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**Analysis of the proteome and the secretome of animal trypanosomes: a standardized analytical method to look for new molecular targets.**

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**ABSTRACT**

The causative agents of animal trypanosomosis are various species of protozoan parasites belonging to the genus *Trypanosoma*, among which *T. congolense* and *T. evansi* are the major pathogenic species. The extra cellular position of the trypanosomes implies to consider both the parasite and its excreted-secreted factors in the course of the physiopathological processes. Proteome (i.e. parasite constitutive proteins) and secretome (i.e. naturally excreted-secreted proteins) analysis need to be conducted in parallel to identify key trypanosome's proteins potentially involved in both virulence and pathogenicity. We developed and standardised a method to produce purified proteome and secretome of the two sub-genus Trypanozoon and Nannomonas. The protocol is based on the production of trypanosome bloodstream forms by infection of naturally immunosuppressed rodents (Nude/SOPF®) and incubated in a defined secretion medium that mimics the blood biochemical environment deprived of cells and macromolecules. Supernatants representing secretome are separated from parasites pellets representing proteome by centrifugation and filtration and both are conditioned for further analysis in two-dimensional gel electrophoresis and mass spectrometry. The defined secretion medium appears to reduced expression of stress proteins. Two-dimensional difference gel electrophoresis (2-D DIGE) analysis of secretomes in particular confirmed both the differences observed in 1-D gels and the high

reproducibility between secretome batches of a same trypanosome strain. The molecular identification of differentially expressed trypanosomes molecules correlated with either the virulence process or the pathogenicity will provide new potential molecular targets.

## INTRODUCTION

Trypanosomes are the causative agents of the Human African Trypanosomiasis (HAT) or sleeping sickness, and of animal trypanosomiasis also called “Nagana”. Nagana slows down the development of breeding in 37 countries located in the most productive areas of the sub-saharian Africa (FAO, 2007). Fifty millions bovines and seventy millions small ruminants are estimated to be exposed. The consequence of this situation is the concentration of the breeding in the semi-arid zones with limited fodder resources.

Mammals can be infected by several pathogenic species of trypanosomes belonging to the three sub-genuses: *Trypanosoma brucei brucei* and *Trypanosoma evansi* for Trypanozoon, *Trypanosoma vivax* for Duttonella, *Trypanosoma congolense* and *Trypanosoma simiae* for Nannomonas. The main symptoms are: hyperthermia, hypertrophy of several organs, anaemia caused by haemolysis and erythropoiesis failure, degenerative, myocarditis and endocrinal troubles, Cachexy and reproduction troubles. The evolution of the sickness depends on the parasitemia, the host's sensitivity and its environment.

The concept of our study concerns the time of the infection in mammals, particularly the cell-cell interactions and the soluble factors released in the host's blood. On one hand, the trypanosome develops strategies to escape the immune response, on the other hand, due to the extra cellular localization; the trypanosome produces constitutive factors and excreted-secreted products (ESP), all factors leading to infection (De Souza, 2006). We distinguish two kinds of ESP: those actively secreted from the cytoplasm by the way of the flagellar pocket, and those directly excreted from the outer membrane. We developed a standardized method to characterize new molecular targets. This approach allowed us analyzing the proteome and the secretome of animal trypanosomes, illustrated here with *T. congolense*.

## METHODS

In a first time the method was tested with two *T. congolense* strains of the savannah type: IL1180 and IL3000, well known for their opposite virulence (Nantulya et al., 1984). This behaviour which has been observed in bovines during several trials in the field was reproduced successfully in rodents, so that rodents could be retained as an infection model.

The first step of the experimental schedule consisted in the infection of Nude SOPF rats (Charles River Laboratories): 250µl of cryoconserved and infected blood containing from a ¼ to ½ million of trypanosomes were injected intraperitoneally. Parasitemia was checked daily by microscopic examination of a drop of blood (Herbert & Lumsden, 1976). Blood was collected as soon as the parasitemia reached  $250 \times 10^6$  parasites per millilitre. The bloodstream forms were purified under sterile conditions using a DEAE cellulose column and phosphate buffer saline plus 1% glucose (PSG) at pH8 (Lanham & Godfrey, 1970). Then, the parasites were washed and concentrated by centrifugation, 3 times successively.

After purification the trypanosomes were incubated during 2 hours at 37°C and 5% CO<sub>2</sub> in a special secretion medium (Holzmuller et al., 2007) at the concentration of 200×10<sup>6</sup> parasites per millilitre. The viability and number of trypanosomes were monitored by flow cytometry every 15 minutes. After incubation the secretome was centrifuged and the supernatant was filtered on a 0,22 µm low-protein-binding filter. Anti-proteases (Complete cocktail, Roche) were added to both parasite pellet and secretome before storage at -80°C.

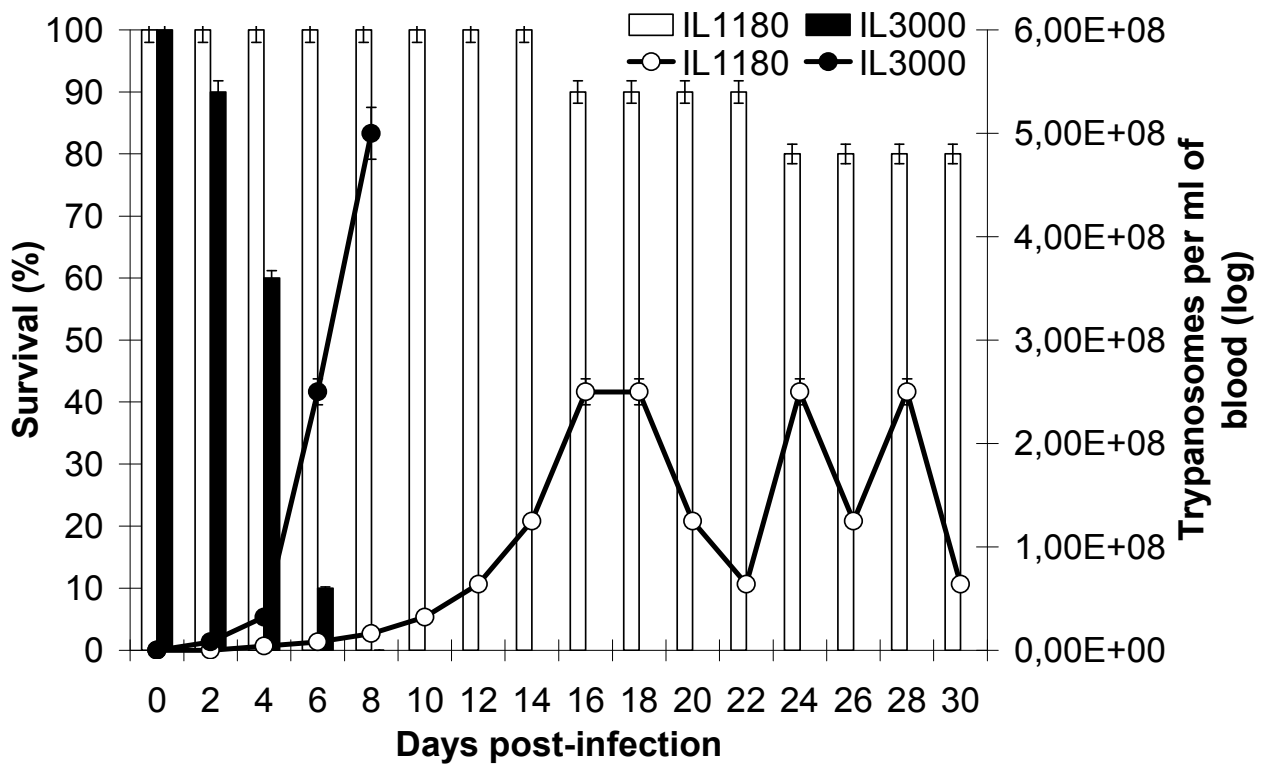
Following production, secretomes and whole parasite extracts (proteome) were submitted to a proteomic analysis. In a first step, the quality of the products has been tested using 1-Dimensionnal electrophoresis in a Trycine 12.5% polyacrylamide gel and the identification by mass spectrometry (MS-MS, Q-Trap 4000®, Applied Biosystem) was performed after automatic picking of the protein bands. In parallel, a 2-Dimensionnal electrophoresis was performed for a comparative mapping issue with DIGE method (Marouga et al., 2005).

## RESULTS

**Difference of virulence and pathogenicity of *T. congolense* strains in bovine and Nude rats.** Infection courses in both trypanosusceptible bovines (Table 1) and Nude rats (Figure 1) were monitored and compared between *T. congolense* IL1180 and IL3000 strains. *T. congolense* IL1180 exhibited a first parasitemia about 2 times delayed and 2-fold lower compared with IL3000. Moreover, the lower virulence of IL1180 was correlated with a survival rate of 80% of the mice in the time course of the experiment (*i. e.* 1 month) compared to full mortality induced by IL3000 (Figure 1).

<i>T. congolense</i> strain	Prepatent period average (days)	Parasitemia average (× 10 <sup>6</sup> trypanosomes per ml of blood)
IL1180	11.20 ± 0.7	71.14 ± 16.36
IL3000	9.50 ± 0.3	121.39 ± 77.39

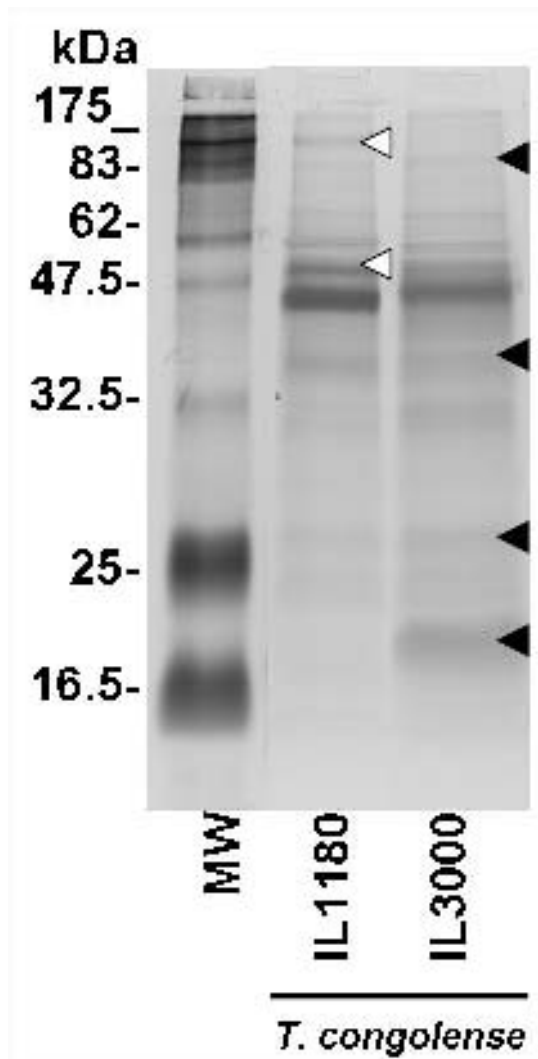
**Table 1.** Clinical follow-up of 1.5-2 old years heifers from north Burkina Faso (trypanosomes-free area) subcutaneously injected (by syringe) with 10<sup>5</sup> tryps/animal derived from in vivo culture in NMRI mice. Parasitemia was evaluated every 2 days on a fresh drop of blood.



**Figure 1.** Clinical follow-up for virulence and pathogenicity of *T. congolense* strains in Nude rats. After intraperitoneal inoculation of about  $100 \times 10^6$  trypanosomes, evolution of infection was monitored considering parasitemia (curves) and survival level (bars) reached in the time course of the experiment (1 month).

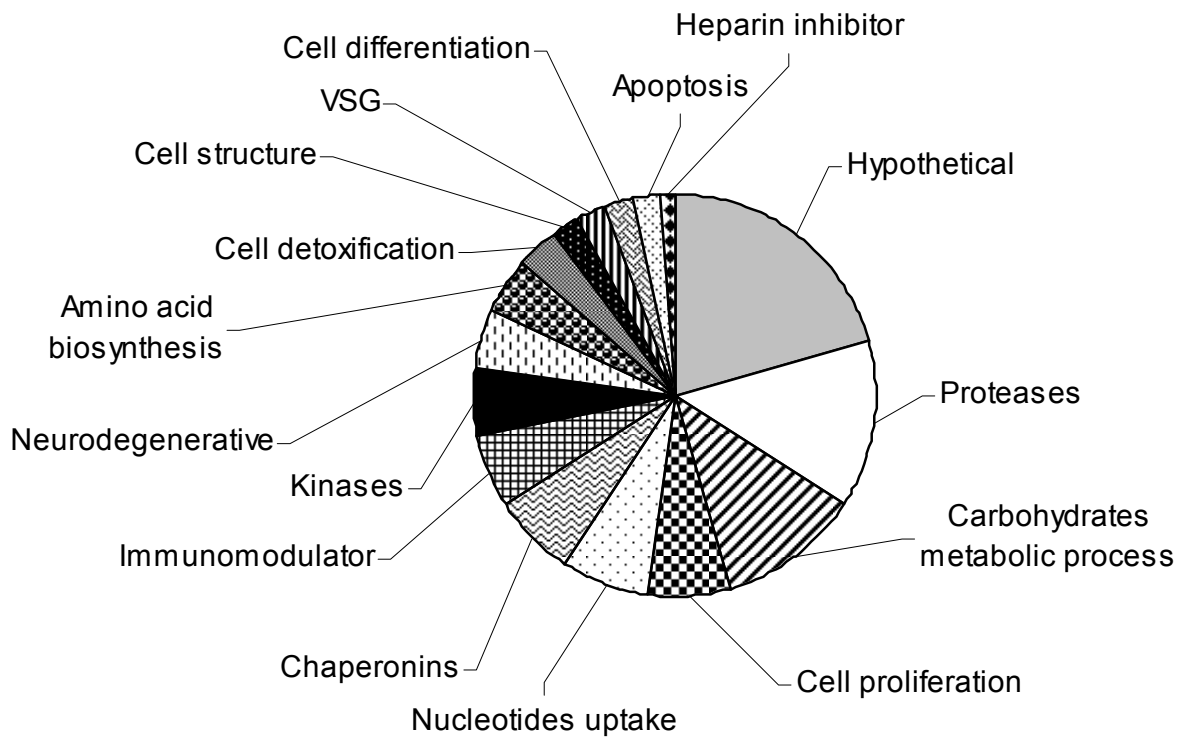
**Differential profiles of *T. congolense* strains secretomes and molecular characterisation**

Viability of *T. congolense* was higher than 97% after 2 hours in the secretion medium, and the parasite concentration remain stable. For each strain, differentially expressed specific bands potentially involved in virulence or pathogenicity are observed in 1D Trycine-PAGE (Figure 2).



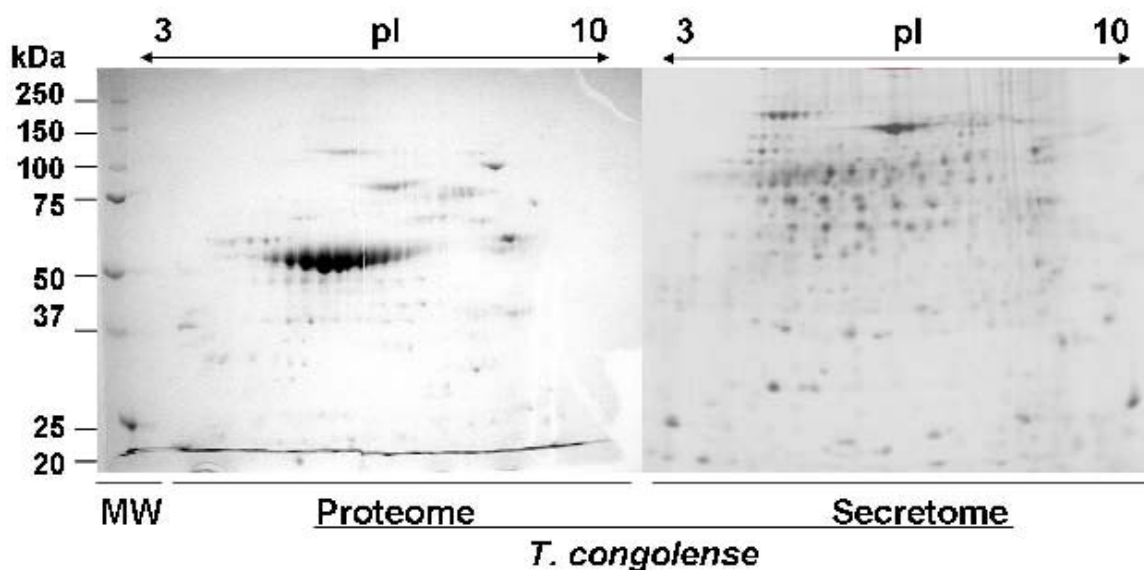
**Figure 2.** 1-D Trycine-PAGE profiles of *T. congolense* secretomes. Arrows indicate protein bands differentially expressed between strains.

Each band was automatically picked and the molecular characterisation by mass spectrometry led to identification of 88 proteins in *T. congolense* secretome. Unfortunately, 18.2% were defined as hypothetical proteins in the databases. Nevertheless, some have been recently described for their vaccine properties or as therapeutic targets, e.g. the eukaryotic translation initiation factor 5A, eIF-5A (Park, 2006) or the triosephosphate isomerase TIM (Olivares-Illana et al., 2006). Interestingly, at least half of the secreted proteins identified are directly involved in the processes influencing parasite virulence with the major parts belonging to important metabolic functions: proteases, chaperonins, carbohydrates degradation, nucleotides uptake or energetic metabolism (Figure 3).



**Figure 3.** Graphic representation of the functions of *T. congolense* strains secretomes components after molecular characterisation by mass spectrometry.

**Comparative mapping of *T. congolense* strains proteomes and secretomes.** Despite different staining methods, *T. congolense* proteome stained with colloidal Coomassie blue exhibit a different 2D map of constitutive proteins compared to secretome stained with fluorescent CyDye (Figure 4).



**Figure 4.** Two-dimensional mapping of *T. congolense* proteome and secretome

Nevertheless, a spot-by-spot comparison demonstrated that the relationship between both *T. congolense* stocks (i) for the area of common protein spots is significant with a high correlation coefficient ( $r = 0.963$ ,  $R^2 = 0.919$ ), and (ii) for the intensity of common protein spots is significant with a low correlation coefficient ( $r = 0.634$ ,  $R^2 = 0.378$ ), suggesting that plasticity in expression of the same molecules could lead to different infectious abilities.

## DISCUSSION-CONCLUSION

Considering the extra cellular localisation of trypanosomes, the success of the infectious process is based on molecular “crosstalk” with the host’s immune cells. By analyzing the secretome of two different strains of *T. congolense* we were able to characterize common and specific proteins used by the parasite for its invading strategy. As in the initial work with *T. b. gambiense* (Holzmuller *et al.*, 2007), in this study we first characterised strains of both *T. congolense* with differing virulence and pathogenicity in the experimental Nude rat model. This constituted a strong base for the use of proteomics analysis to characterise parasite virulence factors. As in recent studies that demonstrated differential protein expression of glycosome’s content during the parasite life cycle (Colasante *et al.*, 2006) or between drug-sensitive and drug-resistant isogenic lines (Foucher *et al.*, 2006) the comparative approach allowed us to evidence quantitative differences between the secretome of the two strains that could be correlated with virulence and pathogenicity, and lead to discriminate new key molecules of the infectious process. Although, our standardized method still requires improvement, especially because most of the identified proteins referred to the genome of *T. brucei*, which is the only available and complete annotated one. Referring to the genome of *T. congolense* should strengthen our model, and may also contribute to highlight molecular differences between Trypanosome species. Another difficulty, knowing that many proteins have several functions, is to choose the most relevant one as a potential therapeutic target. For instance, the kinetoplastid membrane protein KMP11 is involved in the defence response as well as in the positive regulation of cell proliferation. Nevertheless, as shown for Human African Trypanosomosis (Papadopoulos *et al.*, 2004), this technology may contribute also to improve the diagnosis of animal trypanosomosis. To conclude in a prospective manner, ongoing researches on the secretome and proteome of trypanosomes aim at characterising common and species-specific proteins, either differentially expressed in correlation with virulence and pathogenicity to define new therapeutic targets or differentially recognised by host immune system to define new diagnostic tools.

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