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Review

Global protein expression analysis in apicomplexan parasites: Current status

Sabina I. Belli, Robert A. Walker and Sarah A. Flowers

Institute for the Biotechnology of Infectious Diseases, University of Technology Sydney, Australia

Members of the phylum Apicomplexa are important protozoan parasites that cause some of the most serious, and in some cases, deadly diseases in humans and animals. They include species from the genus *Plasmodium*, *Toxoplasma*, *Eimeria*, *Neospora*, *Cryptosporidium*, *Babesia* and *Theileria*. The medical, veterinary and economic impact of these pathogens on a global scale is enormous. Although chemo- and immuno-prophylactic strategies are available to control some of these parasites, they are inadequate. Currently, there is an urgent need to design new vaccines or chemotherapeutics for apicomplexan diseases. High-throughput global protein expression analyses using gel or non-gel based protein separation technologies coupled with mass spectrometry and bioinformatics provide a means to identify new drug and vaccine targets in these pathogens. Protein identification based proteomic projects in apicomplexan parasites is currently underway, with the most significant progress made in the malaria parasite, *Plasmodium falciparum*. More recently, preliminary two-dimensional gel electrophoresis maps of *Toxoplasma gondii* and *Neospora caninum* tachyzoites and *Eimeria tenella* sporozoites, have been produced, as well as for micronemes in *E. tenella*. In this review, the status of proteomics in the analysis of global protein expression in apicomplexan parasites will be compared and the challenges associated with these investigations discussed.

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Correspondence: Dr. Sabina Belli, Institute for the Biotechnology of Infectious Diseases, University of Technology Sydney, P.O. Box 123, Broadway, N.S.W., Sydney, Australia 2007
E-mail: sabina.belli@uts.edu.au
Fax: +61-2-9514-4201

Abbreviations: MudPIT, multidimensional protein identification technology; **NCBI**, National Center for Biotechnology and Information

1 Introduction

The apicomplexan parasites *Plasmodium* spp., *Toxoplasma* spp., *Eimeria* spp., *Neospora* spp., *Cryptosporidium* spp., *Babesia* spp. and *Theileria* spp. are obligate intracellular parasites. A defining characteristic of these parasites is a group of secretory organelles located at the apical tip of the parasite that are involved in attachment and invasion. Species of the genera *Plasmodium*, *Toxoplasma* and *Cryptosporidium* are important medical pathogens that cause serious disease and in some cases death, in humans. Species of *Neospora*, *Eimeria*, *Babesia* and *Theileria* are important veterinary pathogens, which cause severe economic losses to the livestock and poultry meat industries. Apicomplexan parasites have a complex life cycle and pass through asexual and sexual stages of development. *Toxoplasma*, *Eimeria*, *Neospora* and *Cryptosporidium* are intestinal parasites that are collectively referred to as the coccidia [1]. These parasites generate a thick walled oocyst stage that is excreted with feces; transmission is by ingestion of oocysts found in the

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contaminated environment. Unlike the other parasites, the oocyst stage of development for *Toxoplasma gondii* only occurs in felines such as the domestic cat, and in dogs for *Neospora caninum*. Among the coccidia, *Toxoplasma* and *Neospora* also form tissue cysts, where the parasites surround themselves by a wall that protects them from the immune system for years. In humans and animals, ingestion of contaminated meats containing tissue cysts can also lead to disease.

Plasmodium falciparum is transmitted by mosquitoes and is the causative agent of malaria. It is adapted to develop in the liver and erythrocytes of humans, as well as in the gut of mosquitoes. Although it passes through an oocyst stage of development in the mosquito gut, the oocysts are not excreted. Similarly, species of *Babesia* and *Theileria* are transmitted by ticks. They are also adapted to develop in erythrocytes in cattle and in the gut of ticks. It is obvious therefore, that the analysis of the proteomes of apicomplexan parasites is complicated by a number of factors including: (i) intracellular location; (ii) different life cycle stages; (iii) adaptation to vertebrate and invertebrate hosts; (iv) tissue and organ distribution within the host; (v) strain pathogenicity; (vi) host immune status; (vii) environmental factors; and (viii) differences in the morphology of intracellular and excreted stages.

2 *Plasmodium*

Proteome profiles for four different life cycle stages of *P. falciparum* became available in 2002 [2, 3], coinciding with the publication of the entire genome sequence for *P. falciparum* [4] and the rodent parasite, *Plasmodium. yoelii yoelii* [5]. Proteome profiling has advanced more rapidly in *Plasmodium* compared to other apicomplexan parasites as a likely consequence of the urgent need to develop new therapeutics to control malaria, the availability of culture systems for all the life cycle stages of the parasite [6, 7] and the availability of the complete genome sequence for *Plasmodium* spp. The current status of proteomics in apicomplexan research is summarized in Table 1. Recent reviews by Wirth [8] and Ersfield [9] have described the outcomes of the *P. falciparum* genome and proteome projects, and Cooper and Carucci [10] have described the outcomes of both large and small scale proteomic projects in studying drug targets and resistance in *Plasmodium*. Therefore, this subject will only be described briefly here.

Two different approaches, in independent studies, were carried out to analyze global protein expression in the different life cycle stages of *Plasmodium* [2, 3]. Both analyses revealed a large number of differentially expressed proteins. Florens *et al.* [2] used multidimensional protein identification technology (Mud

Table 1. Global protein expression in apicomplexan parasites \

Apicomplexan parasite	Life cycle stage/organelle	Number of unique proteins identified	No. parasites (protein) used/separation[g)]	Protein separation and identification	Reference
P. falciparum[a)]	sporozoites	513	1.6×10^7	MudPIT	[2]; Florens, personal communication
	merozoites	204	2.8×10^9		
	trophozoites	286	4.5×10^9		
	gametocytes	376	4.1×10^8		
P. falciparum[b)]	trophozoites/schizonts	226	–	gel free nanoLC-MS/MS, 1-DE nanoLC-MS/MS	[3]
	gametocytes	315	–		
	gametes	97	–		
T. gondii[c)]	tachyzoites	~30	2.0×10^8 (416 μg)	2-DE MALDI-TOF-MS PSD-MS	[13]
E. tenella[d)]	sporozoites	28	6.5×10^7 (1 mg)	2-DE MALDI-TOF-MS LC-ESI-MS/MS	[28]
E. tenella[e)]	micronemes	3	100 μg	2-DE MALDI-TOF-MS, CAF-MALDI-MS	[29]
N. caninum[f)]	tachyzoites	20	6.8×10^7 (120 μg)	2-DE MALDI-TOF-MS	[36]; Jung, personal communication

a) The total number of proteins identified in this study was 2415
b) The total number of proteins identified in this study was 1289
c) Thousands of spots were resolved in this study, 71 spots were analyzed, and 61% were identified
d) In this study, 460–600 spots were resolved reproducibly by 2-DE. Of these, 130 of the most abundant proteins were analysed and 57 spots were identified corresponding to 28 proteins
e) In this study, 96 spots were analyzed, 37 spots identified and 15 different proteins identified. Of these, two proteins were purification contaminants, and 10 were non-microneme proteins
f) In this study, 359 spots were resolved, 156 spots were analyzed, and 38 spots were identified corresponding to 20 proteins
g) A comparison of cell numbers and quantity of protein used per separation across the apicomplexan parasites is given. In the gel-based system, Coomassie Blue staining was used for detection, except for the analysis of *E. tenella* micronemes. The number of micronemes analyzed was not stated. In the case of *P. falciparum* protein concentrations were not given. – unclear

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PIT), a high resolution LC technique coupled to MS/MS followed by the bioinformatic analysis of peptides, to analyze the proteomes of sporozoites (asexual infective mosquito stage), merozoites (asexual blood stage), trophozoites (asexual blood stage) and gametocytes (sexual blood stage). Among thousands of host proteins, 2415 parasite proteins were identified, and of these 51% were hypothetical proteins when compared to the annotated *P. falciparum* genome sequences, indicating, that these proteins, that have no homology with known proteins to date, might be useful vaccine candidates.

In the study by Florens *et al.* [2], several proteins identified were unique to each developmental stage (see Table 1), and only 6% were common to all stages. Functional profiling of the proteins revealed that the majority of proteins identified in sporozoites were associated with apical organelles, and in merozoites, trophozoites and gametocytes, proteins associated with protein synthesis, protein fate (folding, modification, destination) and metabolism predominated. However, an increase in the number of proteins associated with transcription was detected in trophozoites and gametocytes, and proteins associated with cellular transport also featured highly in the sexual stages. In the study by Lasonder *et al.* [3], high-resolution gel and gel-free separations coupled to LC-MS/MS were used to analyze the proteomes of asexual (trophozoites and schizonts combined) and sexual (gametocytes and gamete) stages of the parasite. In this study, 1289 parasite proteins were identified, and of these, several were stage specific (see Table 1). In this study, 488 proteins were common to the asexual and sexual stages, and 575 proteins were found exclusively in the sexual stages.

In both studies, 54% [2] and ~75% [3] of all predicted gene products were not detected in any of the different life cycle stages analyzed. These figures might reflect technical problems such as sample preparation, or the nature of the proteins, such as their abundance and expression. Alternatively, the unidentified proteins might represent those that are expressed exclusively in the liver stages, and mosquito stages such as the ookinete, zygote and oocyst, which were not studied. An additional consideration is whether the proteome profiles of cultured parasites are true representations of the proteomes of the parasites in an *in vivo* infection, where gene and protein expression is continuously influenced by external stimuli within the microenvironment.

The analysis of the proteome of *P. falciparum* has provided insight into the subsets of proteins that are expressed at the different stages of development of the parasite, however, whether the data is a true representation of protein expression is yet to be determined [9, 11]. Although the number of peptide hits by MS is indicative of the abundance of each protein it is not a quantitative measurement of real changes in protein expression. However, improvements in the quantitative measurement of MudPIT analyses are in progress [12]. In other studies, no correlation was found between the relative abundance of a protein detected by MudPIT and antigenicity [11], highlighting the need for accompanying biological data for the identification of new vaccine, drug or diagnostic targets.

3 *Toxoplasma*

The availability of a 10-fold coverage of sequence for the *T. gondii* genome (www.toxodb.org/ToxoDB.shtml) has given rise to a preliminary analysis of global protein expression in this parasite. Unlike *P. falciparum*, where the proteomes of four developmental stages of the parasite were analyzed, only the proteome of the invasive, tachyzoite stage was characterized in *T. gondii*. 2-DE was used to resolve, reproducibly, over 1000 individual spots and of these proteins, 71 were analyzed by MALDI-TOF-MS and PSD-MS [13]. Only 61% of the proteins were identified by searching *Toxoplasma* sequences or sequences from other organisms. There were ~30 unique proteins identified in the tachyzoites, and no host proteins were detected. The more highly expressed rhoptry, dense granule and structural proteins were easily identifiable.

Proteomic approaches on a smaller scale have been used more extensively in *T. gondii* research, than in any other apicomplexan parasite excluding *Plasmodium*. This might be a consequence of its medical importance, the availability of routine *in vitro* culture systems for the growth of the asexual (tachyzoite) and bradyzoite/cyst stages of development and the availability of a well-established mouse model for toxoplasmosis [14]. There are a number of examples in the literature describing the role of 2-DE coupled to MS or immunoblotting to explore markers of infection and pathogenesis in *T. gondii* [15–17]. For example, in an investigation of the proteomes of virulent and attenuated BK strains of *T. gondii* tachyzoites by 2-DE coupled to quadrupole TOF-MS, ~300 protein spots were resolved, and of the ~200 common spots, the level of expression of 35 of them changed depending on the level of virulence [15]. Subsequently, using specific antibodies or MS, dense granule proteins (GRA2, GRA5, GRA7 and GRA 8), microneme protein 5, NTPase I, catalase and actin were identified to play a role in virulence in this parasite [15].

2-DE coupled to MS or immunoblotting has also been used to identify parasite specific proteins that represent potential vaccine candidates or targets for serodiagnosis. Dlugonska *et al.* [18] resolved 200 spots of *T. gondii* tachyzoite lysates, and identified 11 excretory-secretory dense granule proteins that contained B cell epitopes, and two containing T cell epitopes. Geißler *et al.* [19] resolved ~300 spots in two different strains of *T. gondii* (RH and BK) to evaluate the value of 2-DE to characterize the serological response to infection. These approaches were also used to characterize a number of other proteins involved in invasion, including the excretory protein GRA7 in tachyzoites [20], protein disulfide isomerase [21], the subtilisin-like protein TgSUB1 [22], the microneme protein TgM2AP [23], the rhoptry protein TgROP9 [24] and lactate dehydrogenase [25].

The identification of the entire proteome of *T. gondii* tachyzoites is to be completed, and the proteomes of the bradyzoite/cyst, and oocyst stages of the parasite are yet to be explored. Characterization of the bradyzoite/cyst stage of development for *T. gondii* is a real possibility, given the availability of an *in vitro* culture system for this stage [26]. Analysis of the proteome of the bradyzoite/cyst stage of the para-

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site will reveal pathways involved in the conversion of tachyzoites to bradyzoites, as well as those involved in cyst wall formation. The molecular basis of these pathways could identify novel targets for the design of therapeutics to treat chronic toxoplasmosis. Analysis of the oocyst proteome might be more challenging, due to limitations in the availability of material, given that this stage is only excreted from felines such as the domestic cat. However, given the importance of the wall structure that surrounds both cysts and oocysts in ensuring parasite survival, analysis of the proteomes of these life cycle stages should not be ignored.

4 *Eimeria*

The progress of large scale, high-throughput proteomics-based research in *Eimeria* has been hampered by the lack of a reliable *in vitro* culture system for the generation of all the life cycle stages of the parasite, and also, the limited amount of annotated genome sequence currently available (8.3-fold coverage available; http://www.sanger.ac.uk/Projects/E_tenella/; [27]). Nonetheless, the first proteomic maps of entire parasites [28] and subcellular organelles [29] using modern approaches in proteomics have recently emerged. Proteins were identified against EST and nonredundant databases at the National Center for Biotechnology and Information (NCBI), *Eimeria* clustered EST databases and the *Eimeria tenella* genome assemblies. A reference map for the sporozoite stage of *E. tenella* has been published recently [28]. Initial experiments showed that between 460–600 spots could be resolved reproducibly by 2-DE and stained with silver nitrate. PMF data generated by MALDI-TOF-MS of tryptic digests of 130 of the most abundant proteins were obtained, as well as peptide fragmentation data from LC-ESI-MS/MS. Of these, 57 spots could be matched to 28 proteins, 16 of which were known *Eimeria* proteins, and 12 that were new proteins identified againt the EST and nonredundant databases at NCBI. Of the known *Eimeria* proteins, the most abundant were identified as microneme proteins, proteases, glycolytic enzymes and heat shock proteins.

During the isolation of sporozoites from oocysts, trypsin was used in the excystation medium. The effect of trypsin on the proteome profile of these sporozoites is not known, but an abundance of low M_r proteins was observed on the 2-DE proteome maps. Sporozoites that are excysted from oocysts are a good source of parasite material because they are devoid of host material. An investigation of the protein repertoire of micronemes in *E. tenella* was also carried out using 2-DE coupled to MALDI-TOF-MS or CAF-MALDI-MS with *de novo* sequencing [29]. Micronemes are subcellular organelles found in the apical tip of the parasite that contain proteins involved in attachment and invasion in apicomplexan parasites, and are therefore considered to contain possible vaccine targets. In an earlier study, *E. tenella* rhoptries, another apical organelle involved in establishing infection, was analyzed by 2-DE and found to contain a proteomic profile that was distinctly different to rhoptries from *T. gondii* [30].

Microneme lysates of *E. tenella* sporozoites were separated by 2-DE and although the total number of spots resolved was not indicated, 96 spots were isolated and analyzed by MALDI-TOF-MS [29]. PMFs were identified for 68 of these spots, and 37 spots were positively identified against the nonredundant database at NCBI and *Eimeria* clustered EST databases. In total, 15 different proteins were identified, only three of which were considered to be true microneme proteins; two proteins were identified as components of the purification procedure and the other 10 were identified as non-microneme, sporozoite proteins. These findings reflected the sensitivity of this proteomic approach in the identification of extraneous proteins, given that the micronemes appeared pure by microscopic observation [29].

The first preliminary proteome map for *E. tenella* sporozoites has been produced through modern approaches in sample preparation and identification. De Venevelles [28] commented that a number of protein patterns in these maps were similar to those detected in the pioneering work of Sutton *et al.* [31] who produced the first fingerprints of sporozoites. The identification of the proteomes of the other life cycle stages (merozoites, gametocytes, zygotes) of *Eimeria* are yet to be determined. This will be difficult due to the current lack of *in vitro* culture systems for these life cycle stages. However, methods for the isolation and purification of sufficient quantities of gametocytes for *Eimeria maxima* and *Eimeria tenella* [32, 33] and for merozoites in *E. tenella* [33] from an infected host are available. It is likely that these proteome maps will be contaminated by host material but with the 2004 release of the first draft of the chicken genome sequence (www.genome.wustl.edu/projects/bovine) the contaminating sequences will be easily identified.

Oocysts of *Toxoplasma*, *Eimeria* and *Neospora* are able to persist in the environment for years due to the robustness of the oocyst wall that protects the parasite within from desiccation. A clearer understanding of this structure will help develop new strategies to eliminate the parasites from the environment, as well as give rise to new diagnostic reagents. Unlike *Toxoplasma*, where the analysis of the proteome of the oocyst stage will be limited by the availability of material, analysis of the eimerian oocyst is feasible because the purification of sufficient quantities of eimerian oocysts is routine. Ideally, the proteome of unsporulated oocysts should be investigated to avoid sporozoite proteins complicating the proteome profile, enabling a simpler identification of the molecular composition of the oocyst wall. However, given that the oocyst wall is a structure that is resistant to solubilization, the analysis of this structure will be challenging.

5 *Neospora*

N. caninum is a parasite that is very similar to *T. gondii* in terms of its life cycle, the formation of tissue cysts, excretion of oocysts and the availability of routine *in vitro* culture systems for tachyzoites, bradyzoites and cysts. There is an urgent need for new

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diagnostic tools, as well as drugs and vaccines to control the disease neosporosis that is caused by this parasite, and it is hoped that protein identification in proteomic projects will assist in this area. Like *T. gondii*, only the proteome of the tachyzoite stage of development has been characterized, and although the identification of the proteomes of the oocyst and cyst stages of development might be more challenging they should not be ignored. The small number of reports that have described proteomic approaches in *N. caninum* research have been mostly driven by an interest in identifying new antigens for improved serodiagnosis and to define the molecular difference between *N. caninum* and *T. gondii* [34, 35]. However, in 2003, the first preliminary 2-DE proteome map of *N. caninum* was published [36]. Proteins of *N. caninum* tachyzoite lysates were separated by 2-DE and 359 protein spots were resolved [36]. Of the 156 spots submitted for identification by MALDI-TOF-MS, 38 spots (24%) were successfully identified using the limited genome and EST sequence data available for *N. caninum* (http://genome.wustl.edu/est/) and that of other apicomplexan parasites such as *P. falciparum*, *T. gondii*, *E. tenella* and *Eimeria acervulina*. These spots comprised 20 different proteins including dense granule proteins, microneme proteins, heat shock proteins, glycolytic enzymes and cytoskeletal proteins. No comment was made on the level of host protein contamination in the proteome profile.

6 Other apicomplexan parasites

There are no reports to date that describe the proteome profiles of the human intestinal apicomplexan parasite *Cryptosporidium*, as well as the animal pathogens, *Babesia* and *Theileria*. Some of the difficulties faced in developing these maps include obtaining sufficient quantities of the different life cycle stages of these parasites and the availability of enough sequence data to identify the proteins. However, the current situation is likely to improve now that the complete genome sequences for *C. parvum* and *C. hominis* [37, 38] have become available and that sequencing projects for *Theileria annulata* (www.sanger.ac.uk/Projects/T_annulata) and *Babesia bovis* (www.sanger.ac.uk/Projects/B_bovis) [9] are in progress. The *in vitro* culture of these pathogens has also been a stumbling block for the production of proteomic profiles, yet progress is also being made in this area. An *in vitro*, cell-free culture system for the entire life cycle of *Cryptosporidium parvum* has become available [39], as well as an *in vitro* culture system for the sexual stages of *Babesia bigemina* [40]. Detailed protocols for the *in vitro* culture of different species of *Babesia* [41] and *Cryptosporidium* [42] are also available. While large scale proteomic analyses are being developed for the study of these pathogens, smaller scale projects have used 2-DE to separate parasitic proteins to investigate antigenic variation among parasite isolates [43–46] and to identify immunodominant antigens and virulence markers to develop new reagents for the improvement of the serodiagnosis of these pathogens and to identify new vaccine and drug targets [47–54].

7 Concluding remarks

The first and most extensive proteome maps for all the life cycle stages of an apicomplexan parasite was published in 2002 for *P. falciparum* [2, 3]. Proteome maps have also begun to emerge for the invasive stages of development of the other apicomplexan parasites including the sporozoite of *E. tenella* [28], as well as the tachyzoite of *T. gondii* [13] and *N. caninum* [36]. An attempt has also been made to produce a proteome map of the micronemes of *E. tenella* but the maps have been complicated by the presence of host and other parasite proteins. Clearly, the identification of the protein repertoire of *P. falciparum* was facilitated by the availability of sequence corresponding to the entire genome. Entire sequences at different stages of annotation are now available for *Cryptosporidium spp*., *T. gondii* and *E. tenella*, and genome sequencing projects are in progress for *Babesia bovis* and *Theileria annulata*. Where proteomic maps were generated for *T. gondii* and *E. tenella*, protein sequence comparisons with nonredundant sequence databases at NCBI were shown to be useful in identifying new parasite proteins based on 98% homologies with previously identified sequences. Unlike other organisms such as yeast and bacteria, where pure populations can be analyzed, apicomplexan parasites are intracellular organisms and in some cases, their life cycles take place in vector hosts. As such, it is unlikely that proteome maps will be produced that are completely devoid of contaminating host material. However, with the increase number of genome projects and the 2004 release of the first draft of the chicken (www.genome.wustl.edu/projects/chicken/) and cow (www.hgsc.bcm.tmc.edu/projects/bovine) genome sequences, filtering the extraneous sequences from the proteomes of the poultry parasite *Eimeria* and the cattle parasite, *Neospora*, will be possible.

Although the proteome of many of the life cycle stages of *P. falciparum* has been identified, the question as to whether it will be feasible to identify the proteomes for all the life cycle stages of the other apicomplexan parasites is still debatable. It is currently possible to analyze the proteomes of the sporozoite stage of *Eimeria*, as well as the tachyzoite and bradyzoite stages of *Toxoplasma* and *Neospora*, but the sexual and oocyst stages will be more complicated. *Eimeria* would be the ideal model coccidial parasite to explore the proteomes of the sexual stages, where the isolation of large quantities of gametocytes and oocysts is possible. Perhaps an alternative high-throughput approach for drug/vaccine target discovery in the apicomplexan parasites, or in the case of *Cryptosporidium*, *Babesia* and *Theileria* where the availability of material is limiting, is to use protein microarrays [55, 56].

Apicomplexan parasites have dynamic proteomes to accommodate changes in their microenvironment, such as those induced by the host's immune system during an infection, by invertebrate hosts, and by the variable conditions in the external environment. As such, a careful interpretation of the data is required. In particular, consideration should be made regarding whether the proteome profiles of

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in vitro cultured parasites are a true representation of the parasites in an *in vivo* infection. Consideration should also be made with regard to standardizing analyses for variations in culture conditions, number of passages, and time of harvest, in different experiments and in different research groups. In Table 1, variations in protein yields arising from different extraction procedures is clearly observed among the different apicomplexan parasites. Proteomics is the initial discovery phase in the development of new chemo- and immuno-therapeutic strategies to control apicomplexan diseases, and this technology will identify life cycle stage specific proteins implicated in invasion, survival, virulence, disease pathogenesis and transmission. However, functional and immunological assays will be the key to determining their effectiveness as vaccine and drug targets.

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