepsin B variant in this project, hinting that they are probably inactive. *MCA2* and *MCA3* were shown to be expressed only in the mammalian bloodstream form of *T. brucei*, whereas *MCA5* is also expressed in the insect procyclic form. *T. brucei* metacaspases do not seem to be directly involved in programmed cell death, however, the proteins may have a function associated with RAB11 vesicles that is independent of known recycling processes of RAB11-positive endosomes.

Active, recombinant MCA2 and MCA3 were expressed in an *E. coli* expression system as a His-tagged fusion protein. The N-terminal His-tag was lost during expression, attributed to intrinsic MCA activity (since inactive active site cysteine mutants $MCA2^{Cys\Delta Ala}$ and $MCA3^{Cys\Delta Ala}$ did not lose the His-tag). N-terminal sequencing of the processed peptidases showed that processing occurs on the C-terminal of an Arg residue. This residue was mutated into a Gly to alter the clivage site, and this allowed Ni-chelate affinity purification of MCA2 and MCA3. Autocatalytic cleavage of MCA2 was effected at 37ºC and occurred after a Lys residue, thus confirming the Arg/Lys specificity of MCA. The autocatalytic processing sites were confirmed by site-directed mutagenesis, but it was shown that autocatalytic processing is not essential for activity.

The two metacaspases with substitutions at the cysteine peptidase active site residues, MCA1 and MCA4, were studied as possible metacaspase regulators/modifiers or as being involved in alternative pathways for survival of parasites during deletion of *MCA2, 3, 5* genes. Whereas soluble

MCA1 could not be expressed, soluble MCA4 was readily expressed and purified, but showed no activity against a selection of fluorometric substrates. After site directed mutagenesis of the active site Ser residue to Cys ($MCA4^{S219C}$), peptidolytic activity was obtained. MCA4 expression was shown to be confined to the bloodstream form stage of the *T. brucei* lifecycle, using specific anti-MCA4 antibodies. Further studies will elucidate the P1 substrate specificity, requirement for calcium and its *in vivo* processing in the context of its possible key role in the parasite.

Trypanosomal serine oligopeptidases.

The genes coding for *T. congolense* and *T. vivax* oligopeptidase B were identified by bioinformatics mining of the Sanger trypanosome genome database, and the ORF cloned by PCR. *T. congolense* and *T. vivax* oligopeptidase B were recombinantly expressed as fusion proteins with GST, and the active protease could be cleaved directly on the affinity matrix and purified in its active form for characterisation studies. Recombinantly expressed OpdBTc or OpdBTv were enzymatically characterized and shown to be optimally active and stable at physiological pH and insensitive to plasma serine peptidase inhibitors (serpins) that confirm the peptidase's role as a pathogenic factor in the host's bloodstream. The substrate specificity regarding preferred amino acid residues in P1, P2 and P3 was determined as well as the influence of different class-specific inhibitors on the peptidolytic activity of OpdB. Leupeptin, antipain, TLCK, DCI and AEBSF were the most effective inhibitors, thus confirming that the enzymatic characteristics of OpdB from different trypanosomal species are broadly similar.

The translated amino acid sequence was used to identify immunogenic epitopes using a computer algorithm. Corresponding synthetic peptides were synthesized and anti-peptide antibodies were raised and affinity-purified. They recognise recombinant OpdBTc and OpdBTv to varying degrees in ELISAs and western blots. None of the anti-oligopeptidase B peptide antibodies however inhibited the activity of either OpdBTc or OpdBTv, in contrast with the antibodies produced against the whole recombinant OpdBTc or OpdBTv, that showed almost complete inhibition of ObdB activity. This suggests that OpdB is sufficiently immunogenic in animals to produce antibodies that inhibit the activity of OpdB.

Recombinant OpdBTc or OpdBTv was also used to immunize mice that were challenged with either *T. congolense* IL 1180 or *T. vivax* Y486 depending on antigen used for immunization. This work was conducted in Uganda. The challenged mice were monitored and analyzed for prepatent period, parasitaemia, PCV (anaemia development indicator) and survival/death. Although the parasitaemia levels were similar in control and trypanosome immunized mice, infection was established later in immunized mice, anaemia was less pronounced and these mice survived for longer. The work was repeated in South Africa, only with *T. congolense*, and leaded to the same results. A more thorough immunological analysis was however conducted in that case. Antibody levels peaked one month after the first inoculation and remained high over a 5-month period. Challenge with *T. congolense* (IL 1180) gave a boost in the immune response and the antibodies recognised recombinant and native OpdB in western blots. The anti-OpdB antibodies were also able to inhibit the peptidase activity of OpdB, with increased inhibition observed following trypanosome challenge. Like in the previous experiment, no substantial difference was observed in parasitaemia between mice immunized with OpdB and an irrelevant protein, VP2. However the prepatent period was slightly longer in OpdB immunized mice and these mice showed a survival time 40% higher, while their PCV increased slightly over the two-month period following infection. For this reason, Oligopeptidase B was incorporated in the multicomponent vaccine (see after), ultimate goal of the project.

The adjuvant potential of α_2-macroglobulin (α_2-M), already validated with congopain, was investigated in rabbits, through co-administration with recombinant oligopeptidase B (OpdB), in order to produce antibodies that inhibit OpdB. Recombinant catalytic domain of congopain, C2, was used to complex OpdB with α_2-M, resulting in a vaccine model that includes two pathogenic factors. Similar antibody levels were induced by immunisation with *T. congolense* and *T. vivax* OpdB in combination with alum or α_2-M. However, only those antibodies produced with alum

inhibited 100% of the activity of OpdB. Alum was also the most efficient adjuvant for anti-C2 antibody production, but the best inhibition of C2 activity was achieved using α_2-M as an adjuvant, confirming previous results.

RNAi and gene knock-out studies were undertaken to establish the roles of OpdB and related enzymes in the host-parasite relationships. Parasite transformed with RNAi-OpdB constructs (*p2T7^{ti}* / TREU183 29-13 system) showed down-regulation of the gene as shown by RT-PCR and quantitative PCR as well as western blot over a 15 day period. However, no particular phenotype seemed associated with this down-regulation, indicating that gene knock-out ought to be possible. For this purpose, both OpdB flanking regions were amplified from genomic *T. b. brucei* and *T. congolense* DNA and cloned into knock-out vectors. Several transfections for OpdB knockouts had to be done in bloodstream and procyclic forms of *T. brucei* and *T. congolense,* and finally a clone with a single copy of the OpdB gene knocked out was identified. An initial growth analysis of single knock-out parasites showed an insignificant effect of having a single copy OpdB modified in trypanosome cultures. At the end of the project, a double knock-out clone was obtained, showing no obvious effect on cultured parasites. These *opdb*$^{-}$ parasites will be used to study the involvement of OpdB in host-parasite relationships in *in vivo* experiments.

Natural inhibitors of trypanosomal cysteine and serine proteases.

The general objective within the project was to characterise the pathogenic roles and examine the "protective potential" of natural peptidase inhibitors of parasite origin: inhibitors of cysteine peptidases (ICP) or inhibitors of serine peptidases (ecotin, also known as ISP).

Inhibitor of cysteine protease (ICP): The *T. brucei ICP* gene was identified by searching the genome databases at the Sanger Institute with the chagasin sequence of *T. cruzi*. Using the *T. brucei* gene, we were able to identify the *T. vivax* and *T. congolense ICP* genes. The *T. brucei* ICP had been successfully expressed in *Escherichia coli* using the pET28a expression plasmid. We took the same approach for the other trypanosome ICPs. Soluble ICP was purified by using FPLC techniques, with affinity chromatography using Ni-NTA agarose and ion exchange. Up to 3 mg of TbICP or TvICP could be prepared in each FPLC run from 1 litre of *E. coli.* This protein was tested for inhibitory activity towards Clan CA, family C1 cysteine peptidases such as papain and *L. mexicana* CPB. This showed that TvICP was a potent inhibitor of these cysteine peptidases.

Targeted deletion of the diploid *T. brucei ICP* (Tb*ICP*) locus was achieved by homologous recombination (gene Knock-out) in the Lister 427 strain. The presence of functional ICP in parasite lysates was assessed by measuring inhibition of CP activity. Taking advantage of the fact that ICP is thermostable, lysates were boiled in order to inactivate endogenous peptidases prior to incubation with papain. As expected, lysates of WT and Δ*icp*:*ICP* (single knock-out) inhibited about 60% of papain activity while no inhibitory activity was detected in Δ*icp* lysates, indicating that functional ICP is absent from Δ*icp*. We next assessed the amounts of functional CPs in lysates of BSF by enzymatic assays using fluorogenic substrates. The CP activity present in Δ*icp* lysates was 3-fold higher than in WT or Δ*icp*:*ICP*. Growth rate analysis of Δ*icp* in culture indicated that it grew more slowly than WT or Δ*icp*:*ICP* over a 5 day period. Δ*icp* did not have apparent alterations in morphology or in the cell cycle progression as compared to WT, suggesting that the reduced growth rate might be due to changes in the parasite's metabolism. WT and *ICP* mutant cell lines were inoculated in Balb/c mice and parasite density in the blood was examined from days 3-6. Surprisingly, Δ*icp* parasites grew better than WT parasites *in vivo*, reaching a significantly higher parasitemia than the WT or Δ*icp*:*ICP* lines. In addition, approximately 50% of the mice infected with Δ*icp* died at day 7, while the mice infected with WT survived until day 10 post-infection, indicating that deficiency in ICP increased the parasite's virulence in the mammalian host. If these results seem to argue against ICP as a pathogenic factor in trypanosomosis, they however seem to confirm that indeed, cysteine proteases such as cathepsins (cathepsin B, cathepsin L) are.

Synthetic CP inhibitors have been shown to kill *T. brucei* BSF in culture and their trypanocidal effect was associated with the inactivation of the cathepsin L-like CP of the parasite. Considering

that Δ*icp* parasites have higher CP activity, we decided to test if that could have an impact on their sensitivity to synthetic CP inhibitors. We observed that the growth of WT parasites was inhibited by 50% in the presence of 0.25 μM of N-Pip-F-hF-VSPh, while it was necessary to increase drug concentration 4-fold (to 1 μM) to observe a similar effect in Δ*icp* parasites. Δ*icp*:*ICP* had similar drug-sensitivity as WT parasites, confirming that the increased resistance displayed by Δ*icp* was due to lack of *ICP*. These results show that ICP levels affect BSF sensitivity to the trypanocidal effect of synthetic CP inhibitors and suggest that ICP modulates the availability of active CPs in the parasite.

We also generated an ICP deletion mutants in pleomorphic *T. brucei* cell line (STIB247). The plasmids used to generate ICP knockout mutants in Lister 427 were used to generate STIB427 null mutants, using standard transfection technology. PCR analysis confirmed the correct integration of the BSD and HYG integration cassettes. A full phenotype analysis of this line was not performed due to time constraint in the course of this project.

Inhibitor of serine protease (ISP): *T. brucei* encodes inhibitors of serine peptidases (ISPs) similar to ecotins, which are small proteins normally found in only a few bacterial species, including *E. coli.* Ecotins are inhibitors of trypsin/chymotrypsin-like serine peptidases (Clan P(S), family S1), which are notably absent in *T. brucei*. However, as these peptidases are abundant in both mammals and insects, ISPs very likely play an important role in host-parasite interactions. Two *T. brucei* ecotin-like gene (*TbISP1 and TbISP2*) were identified and cloned. Using the TbISP1 sequence for comparison, ISP gene sequence fragments in the *T. congolense* and *T. vivax* genome databases were identified and then cloned.

The same strategy as used to generate ICP *T. brucei* null mutants was employed with ISP1 and ISP2. New constructs for deletion of ISP1 with Blasticidin and Puromycin (pGL1947 and pGL1948) and for deletion of ISP2 with Hygromycin and Neomycin (pGL1959 and pGL1960) were created. The independent selectable markers allowed for selection of individual ISP1 and ISP2 null mutants, as well as having the potential to be used to generate double null mutants. The phenotype analysis of the mutants will have to be carried out however after the completion of the project.

Monoclonal antibodies: *Female* Balb/c mice were immunized with recombinant ICP, ISP1 and ISP2 (10μg/mouse/inoculation) emulsified in incomplete Freund adjuvant, with 6 intraperitoneal injections at intervals between 15 to 21 days. Serum antibody titer was determined by ELISA. Cell fusion was performed between spleen cells of the immunized mice and the myeloma cell line Sp2/0 using PEG-DMSO as fusogenic agent in a proportion of 10:1 cells respectively. Hybridoma selection was done in HAT medium by incubation at a 37ºC, 5% CO_2, 95% humidity. The supernatant screening was done by ELISA. Cells from positive supernatants were expanded in HAT medium and cloned by limiting dilution method. The specificity of each of the antibodies present in supernatants positive by ELISA, was confirmed by Western blotting using recombinant peptidase inhibitors and *T. brucei* extracts. From tested supernatants eleven of them were positive for ICP, eight for ISP1 and fifteen for ISP2. The Western blotting results for selected positive supernatants showed that only three of those towards ISP2 recognize the respective recombinant antigen (Mw circa 19,7 kDa) and were negative for ISP1. The polyclonal antiserum anti ICP, ISP1 and ISP2 recognize as expected the recombinant antigen as well a band with the expected MW on the parasite extracts.

Immunization-challenge experiments in mice: To test ICP as a vaccine, test animals were immunized with *Tb* ICP and the control animals received only PBS emulsified in titermax adjuvant, the animals were then challenged with *T.b.b* strain GVR-35 and then monitored for parasitemia, PCV, prepatent period and survival span post-infection. The course of infection was similar in both *Tb*ICP-immunized and non-immunized mice which suggests that ICPs may not be a good candidates for use as anti-disease vaccine development.

2. Proteomic studies – (WP6)

In this part of the project we endeavoured to identify, using proteomics tools, novel pathogenic factors from *T. congolense* and *T. evansi* to use as candidate antigens in an anti-disease strategy. The electrophoretic separation of the trypanosomal proteins and mass spectroscopy analysis of the separated protein bands were conducted in Partner 1's laboratory in collaboration with the INRA proteomic platform in Montpellier. Partner 1 and Partner 8's students at UKZN recombinantly expressed and characterised the identified proteins as diagnostic and vaccine candidates. Partners 9 and 12 identify strains of *T. congolense* and *T. evansi* of differing virulence and pathogenicity that underpins the identification of candidate pathogenic factors.

Scheduled objectives of the proteomic part of the project involved first to identify strains of *T. congolense* and *T.evansi* of differing virulence and pathogenicity, then to characterise them at the molecular level through mapping of the secretome (naturally excreted-secreted proteins) and proteome of defined strains, the final aim being to identify novel immunodominant antigens of *T. congolense* and *T.evansi* for use as diagnostic tools or therapeutic targets.

Biological and genetic characterisation of parasite strains of *T. congolense* and *T. evansi*/*T. equiperdum* of differing virulence and pathogenicity were done on a sampling of strains by experimental infection, and the most and least pathogenic selected for each species.

The first step to perform proteomics studies of trypanosome's pathogenic factors was to optimize the secretome production in *T. congolense* and *T. evansi*/*T. equiperdum* by using the "secretion medium" (patent pending) initially developed for *T. b. gambiense*. After successfull adaptation, we were able to produce reproducible batches of secretion and parasite pellets. We validated the procedures of protein extraction and purification of both the secretome and the whole proteome of *T. congolense* and *T. evansi*/*T. equiperdum* for further mapping and molecular characterization.

T. congolense secretome exhaustive molecular characterization:

Using 1-D 15 % acrylamide preparative gel in Trycine buffer as a secretome screening method coupled to the automated picking of protein bands for mass spectroscopy analysis (MS-MS, Q-trap 4000, Applied Biosystem), we identified 141 polypeptide sequences corresponding to 108 different proteins. More than 75 % of these proteins are predicted to be secreted, mainly through nonclassical pathway. Among the secretome identifed proteins, 87 % are enzymes (ProtFun server) and 86 % were allocated enzymatic or binding function, suggesting an active compartment dedicated to interaction with the host immune system. We selected 5 candidates for functional analysis with a particular attention to their "anti-disease vaccine" potential: paraflagellar rod protein and β tubulin (vaccine properties), cystathionine β synthase (drug target of DFMO, second line drug against trypanosomosis), tryparedoxin peroxydase and S-adenosyl homocystein hydrolase (drug response and parasite virulence). These proteins are still under analysis.

T. congolense secretome and proteome comparative mapping:

The 2-D DIGE technique allowed to establish a comparative map of proteins belonging to the secretome or to the whole proteome of *T. congolense*. Normalised protein quantification data of both secretomes and proteomes from the two parasite strains were used to group the proteins into clusters as a function of their differential expression (k-means method, MeV software). For secretomes, 3 clusters grouped the proteins exhibiting a differential of expression more marked for IL1180 (62 spots) and 3 clusters grouped proteins with a differential expression more marked for IL3000 (35 spots). For proteomes, 5 clusters grouped the 66 proteins exhibiting the more marked differential expression between the trypanosome strains: 2 clusters for IL1180 (17 spots) ; 3 clusters for IL3000 (36 spots). Interestingly, cysteine proteases were expressed as different isoforms as a function of their location (cellular or secreted) what strengthen their definition as candidates in an "anti-disease" strategy.

Identification of novel immunodominant antigens of T. congolense: the immuno-precipitation technique was performed using IgG that was isolated from the sera from a N'Dama

(trypnotolerant breed from West Africa) that had been infected multiple times with *T.* congolense and from sera of a naïve N'Dama. The isolated IgG was subsequently coupled to an affinity resin. Antigens which bound specifically to the infected IgGs were identified by mass spectrometry using the *T. congolense* protein database. (http://www.expasy.ch, "Trypanosoma congolense" entries). Some of the more interesting antigens identified include a phosphoglycerate kinase, a protein disulfide isomerase and an aminopeptidase, which is the most promising, alongside the ubiquitopus cystein proteases and tubulins. Upon database analysis of the *Trypanosomatidae* aminopeptidase genes, it appeared that these genes, specifically Family M1 (alanyl) aminopeptidases, occur as a family of three distinct genes. Further phylogeny analysis showed that one of these genes is unique to *T. congolense* and has no homologue in *T. brucei*. This could be of possible diagnostic use in differentiating between the species. Expression in recombinant systems and purifications of the corresponding proteases are ongoing.

T. evansi/T. equiperdum secretome molecular characterization, secretome/proteome comparative mapping and immunodetection: the secretome of three Venezuelan *T. evansi/T. equiperdum* isolates (Teva1, El Frio and TeGub 323) of differing virulence/pathogenicity has been compared. In agreement with the analyses of microsatellites carried out in collaboration with P2, Teva 1 was found to be distinct from the *T. evansi* group. Comparative mapping and immunodominant antigens characterization are ongoing through new collaborative project's grants.

3. Immunological and pathophysiology studies on anaemia - WP5

This theme focused on glycosyl phosphatidyl inositol (GPI) molecules that are cell membrane anchor molecules, shown to have pro- as well as anti-inflammatory activities. Regarding trypanosomosis a number of infection-linked pathological features are a direct result of the pro-inflammatory activity of GPI molecules. Consequently, vaccination with GPI was attempted to prevent infection-associated complications such as the incidence of severe anemia as well as host fat and energy metabolism disturbances. Specifically, the objective was to assess the protective capacity of a GPI vaccination for cattle to provide protection against *T. vivax* and/or *T. congolense* induced pathology.

Analysis of immune effector mechanisms in the pathology of experimental *T. vivax* infections was addressed using a battery of gene deficient mice. Results showed that parasitemia data can be translated into survival data, showing clearly that B-cells, CD4 T-cells, MHC-II, TNF, TNF-R1 and iNOS are all involved in the control of infection, while CD8, MHC-I and TNF-R2 are not. Using a battery of TNF and TNF-receptor gene deficient mice, we have shown that the TNF-mediated macrophage activation pathway is not involved in the induction of *T. congolense* or *T. vivax* associated anaemia, and this in contrast to the *T. brucei* induced anaemia. The results confirmed the importance of TNF and TNF-receptor 1 (TNF-R1) signalling in parasitaemia control, and confirmed the role of iNOS and B-cells as well. These results suggested the involvement of macrophages in the first weeks of parasite infection. This hypothesis was tested with mice deficient in different 'pattern recognition receptors'. Results showed the lack of TLR2 and 4 signalling in these events, and the lack of any correlation between macrophage activation signals and the development of infection-associated anaemia. Only the involvement of TLR9 (receptor for parasite DNA recognition) was found to play an additional crucial role in parasitaemia control (regular and efficient parasite clearance from the circulation).

Preliminary results of immunisation studies in mice with *T. brucei* derived GPI were encouraging. However, since *T. brucei* is not very pathogenic in mice, a further study was required to develop a GPI-based anti-disease vaccine for *T. congolense* and *T. vivax* that infect livestock. Work was conducted to confirm the possibility of generating a GPI-liposome based anti-disease strategy for experimental *T. congolense* infection and assess the possibility of generating a similar anti-disease strategy for *T. vivax* infections in mice. The results obtained regrettably showed that immune regulation of *T. brucei* infections differs substantially from those of *T. congolense* and *T. vivax*, and no GPI-driven immune modulation was derived from vaccination. Also cross-protection of *T. congolense*/*T. vivax* pathologies with *T. brucei* GPI was not achievable. Hence, the conclusion was

that although GPI-vaccination does protect the host against *T. brucei* infections, this could not be mirrored in *T. congolense/T. vivax* infected animals. Consequently GPI vaccination was not started in cattle and the budget reverted to the coordinator for redistribution.

4. Antibody and antigen detection tests based on previously identified molecules - WP7

In this part of the project we aimed to develop serologic diagnostic tests based on antigens previously identified, namely BiP (immunoglobulin heavy chain binding protein) subfamily of heat shock proteins 70 (Hsp 70), congopain, evansain and oligopeptidase B. The bulk of the development work was carried out at CIRAD in Montpellier by Partner 1 and while other partners were involved in providing field samples (Partners 9, 11 and 12 mainly), expertise (Partner 4), monoclonal control antibodies (Partner 6), or antigens (Partners 8 and 12). For various reasons developed below, the work has focused mainly on BiP, an immunodominant antigen in trypanosome infections, and at a late stage of the project also on evansain (Partner 12).

BiP-based inhibition ELISA (i-ELISA) :

Following the development of an indirect ELISA test based on a recombinant fragment of BiP, called C25, that showed good potential, but low sensitivity in primary infection (Boulangé et al. 2002), we attempted to improve the test through its adaptation into inhibition ELISA. The test is based on the use of recombinant MBP-BiP fusion protein and a mouse monoclonal antibody directed against a major epitope on the BiP molecule, reagents developed before the onset of the project. Using the fusion protein instead of a highly purified recombinant fragment, and a monoclonal antibody (mAb) such a test would allow 1/ to free oneself from the costly and time-consuming preparation and purification of C25, and 2/ to utilize the same set of reagent for testing all species. The initial development was conducted using bovine sera from both experimental and natural infections.

The BiP i-ELISA detects antibody in animals infected with either *T. vivax, T. congolense, T. brucei* and *T. evansi*, consistent with the fact that the epitope recognized by the mAb is conserved among all species of African trypanosomes and the BiP i-ELISA. Therefore the current test is a pan-trypanosome test. The test accurately detects re-infections and importantly does not show any cross-reactivity with putative homologues in tick-born pathogens. The BiP i-ELISA was validated with field sera and compared to other diagnostic methods including the standard buffy coat technique, PCR using specific primers and an indirect ELISA based on trypanosomal lysates. The sensitivity of the BiP i-ELISA was superior to all these tests.

Compared to indirect ELISAs using whole parasite extracts, the BiP i-ELISA shows a relatively rapid decline of antibody after treatment. The lack of persistence of anti-BiP antibody following trypanocidal treatment is a crucial aspect of the BiP i-ELISA performance.

The test was further validated with a wide range of field sera provided by the project partners, and the investigation of the efficacy of the test in other host species (sheep, camel, horse and even human). The cut-off values for each species had to be determined.

Species-specific indirect ELISA:

A species-specific indirect ELISA was designed based on peptide sequences unique for *T. congolense* and *T. vivax* BiP. The first set of peptides gave very good reactivity with infected sera, appearing indeed as major epitopes of BiP in natural infections. However, they did not appear species-specific, as sera from both *T. congolense* and *T. vivax* -infected cattle react strongly with both, *T. congolense* or *T. vivax* "specific" peptides. It was soon made obvious using irrelevant peptides that trypanosome-infected cattle, particularly in early infection, tend to react with all peptides, a common feature called polyclonal activation, and which render the development of

serologic tests for trypanosomosis so difficult. The peptides were used to raise anti-peptide antibodies in chickens, and their potential in species-specific capture-ELISA was examined.

Capture ELISA:

The anti-peptides antibodies raised in chicken did not prove useful in capture ELISA. While they seem able to capture the peptides against which they were raised, they did not capture the protein in native conformation, and the project had to be abandonned. Likewise, we experienced problems to revive anti-BiP and anti-congopain antibodies raised in the late 80s. Some new mAbs were raised in the frame of the project against the recombinantly-expressed proteins, but at too late a stage to allow developing capture ELISA tests. MAb against recombinant FL_{SYN} (a stable variant of full-length congopain) and recombinantly expressed Oligopeptidase B. Several MoAbs were produced against the three forms of FL_{SYN}, i. e a glycosylated form, the proenzyme and a mature form with most of the MoAbs recognising the proenzyme (and its glycosylated form). Sufficiently high levels of anti-OpdB antibodies were not produced in mice to conduct a hybridoma fusion for MoAb production. Hence, phage display library technology was used to produce recombinant antibodies (scFv) binding to OpdB and the scFvs are currently under characterization.

Test based on native evansain:

Native evansain purified from *T. evansi* trypanosome lysates (STev), called Ev-ConA, Ev-Bac as well as recombinant evansain was used in ELISA to evaluate the antigenic potential of native evansain in *T. evansi* infected horses. Horse sera from between 10 and 48 days post-infection were able to recognize STev, Ev-ConA or Ev-Bac coated in indirect ELISA. In a competitive PINC-ELISA two major peaks of antibody recognition of evansain was observed around 25 and 45 days post infection. Both native and recombinantly expressed evansain were found to be antigenic in horses. Two positive horse sera recognised purified 28 kDa evansian between 13 and 43 days post-infection, while sera produced later (27 to 41 days) recognized the 42 kDa recombinant evansain was recognized in western blots.

5. Antibody detection tests based on newly identified antigens – WP8

To develop serodiagnostic tests for *Trypanosoma congolense, T. vivax, T. brucei s.l.* and *T. evansi* several antigens and corresponding antisera were collected. Representative sera were selected from a collection of sera from natural and experimental infections of different mammal host species (table 1). Nineteen recombinant and three native antigens were provided by Partners on the projects (table 2).

A generic protocol for an indirect ELISA for the detection of IgG in serum or plasma samples was chosen for the evaluation of the putative antigens. It is excellent for water-soluble antigens and readily adaptable for different antigens and sera from different host species. For each antigen and host species, extensive cross titration generates data that allow defining optimal test conditions. This generic ELISA can further form the basis for an eventual inhibition ELISA that per definition is applicable to all different host species. Indices were defined to allow comparison of the different antigens and identify the antigen(s) best for distinguishing infection from non-infection sera.

Table 1. List of serum collections made available to the project to evaluate the antigen candidates

HOST	TRYP. SPECIES	ORIGIN	INFECTION	PROVIDER	PARTNER
Bovine	*T. b. brucei*	Belgium	Experimental	V.D. BOSSCHE	ITM
Bovine	*T. b. brucei*	Burkina Faso	Experimental	CIRDES	CIRDES
Bovine	*T. b. brucei*	Zambia	Experimental	MAATA	ITM
Bovine	*T. b. brucei*	Benin	Experimental	DOKO	ITM
Bovine	*T. congolense*	Belgium	Experimental	V.D. BOSSCHE	ITM
Bovine	*T. congolense*	Burkina Faso	Experimental	CIRDES	CIRDES
Bovine	*T. congolense*	Belgium	Experimental	KONE	ITM
Bovine	*T. congolense*	Gambia	Natural + experimental	MATTIOLI	ITM
Bovine	*T. vivax*	Burkina Faso	Experimental	CIRDES	CIRDES
Bovine	*T. vivax*	The Gambia	Natural + experimental	MATTIOLI	ITM
Bovine	*T. co. + T. vivax*	The Gambia	Natural + experimental	MATTIOLI	ITM
Bovine	*End. controls*	Mozambique	Natural	NEVES	UEM
Bovine	*T. evansi*	Venezuela	Natural	GONZATTI	USB
Goat	*T. b. brucei*	Belgium	Experimental	NDAO	ITM
Goat	*T. b. brucei*	Belgium	Experimental + vaccin.	CRISTA	ITM
Goat	*T. congolense*	Belgium	Experimental	NDAO	ITM
Goat	*T. congolense*	Mozambique	Experimental + vaccin.	NEVES	UEM
Goat	*T. vivax*	Belgium	Experimental	NDAO	ITM
Goat	*T. evansi*	Gran Canaria	Experimental	GILLINGWATER	ITM
Horse	*T. evansi*	Venezuela	Natural	GONZATTI	USB
Horse	*T. evansi*	Gran Canaria	Natural	GUTIERREZ	ITM
Camel	*T. evansi*	Gran Canaria	Natural	GUTIERREZ	ITM
Camel	*T. evanis*	Niger	Natural	VERLOO	ITM
Human	*T. b. gambiense*	DR Congo	Natural	THARSAT	ITM
Human	*T. b. gambiense*	DR Congo	Natural	THARSAT	ITM
Human	*T. b. gambiense*	DR Congo	Natural	BWA	ITM
Human	*T. b. gambiense*	Sudan	Natural	MSF	ITM

A total of 22 antigens provided by different partners were tested with sera from several host species (bovine, goats, sheep, camel and human) infected experimentally or naturally with different Trypanosoma species (*T. brucei, T. congolense, T. vivax, T. evansi*). From the recombinant antigens, seven show clear potential as diagnostic antigens (indicated by X in the table). Unexpectedly, no antigen showed specific reaction with sera from *T. vivax* infections in goat and bovine.

From the native antigens, the diagnostic value of VSG RoTat 1.2 and AnTat 1.1 for respectively *T. evansi* and *T. brucei* infections in camel, goat and bovine was known. They were used merely as a reference with which the other antigens could be compared.

For each host species (bovine, goat, sheep, camel, human) a standardised ELISA protocol has been established that allows not only to test the diagnostic value of unknown antigens but that can be used as serodiagnostic antibody test format to test for the different *Trypanosoma sp*. infection, except for *T. vivax.*

Table 2: List of antigens tested with indication of diagnostic potential.

					Diagnostic potential for infection with			
	Taxon	**Protein**	**Origin**	**Expression system**	**T. brucei**	**T. evansi**	**T. congolense**	**T. vivax**
1	***T. brucei gambiense***	ISG75	rec	*E. coli*	X	X		
2	***T. brucei brucei***	ISG75	rec	*P. pastoris*	X	X		
3	***T. congolense***	FL28	rec	*P. pastoris*	X		X	
4	***T. vivax***	OpdB	rec	*P. pastoris*	X		X	
5	***T. evansi***	VSG RoTat 1.2	rec	*P. pastoris*		X	X	
6	***T. evansi***	cathepsin L fr 1	rec	*P. pastoris*			X	
7	***T. congolense***	ICP	rec	*P. pastoris*			X	
8	***T. brucei***	ICP	rec	*E. coli*				
9	***T. brucei***	ISP1	rec	*E. coli*				
10	***T. brucei***	ISP2	rec	*E. coli*				
11	***T. vivax***	ICP	rec	*E. coli*				
12	***T. evansi***	cathepsin L fr 2	rec	*P. pastoris*				
13	***T. evansi***	cathepsin L fr 3	rec	*P. pastoris*				
14	***T. evansi***	Cysteine peptidase (evansain)	rec	*P. pastoris*				
15	***T. congolense***	OpBTc2	rec	*P. pastoris*				
16	***T. congolense***	OpBTc3	rec	*P. pastoris*				
17	***T. congolense***	HPCBc1 (cathepsin B)	rec	*P. pastoris*				
18	***T. vivax***	TvHPCB (cathepsin B)	rec	*P. pastoris*				
19	***T. vivax***	HSP 70 pept 4	synth					
20	***T. brucei***	VSG AnTat 1.1	native		X			
21	***T. evansi***	VSG RoTat 1.2	native			X		
22	***T. evansi***	VSG p64	native			X		

T. evansi cathepsin L recombinants, unexpectedly, only detected *T. congolense* infections in goats and cattle. No antigen has been identified to date that specifically detects *T. vivax* infections. An extensive collection of animal and human sera from trypanome infected individuals are now available as well as a number of antigens that show potential as diagnostic agents for *Trypanosoma* (with the exception of *T. vivax*) infection.

The most promising antigens for detection in the respective species were found to be *T. congolense* cathepsin B and sera of goats and cattle infected with *T. congolense*, native RoTat1.2 VSG and goats experimentally infected with *T. evansi* and *T. b. brucei*, VSG p64 with Venezuelan cattle infected with *T. evansi*, recombinant *T. b. gambiense* ISG75 and camel sera, *P. pastoris* expressed *T. b. gambiense* and *T. b. brucei* ISG75 and goat sera. Seven of the nineteen recombinant and three native antigens show clear potential as antigens for the diagnosis of *T. b. brucei*, *T. evansi* and *T. congolense* infection in several host species. However, not a single antigen showed potential for specific diagnosis of *T. vivax* infection. A standarised ELISA protocol was developed to test goat, sheep, bovine (and buffalo), camel and human sera. An important finding was the use of yeast culture medium as diluent for sera tested with *Pichia* expressed proteins.

Stability of the recombinant antigens should be studied in detail before considering further development. Since not all sera reacted positively with these antigens, combinations of antigens will remain necessary for a broad spectrum diagnostic test.These antigens will now be used to test the operational performance of these ELISA tests on target populations and to incorporate the best performing antigens into rapid lateral flow or agglutination tests.

6. Assessment of the “anti-disease” protective potential of a pathogenic factor cocktail vaccine (across workpackages).

Six antigens developed during the project under workpackages WP1, WP2, WP3 and WP4 (CB1, CB12, C2, FL28, OpdB and ICP) were selected for testing in an immunization/challenge field trail.

The immunization/challenge experiment in large ruminants was conducted in three sites: Partner 10 (Makerere University, Uganda), Partner 9 (CIRDES, Burkina Faso) and Partner 11 (UEM, Mozambique) as per an agreed common protocol, while Partner 4 (ITG, Antwerp) provided all the consumables required for sample collection and storage and advised on standardization of

procedures. Cattle/goats selected tested negative against trypanosome lysate and the six test antigens. They were adapted to housing conditions, treated prophylactically against worms and ticks and it was ensured that pre-immunisation mean weight and PCV fall within a 10% range.

Animals were randomly assigned to a test antigens (7), control antigens, VP4 (bacterial-expressed viral protease) and OVA (chicken egg ovalbumin) (7) or control- no antigens (4) group. Protease antigens were administered at separate sites with a mix of QuilA (saponin) and Adjuphos (aluminium phosphate) adjuvant three times at three week intervals. Animals were bled weekly and serum stored for testing. Animals were infected with a chronic *T. congolense* strain 3 weeks after the last booster (intravenously 10^3 parasites or bite of 3 to 5 infected tsetse flies). Animals were bled daily to determine prepatent period, PCV and parasitaemia and treated/removed when PVC dropped below threshold.

Heifers from an area in Burkina Faso that is exempt of tsetse-flies showed that the antigens are very immunogenic, but it does not seem that the immune response is enough to protect the animals against infection and its deleterious effects. One hypothesis for this non-protection could be too much delay between injections. The dose of parasite (10^3) used for infection could also have played a role. Cyclic infection (through the bite of infected tsetse flies) would be recommended, to mimic natural infection. The results obtained are nonetheless encouraging and deserve to pursue the experimentations, taking into account the above observations.

Some disease protection was afforded by immunisation of Ankole cattle in Uganda with the test antigens, but although not fully protected, it contrasts with the control animals that experienced serious disease effects. These results were confirmed after re-challenge with another *T. congolense* strain after cattle self cured after the first challenge. There were no significant differences in parasitaemia and temperature fluctuations, but test antigen immunised animals maintained weight as opposed to control groups that lost some weight. Antibodies from test antigen immunised animals inhibited the enzymatic activity of C2 and to a lesser extent CB1, further suggesting an anti-disease effect of the vaccination.

Goats immunized in Mozambique with the cocktail of pathogenic factors showed a greater recovery in PCV after infection than the control groups. These goats also showed lower parasitaemia levels and weight gain following infection indicating that the vaccine cocktail had a positive effect on disease development.

Deliverables

For all workpackages, most of the objective were reached, and in some cases, exceeded. The table 3 shows the list of deliverables and their state at the end of the project, while the status chart on table 4 shows the level of achievement of project tasks.

Concerning the anti-disease strategy, objectives were not reached in GPI-anchor vaccination experiments in cattle, the reason being no protection was observed in mice, there was hence no need to embark on costly cattle experiments. Some delays were incurred in the cristallisation of congopain, but results are pending. Likewise, progresses were made in the obtention of null mutants for cathepsin L (congopain), cathepsin B, oligopeptidase B, and ISP 1 and 2, but the analysis of phenotypes *in vitro* and *in vitro* is ongoing.

On the diagnostic tests front, a large number of antigens were tested, and some tests validated. Species-specific indirect-ELISA tests could not be achieved, due to the close-proximity of the antigenic fabric of the three main species of parasites. The preliminary results regarding the use of cathepsin B as a *T. congolense*–specific test however are encouraging for this particular theme. Antigen- (capture-) ELISA, that has occupied scientists in the field for the past 20 years, still remains elusive, in our case due to problems encountered to obtain monoclonal antibodies in a timely manner. Finally, the ultimate withdrawal of DiaMed in year 3, the private partner whose role was to adapt test to rapid format, implies that this objective could not be reached.

Table 3 – List of deliverables

Del. no.	Deliverable name	WP no	Date due (project month)	Lead contractor
D1	A Web-based Presentation of the project and communication site	WP9	3	P12
D2	Recombinant oligopeptidases B	WP2	6	P8, P1
D3	Synthetic peptides of oligopeptidases B and specific antibodies	WP2	12	P8
D4	Recombinant CPs from *T. vivax* and *T. evansi*; specific antibodies	WP1	12	P12
D5	Novel trypanosome proteases; corresponding gene sequences; recombinant molecules	WP3,WP1	24	P2
D6	Purified GPI from VSGs of *T. congolense* and *T. vivax*	WP5	12	P5
D7	A collection of sera from naturally infected cattle	WP6,WP7,WP8	12	P9, P11
D8	3-D structure of congopain	WP1	18	P1
D9	Vaccine strategy with mixed GPI	WP5	24	P5
D10	Novel pathogenic factors of *T. congolense*	WP6	24	P1
D11	Novel pathogenic factors of *T. evansi*	WP6	24	P12
D12	Recombinant inhibitors of cysteine and serine proteases (ICP, ECO)	WP4	24	P3
D13	A list of antigens with confirmed diagnostic potential	WP8	24	P4, P1
D14	Pan-species antibody detection tests	WP7, WP8	24	P1, P4, P11, P13
D15	*T. congolense* CP null mutants (KO or RNAi)	WP1	24	P1
D16	Null mutants (RNAi) for novel proteases of interest	WP3	24	P2
D17	*T. brucei* null mutants for ICP and ECO (KO or RNAi)	WP4	24	P3
D18	Monoclonal antibodies against trypanosome proteases and inhibitors	WP1,WP3, WP4	30	P6
D19	Species-specific antibody detection tests	WP7,WP8	30	P1, P4, P13
D20	Antigen detection ELISAs	WP7	36	P1
D21	A list of antigens with confirmed protective potential for development of a multicomponent anti-disease vaccine	WP1,WP2, WP3,WP4,WP5, WP6	36	P1, P2, P3, P5, P8, P9, P10, P11, P12
D22	Annual and final reports of the project	All WP	12, 24, 36, 48	All P
D23	Final plan for using and disseminating knowledge	All WP	48	P1

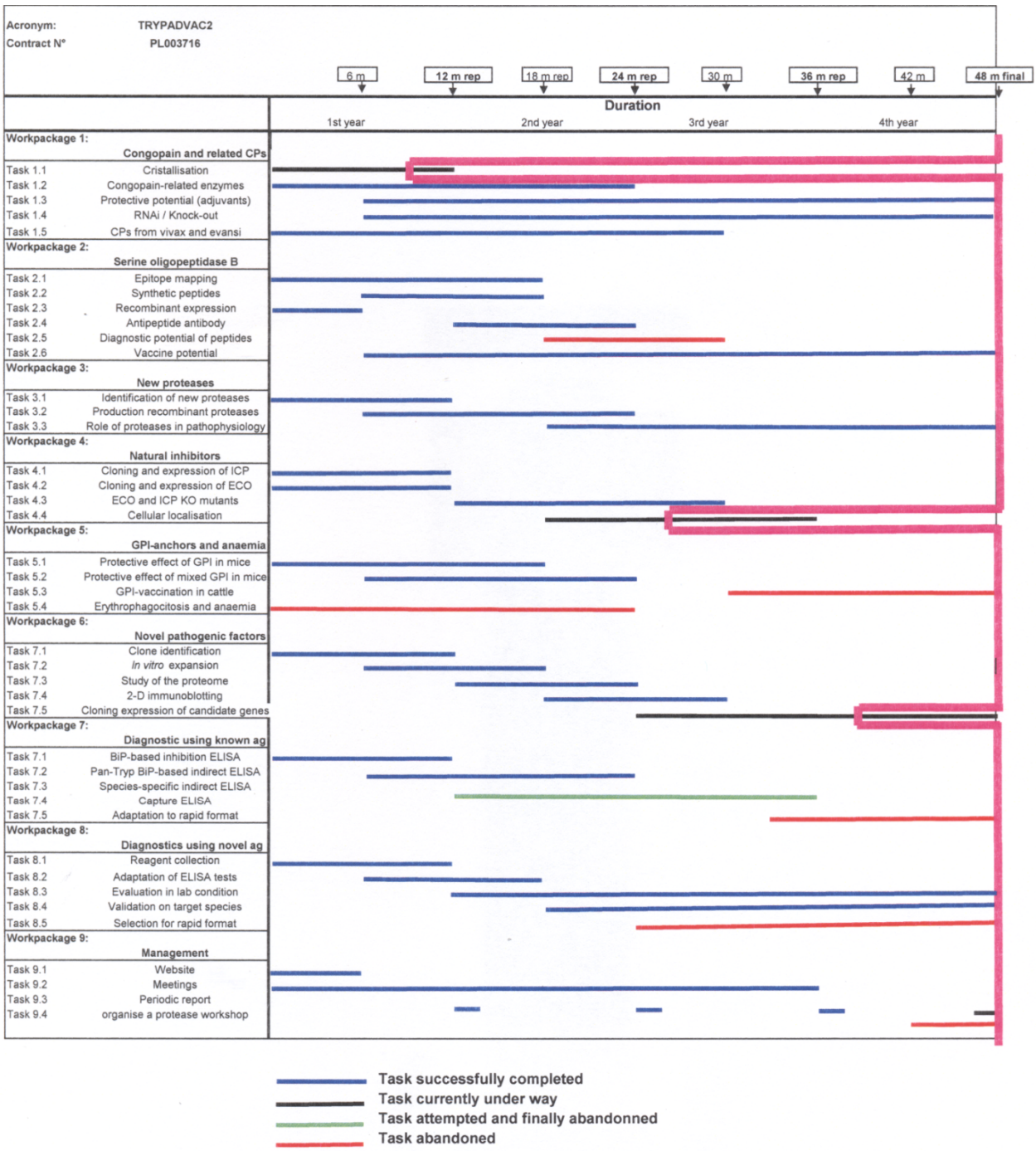
Completed and delivered earlier

Completed and delivered in this report

Failed, or abandoned

Ongoing, completion after the end of the project

Table 4 – bar chart for the project tasks.



Conclusions

In the course of this project, significant progresses have been made towards the development of an anti-disease vaccine and diagnostic tests for trypanosomosis. For both themes, the results at the end of the project may not appear sensational. One must keep in mind though that we are dealing here with a particularly shrewd parasite, with highly evolved mechanisms to evade host immune response, making the development of a vaccine, and as a corollary development of serologic tests, a particularly daunting task.

We have seen that animals immunized with a cocktail of pathogenic factors did generally better than controls, validating the concept of an anti-disease vaccine, but at a level that would not warrant pursuing commercially the venture in the present form. Likewise, some antigens with a diagnostic potential were characterized, and some tests validated, but sensitivity often remained low, and optimization work is required. On upstream research level, positive outcomes are patent. A large number of trypanosomal pathogenic factors were characterized, through cloning, expression and enzymatic characterisation, and for some crystallization studies, and gene-silencing, a very important emerging technology. New tools were developed regarding *in vitro* culture and cyclical transformation of trypanosomes, analysis of parasite proteome and secretome, and molecular adjuvants.

The project has generating close to twenty papers in peer-reviewed, with another ten submitted or in preparation. It should also not be understated that the project served as the basis of twenty Master's theses and eight PhDs, and further strengthened North-South collaborations through important movement of personnel for technology transfer and capacity building.

A vaccine against trypanosomosis has been a venture of many decades, and the road may be still long. Our results, as disappointing as they may look, clearly bring some stones to the edifice, and prove worthwhile the efforts of the consortium and the support of the European Commission.

2 – Plan for using and disseminating the knowledge[1]

Section 1 - Exploitable knowledge and its Use

Overview table

Exploitable Knowledge (description)	**Exploitable product(s) or measure(s)**	**Sector(s)** of application	Timetable for commercial use	Patents or other IPR protection	**Owner** & Other Partner(s) involved
Pathogenic factors involved in the disease process of trypanosomosis	Multi-component anti-disease vaccine	Veterinary medicine	2010	Given the results, it is unlikely that a patent will be requested for this product	**All partners involved in the development phase: P1, P2, P3, P5, P8**
BiP as a diagnostic antigen	BiP-based inhibition ELISA; BiP-based indirect-ELISA	Veterinary diagnostics	2009	No patent foreseen	**P1**
Novel antigens with diagnostic potential	Indirect-ELISA, inhibition ELISA	Veterinary diagnostic, Human diagnostics	2010	No patent foreseen	**P4, P1, and each antigen developer**
Validated diagnostic antigens	Fast format tests (lateral flow, dip-stick, …)	Veterinary diagnostics, Human diagnostics	2011	According to further development, patent may be obtained	**P13, P4, and each particular antigen developer**

A cocktail of pathogenic factors has been tested as anti-disease vaccine in the course of year 4 at three locations in Africa. The outcome of these experiments, although generally encouraging, do not allow to foresee that a potentially commercially viable product may emerge, or if some more studies are necessary. It is important to keep in sight that this project is essentially upstream research,

Similarly, the development of diagnostic tests, if generally more advanced that its anti-disease vaccine counterpart, could become exploitable commercially only if it proves more sensitive and/or specific than already existing tests. At the end of the project, results are encouraging with some of the antigens, but more work is needed in the validation of these tests before a commercial venture could be considered.

Section 2 – Dissemination of knowledge

Overview table

Planned /actual Dates	**Type**	**Type of audience**	**Countries addressed**	**Size of audience**	**Partner responsible /involved**
September 2005	Project web-site	Higher education/ Research	**All**	Ind.	P12
October 2005	Scientific publication	Higher education/ Research	**All**	General	P12
May 2006	Scientific publication	Higher education/ Research	**All**	General	P3
June 2006	Scientific publication	Higher education/ Research	**All**	General	P5
June 2006	IVVDC Conference, Norway: 1 oral presentation and 1 poster	Higher education/ Research	**All**	150	P1
July2006	XXth SASBMB Conference, South Africa: 2 Posters	Higher education/ Research	**South Africa**	350	P8, P1
Aug 2006	IXth ICOPA Conference, Scotland: 3 posters	Higher education/ Research	**All**	2000	P1, P3, P8
Feb 2007	Book	Higher education/ Research	**All**	General	P3
March 2007	5ème Colloque « Protéolyse Cellulaire » SFBBM, France : 1 oral presentation	Higher education/ Research	**France**	400	P2
June 2007	STVM Conference, Mexico; 3 presentations	Higher education/ Research	**All**	600	P1
Sept 2007	scientific publications	Higher education/ Research	**All**	General	All partners
Nov 2007	Scientific publication	Higher education/ Research	**All**	General	P5
Dec 2007	Scientific publication	Higher education/ Research	**All**	General	P3
Jan 2008	Conference SASBMB; Grahamstown 2 oral presentations 1 Poster	Higher education/ Research	**South Africa**	350	P1, P8
Feb 2008	Scientific publication	Higher education/ Research	**All**	General	P8

Planned /actual Dates	**Type**	**Type of audience**	**Countries addressed**	**Size of audience**	**Partner responsible /involved**
Apr 2008	Scientific publication	Higher education/ Research	**All**	General	P2, P1, P8
June 2008	Scientific publication	Higher education/ Research	**All**	General	P4
Nov. 2008	Scientific publication	Higher education/ Research	**All**	General	P5
Dec. 2008	Scientific publication	Higher education/ Research	**All**	General	P1, P9, P12
Dec 2008	*BIT Life Sciences' 1st Annual World Congress of Vaccine (WCV-2008). Foshan, China.* 2 oral presentations	Higher education/ Research	**All**	900	P1, P2, P3, P8, P10, P11
Dec. 2008	Scientific publication	Higher education/ Research	**All**	General	P1
Feb. 2009	Course	Higher education/ Research	**Mozambique**	15	P1, P8, P11
Apr. 2009	Scientific publication	Higher education/ Research	**All**	General	P4
May 2009	Scientific publication	Higher education/ Research	**All**	General	P12
Jul. 2009	Scientific publication	Higher education/ Research	**All**	General	P4
Aug. 2009	Scientific publication	Higher education/ Research	**All**	General	P1, P9
Oct. 2009	Scientific publication	Higher education/ Research	**All**	General	P5
Nov 2009	Scientific publication	Higher education/ Research	**All**	General	P8; P1

The project website became available on line by the end of September 2005, and is regularly updated. Its content was extensively discussed during the first annual meeting, and improvements as well as added content are made on a monthly basis. Designed and maintained by partner 12 (University Simon Bolivar, Caracas, Venezuela), it is available at the address http://trypadvac2.eventos.usb.ve/.

The trypadvac2 consortium produced so far 18 papers in peer-reviewed journals, one from the first reporting period, two for the second reporting period, five for the third reporting period, et finally not less than 10 papers for the last reporting period. Six additional papers have been submitted, and about as many are in preparation. To be noticed is that several of these papers are co-publication between two or more groups being part of the consortium.

In February 2009, a two-weeks biotechnology course was co-organised by CIRAD (Partner 1), UEM (Partner 11) and Partner 8 (UKZN), and run at UEM in Mozambique, by the coordinator, A. Boulangé, two Ph.D student of Partner 8, D. Pillay and R. Kangethe, and a M.Sc. student of partner 11 (registered at UKZN, Partner 8), H. Mucache. It was co-funded by the Project (through an invoice to CIRAD) and the French Embassy in Pretoria (South Africa).

List of publications (peer-reviewed journals) deriving from the project

Published Book:

Trypanosomes. After the genome., **J.D.Barry, R.McCulloch, J.C.Mottram, and A.Acosta-Serrano,** eds. (Wymondham, Norfolk, UK: Horizon Bioscience). (2007).

Papers in peer-reviewed journals

González LE, García JA, Núñez C, Perrone TM, González-Baradat B, Gonzatti MI, Reyna-Bello A. Trypanosoma vivax: a novel method for purification fromexperimentally infected sheep blood. *Exp Parasitol*. 2005 Oct;111(2):126-9.

Helms MJ, Ambit A, Appleton P, Tetley L, Coombs GH, Mottram JC. Bloodstreamform Trypanosoma brucei depend upon multiple metacaspases associated withRAB11-positive endosomes. *J Cell Sci*. 2006 Mar 15;119(Pt 6):1105-17.

Magez S, Radwanska M, Drennan M, Fick L, Baral TN, Brombacher F, De Baetselier P. Interferon-gamma and nitric oxide in combination with antibodies are key protective host immune factors during trypanosoma congolense Tc13 Infections. *J Infect Dis*. 2006 Jun 1;193(11):1575-83.

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Antoine-Moussiaux N, Büscher P, Desmecht D. Host-parasite interactions in trypanosomiasis: on the way to an antidisease strategy. *Infect Immun.* 2009 Apr;77(4):1276-84. Review.

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