Fernanda Ludolf Ribero

Fernanda Ludolf Ribero has been developing her scientific career at the Research Institute René Rachou–Fiocruz/Brazil, working on the molecular biology of the Schistosoma mansoni *parasite. She started at the Institute as an undergraduate student, got her master's degree and presently is in the last year of her doctorate. Prior to starting her doctorate she had the chance to expand her knowledge on proteomics by spending one year at the Center for Tropical and Emerging Global Diseases, at the University of Georgia. Now she has had the opportunity to improve her skills by going to the University of Nottingham to work with in vitro expression and protein microarray.*

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ISID/ESCMID Fellowship Report

Profiling the Humoral Immune Response to *Schistosoma mansoni* using a New Immunoproteomic Approach—protein microarray.

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Training Program at the University of Nottingham

PhD Current Research Project

Schistosomiasis is an important parasitic disease, which affects more than 200 million people in 74 countries around the world. The schistosomiasis control strategy has been mainly based on the treatment of infected individuals by chemotherapy with the drug Praziquantel. While the use of this drug has an effect on morbidity, it does not prevent the re-infection, especially for those people who live in endemic areas. Therefore, the development of long term protection based on vaccination would be a significant benefit for disease control. Although many *Schistosoma mansoni* potential vaccine antigens have been identified until now, none was fully protective. Strategies used for vaccine development have changed as the genomic data for schistosomes have become increasingly available and post-genomic technologies have matured, proposing a more rational discovery of new vaccine candidates. The combination of conventional proteomic analysis and serologic analysis has been employed as a novel experimental approach in the discovery of serological markers for different diseases. The identification of individuals living in endemic areas that are naturally resistant to infection suggests the existence of protective immunity. So it was proposed by us to use endemic area individuals sera associated to proteomics approaches in order to characterize an antigenic protein profile of the parasite and to relate it to a protective profile. The association of two-dimensional electrophoresis (2-DE) and western-blot, had enabled a pre-screening of immuno-antigenic proteins of the parasite. The list of proteins screened by the bi-dimensional western-blot of *S. mansoni* protein against sera of residents of endemic areas would then be synthesized *in vitro*, and scanned against a panel of sera (pooled and individually / infected and naturally resistant) using the protein microarray approach.

Introduction

Protein microarray is another methodology that has also been used to identify biomarkers, allowing the analysis of hundreds or thousands of proteins at once being also able to quantify simultaneously the levels of different antibody classes/subclasses to any individual antigen represented on the array. This project aims to refine the list of potential candidates for subsequent testing as protective or diagnosis antigens. Understanding which immune responses are associated with protection would represent a huge step forward towards designing a functioning vaccine against schistosomiasis, looking not only as a single vaccine candidate but as a complex of vaccine candidates.

Cell-free protein synthesis has emerged as a powerful alternative to cell-based protein expression systems for functional and structural proteomics. *In vitro* expression systems offer significant time savings over in vivo systems, which is important when performing high-throughput screening. *In vitro* systems have also the ability to express toxic, proteolytically sensitive, or unstable gene products, increasing the chances of a successful expression of a high number of proteins.

Training Methods

Cell-Free (CF) expression optimization was conducted by testing and comparing different available systems and strategies. The best performing CF expression system were selected based on protein yields and recognition of antigens by immune sera. The wheat-germ system (Promega's TnT SP6 High yield wheat germ protein expression system) was the system selected because of the expected higher yield comparing with the others systems from the same brand. For optimization of the CF expression system it was chosen two, of the proteins previously identified at the 2D-WE, called here as SmM and SmH. Primers were designed as suggested by the manufacturer and the option of adding a histidine c-terminal tag was implemented by us for subsequent purification.

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The amplification annealing temperature was determined empirically by a temperature gradient PCR. Cloning and expression were conducted as suggested by manufacturer. After the successful optimization of the CF expression system using the two model proteins, the same protocol was used for the expression of the 43 proteins selected as antigenic by the 2D-WE pre-screening. 50 other proteins, selected by another PhD student from Brazil, in a bioinformatic pre-screening of potential epitopes, were also included to be expressed and analyzed in the microarray.

Results and Discussion

In vitro coupled transcription-translation, using a TNT expression system, is a suitable means for producing Schistosome proteins as proved by the positive expression of the SmM and SmH. A Western blot, using pooled human sera, suggested that the two translated proteins are still fully antigenic. Those sera were obtained from individuals from a schistosomiasis endemic area and who had, therefore, raised antibodies to the natural protein. Consequently, results presented here suggest that in vitro synthesized proteins are as antigenic as its natural counterpart, confirming the 2D-WE result. After the efficacious optimization, the same procedure was conducted for all the other proteins identified by the 2D-WE and by the bioinformatics approaches.

PCR amplification of the genes corresponding to the proteins selected by 2D-WE was highly effective (95%), however, the ones selected by bioinformatics showed a low efficacy of amplification (40%). Transformation, in particular, was very inefficient and consumed much of the time spent in the laboratory specially when doing it for around 65 proteins amplified before. The issues with transformation were solved after many different attempts concluding that inactivation of the T4 ligase at 65 degrees for 5 min would increase the number of clones (not as specified in the manufacturer's protocol). In the end, the wheat-germ protein expression system (Promega's TnT SP6 High yield) showed to be a good system, expressing almost all the proteins.

Issues arose, however, when the system (TNT-wheat germ) is combined with the multiple protein extract microarray technology employed. Proteins of the wheat-germ produced high levels of background fluorescence that rendered microarray data inadequate. This would suggest that incubation of serum with the wheat germ protein extract was insufficient to prevent non-specific binding. The presence of endogenous biotinylated proteins in the wheat germ lysate also proved to be an issue when imaging IgE (IgE detection uses streptavidin in our system). So to design the primers including polyhistidine-tags (His-tags) and to integrate a His-tag purification step into the protocol would be an essential step. This additional step should remove all wheat germ proteins, preventing non-specific binding and background fluorescence.

Conclusion

The pre-screening using the 2D-WE were conducted in Brazil while the protein microarray with the *in vitro* expression of all the proteins were conducted during the visit of the PhD student, Fernanda Ludolf Ribeiro, to the laboratory of the collaborator Dr. Franco Falcone, at the University of Nottingham, and was only possible because of the generous support by ISID/ESCMID. The PhD student not only optimized the cell-free expression protocol and produced most of the proteins in a high throughput way, but also was involved in the printing process of the slides and started the optimization of the microarray of the *Schistosoma* proteins. The PhD student met some unexpected technical problems associated with the printing and unfortunately was unable to come back from this training with the microarray final results. However, the PhD student is pleased to have met the main objective of this training, which is to promote collaboration, to take home new ideas, and to transfer technology.

The expectative was well fitted just because of all the effort of the PhD student and her supervisors. The good atmosphere in the lab and between all the lab mates helped a lot in the development of the project. The University of Nottingham supervisor was always opened to talk about results. The lab has all the facilities needed for the work and the reagents were in a short time in the lab. The PhD student could meet many new other scientists, increasing her scientific network. Therefore, proposal brings advances in the development of two theses, in the potential of the incorporation of new technology in the endemic country, strengthening of work in cooperation with institutions abroad, and great potential for high impact publications.

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