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Analysis of the *Babesia microti* proteome in infected red blood cells by a combination of nanotechnology and mass spectrometry

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ABSTRACT

Proteomics of *Babesia microti* has lagged behind other apicomplexans despite recent genome and transcriptome studies. Here, we used a combination of nanotechnology and mass spectrometry to provide a proteomic profile of *B. microti* acute infection. We identified ~500 parasite proteins in blood with functions such as transport, carbohydrate and energy metabolism, proteolysis, DNA and RNA metabolism, signaling, translation, lipid biosynthesis, and motility and invasion. We also identified surface antigens with roles in the immune response to the parasite. This first evaluation of the *B. microti* proteome in erythrocytes provides information for the study of intracellular survival and development of diagnostic tools using mass spectrometry.

Tick-borne diseases (TBD) among humans are on the rise in North America due to a number of factors such as continuous urbanization along previously unpopulated areas and changes in vector ecology due to climate change. Among TBDs, babesiosis has been recognized in recent years as an emerging infectious disease. Babesiosis is caused by red blood cell (RBC) infecting protozoan parasites of the genus *Babesia* (Vannier et al., 2015). Similar to Lyme disease, transmission of babesiosis occurs primarily by *Ixodes* ticks; however, incidental transmission may occur through maternal-fetal transmission and blood transfusion (Lobo et al., 2013). The latter is of particular concern, especially among carriers of *Babesia*, as to this date there is no Food and Drug Administration (FDA)-approved assay to test for the parasite in the U.S. (Gubernot et al., 2009).

Babesia microti is a member of the phylum Apicomplexa and the causative agent for the majority of human cases of babesiosis in the U.S., with higher prevalence in the northeastern and upper midwestern regions of the country (Centers for Disease and Prevention, 2012). Infection is usually asymptomatic, or results in mild symptoms that resolve within a few days. Severe cases feature acute anemia, thrombocytopenia, organ failure, or even death among elderly, splenectomized, and immunocompromised individuals (Vannier et al., 2015). Diagnosis is performed primarily by microscopy of blood smears, which requires skilled and experienced laboratory personnel and lacks sensitivity. Serological methods and molecular assays based on conventional and real-time PCR have also been developed for detection of *B. microti* infections but are not broadly available (Mosqueda et al., 2012; Wilson et al., 2015).

The genome sequence of *B. microti* R1 strain was reported in 2012 (Cornillot et al., 2012). Results revealed that the genome consists of four nuclear chromosomes and a linear mitochondrial genome (Cornillot et al., 2013). The overall size of the nuclear genome is ~6.5 Mbp, encoding ~3500 polypeptides, and it is the smallest apicomplexan genome sequenced to date. Reconstruction of metabolic pathways from the annotated genome revealed limited metabolic machinery and lack of enzymatic processes known to be important for *Plasmodium* (Cornillot et al., 2012). In addition, phylogenetic analyses revealed that *B. microti* belongs to a new apicomplexan lineage distinct from the families represented by *Babesia bovis* and *Theileria* spp. A recent study based on genomic and transcriptomic analyses of various *B. microti* isolates resulted in the re-annotation of the entire *B. microti* R1 strain genome (Silva et al., 2016). In addition to characterizing the genomic diversity among the isolates, the authors identified the full complement of genes encoding the *B. microti* secretome and surface proteome.

The application of emerging technologies in proteomics and mass spectrometry (MS) has contributed significantly to the validation of genome annotation studies in other apicomplexan

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parasites such as *Plasmodium*, *Toxoplasma* and *Neospora* (Bautista et al., 2014; Silmon de Monerri and Weiss, 2015). Of note, the great majority of proteomic studies performed with these pathogens have relied on material derived from in vitro cultures. For malaria, such experiments take advantage of synchronized culture and enrichment methods for specific parasite stages, minimizing host cell contamination and reducing background noise from the host proteome during analysis of blood stage parasite proteins (Bautista et al., 2014). Contrary to this, there has been little if any research on the characterization of the *B. microti* proteome, due in part to the absence of a stable long-term in vitro culture system for the parasite. Alternatively, proteomic studies using in vivo models of babesiosis are appealing but can be challenging, even with the support of advanced mass spectrometry instrumentation due to the masking of parasite proteins by highly abundant host proteins found in blood.

A recently developed nanotechnology approach based on hydrogel affinity nanoparticles circumvents some of the analytical and physiological roadblocks mentioned above and allows protein discovery directly in complex biological fluids with increased sensitivity. The applicability of this technology has been extended to other vector-borne infections such as Lyme disease (Douglas et al., 2011; Magni et al., 2015) and Chagas disease (Castro-Sesquen et al., 2014, 2016). The nanoparticles used in these studies are polymeric buoyant networks of cross-linked poly(N-isopropylacrylamide) (NIPAm) functionalized with high affinity chemical baits. Once incubated with a biological fluid, the nanoparticles act as a molecular sieve and rapidly capture, concentrate and preserve low abundance biomarkers, while excluding unwanted high abundance, high molecular weight proteins such as albumin and immunoglobulins that often interfere with mass spectrometry analysis (Longo et al., 2009; Douglas et al., 2011; Magni et al., 2015). While size and porosity can be engineered for size-exclusion of interfering substances, different high affinity capturing baits such as Remazol Brilliant Blue (RBB), Cibacron Blue (CB), Reactive Blue 221 (RB221), Reactive Black 31 (RB31), and other synthetic organic dyes can be incorporated to entrap different classes of low-abundance proteins and peptides through a combination of ionic, hydrophobic and electrostatic interactions (Shafagati et al., 2014). After capture, proteins can be eluted from the nanoparticles and analyzed by mass spectrometry, Western blotting, and other immunoassays.

The primary objective of this study was to perform a proteomic analysis of *B. microti* in whole blood using the aforementioned nanotechnology combined with MS. Using the hamster model of babesiosis, our methods resulted in the identification of $\sim$500 *B. microti* proteins with functions as broad as intracellular signaling, protein translation and modification, protein transport, DNA replication, cytoskeleton support, and lipid and glucose metabolism. We also identified members of the *B. microti* secretome and surface proteome previously described by genomic studies (Cornillot et al., 2016; Silva et al., 2016). This first evaluation of the *B. microti* proteome complements recent genomic and transcriptomic studies to significantly advance our knowledge of metabolism and pathogenesis of the parasite. In addition, our results provide scientific evidence to support the use of a nanoparticle-MS method for the identification of *Babesia* in blood samples, thus contributing towards the development of newer diagnostic tools.

Babesia microti GI strain (BEI Resources, USA, Cat No. NR-44070; ATCC®, USA, Cat. No. PRA-398™) was originally isolated from blood obtained from a human case of babesiosis in Nantucket, Massachusetts, USA, in 1983 (Piesman et al., 1986; Gray et al., 2002). The isolate was propagated in Golden Syrian hamsters (Harlan Laboratories, USA, stock: HsdHan:AURA) according to protocols approved by the ATCC® Institutional Animal Care and Use Committee. Parasitemia was determined in a minimum of 500 RBCs by microscopic examination of blood films stained with Giemsa. This included all parasitized RBCs regardless of intra-erythrocytic stage or number of parasites per cell. Blood samples for proteomic studies were collected by peri-orbital route 14 days p.i. and exhibited 30% parasitemia (Fig. 1A).

Detailed procedures for the synthesis of hydrogel nanoparticles, chemical functionalization, and validation of nanoparticles are described in Supplementary Materials and Methods S1. A 0.5 ml aliquot of hamster blood infected with *B. microti* was centrifuged at 2000*g* for 10 min. One ml of deionized water and 0.1 ml of Remazol Brilliant Blue (RBB)-functionalized nanoparticle suspension (5 mg/mL of dry weight concentration) were added to the RBC pellet and incubated at room temperature for 45 min. The RBC/RBB-nanoparticle suspension was subjected to three freeze/thaw cycles at −80 °C and centrifuged at 16,100*g* for 5 min. The resulting suspension was centrifuged at 2000*g* for 10 min in order to remove cellular debris. The supernatant containing affinity nanoparticles was centrifuged at 16,100*g* for 25 min and washed

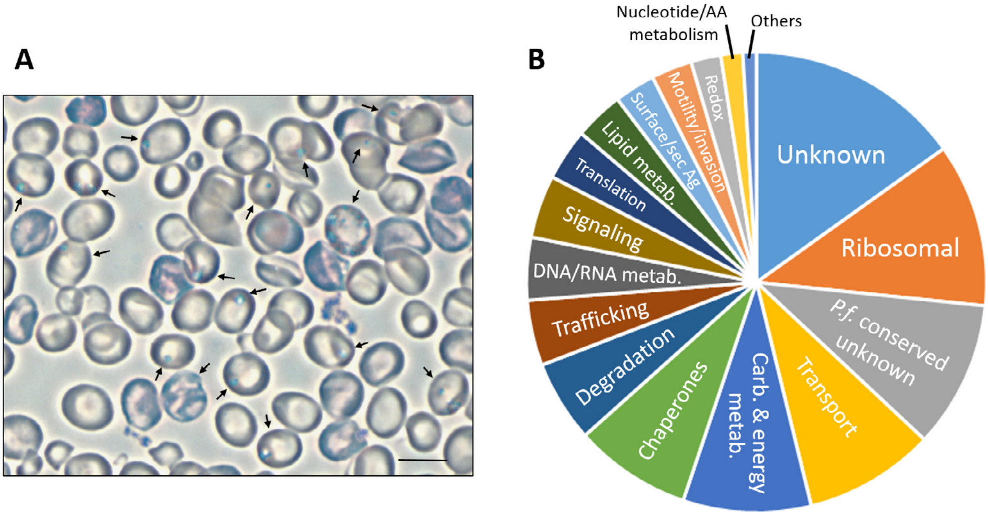


Fig. 1. Proteomic map of *Babesia microti* infection in blood. (A) Light-microscopy image from Giemsa-stained hamster blood infected with the *B. microti* GI strain. Bar, 6 µm. Arrows indicate red blood cells harboring ring-form trophozoite stages of the parasite. (B) Classification of identified proteins based on biological function.

three times in ultrapure water (18 MΩ-cm). The nanoparticle pellet was re-suspended in 20 μl of elution buffer (RapiGest™ SF surfactant, Waters, USA, with 50 mM ammonium bicarbonate and 5% Tris(2-carboxyethyl)phosphine hydrochloride reducing agent) and incubated at room temperature for 20 min. Nanoparticles were centrifuged at 16,100*g* for 20 min and the post-elution supernatant was subjected to MS analysis as described in Supplementary Materials and Methods S1. Blood from an uninfected hamster was processed exactly as above for use as a negative control.

Five separate injections of the *B. microti*-infected hamster blood sample and the uninfected control were analyzed by MS. Tandem mass spectra obtained from this analysis were searched against theoretical spectra derived from an annotated database of protein sequences from *B. microti* R1 strain (PiroplasmaDB.org) using the SEQUEST algorithm and tryptic cleavage constraints. Peptide hits were combined within the five technical replicates. High-confidence peptide identifications were obtained by applying the following filter criteria to the search results: (1) Xcorr > 2.0, >3.0 and >4.0 for 2+, 3+, 4+ precursor ions; (2) *q*-value < 0.05; and (3) probability of randomized identification: e0.01. The acceptable false discovery rate (FDR) based on forward-reverse decoy was <1%. In addition, post-analysis filtering criteria were applied as follows: (1) peptide length >7 amino acids; (2) absence of carryover as determined by analyzing a blank sample with a 90 min gradient; (3) manual validation of each peptide spectra; and (4) rejection of peptides showing >90% similarity with any *Mesocricetus auratus* (Golden hamster) protein and any other organism contained in the non-redundant database. The functional descriptions of the identified *B. microti* proteins were performed by searches in UniProt. In addition, searches for orthologues in other apicomplexans were performed in *B. bovis*, *Theileria annulata* and *Plasmodium falciparum* using PiroplasmaDB and EuPathDB.

Using a combination of nanoparticle harvesting technology and mass spectrometry, we identified 522 parasite proteins from whole hamster blood harboring acute *B. microti* infection (Supplementary Table S1). The 14 day-infected sample exhibited 30% parasitemia and consisted largely of ring-form trophozoite stages of the parasite (Fig. 1A). Identifications based on five or more unique peptides were observed in 106 proteins (20% of total); those based on two to four unique peptides were observed in 200 proteins (38%), and single unique peptide identifications were observed in 216 proteins (42%) (Supplementary Table S1). No parasite proteins were detected in the uninfected control.

Categorization of proteins based on biological function determined that ~75% were associated with common cellular and metabolic activities (Fig. 1B and Table 1). Nearly all of these proteins had orthologues in *B. bovis* (357 proteins), *T. annulata* (353 proteins), and *P. falciparum* (366 proteins) (Supplementary Table S2). The ascribed biological functions included ribosomal (11%), transport (9%), carbohydrate and energy metabolism (9%), chaperones (8%), proteolysis (6%), trafficking (5%), DNA and RNA metabolism (4%), signal transduction (4%), protein translation and modification (4%), lipid metabolism (4%), surface/secreted antigens (3%), and motility and invasion (3%). Many of these proteins (148 in total) were previously found to be among the top 10% of most expressed genes in *B. microti* by transcriptomic analysis (Silva et al., 2016). Regarding cellular location, 147 identified proteins were predicted to localize to the mitochondrion, 52 to the apicoplast, 34 to the endoplasmic reticulum/Golgi, 28 to the nucleus, and five to secretory organelles (i.e., rhoptries and micronemes) (Supplementary Table S2). Approximately 25% of the 522 *B. microti* proteins in our analysis were classified as ‘uncharacterized’ (i.e., matching to hypothetical genes in the *B. microti* genome). Of these, 53 were designated as conserved uncharacterized proteins in *P. falciparum* (Fig. 1B, Table 1, and Supplementary Table S2). With the purpose of supplementing previous genome annotation studies and transcriptomic analysis, we provide below an overview of components of lipid biosynthesis, carbohydrate and energy metabolism, and sero-reactive surface or secreted antigens identified in our analysis of the *B. microti* proteome during acute infection.

The analysis of the *B. microti* genome shows the presence of enzymes for the de novo synthesis of phospholipids and sphingomyelin as well as glycosylphosphatidylinositol (GPI) biosynthesis (Cornillot et al., 2012). In our study, the majority of the 19 identified proteins involved in lipid metabolism have roles in synthesis and catabolism of sphingomyelin, phosphatidylethanolamine, phosphatidylcholine, and recycling of phospholipids (Supplementary Table S2). We also detected two of the

Table 1
Classification of identified proteins based on biological function.

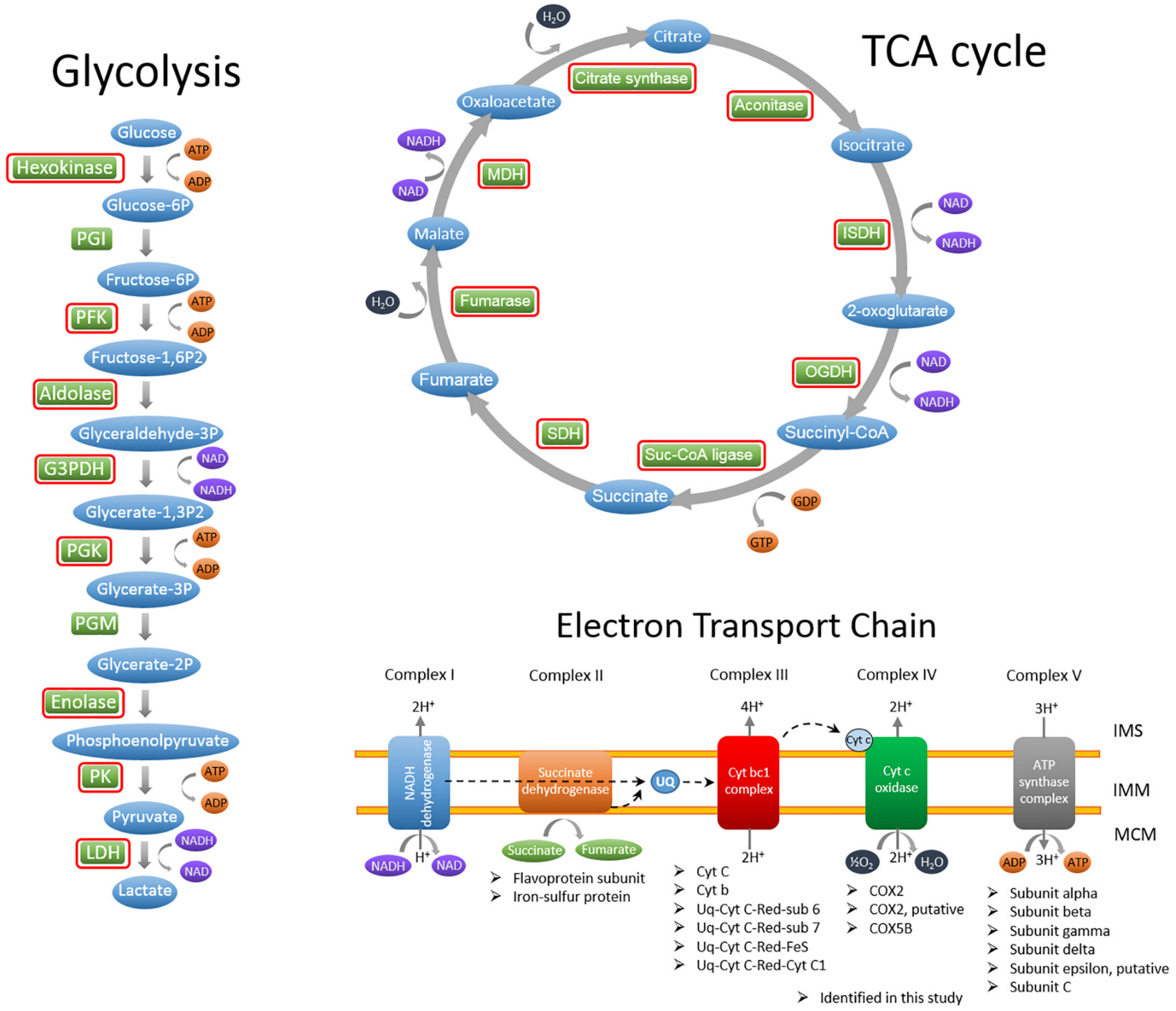
Biological function	No. of proteins	Examples[a]
Unknown	79	Uncharacterized proteins matching to hypothetical genes in the genome
Ribosomal	60	Components of small subunit and large subunit ribosomal proteins
Plasmodium conserved, unknown	54	Uncharacterized *Plasmodium* conserved proteins
Transport	48	Ion transporters, carbohydrate transporters, protein transporters, ABC transporters
Carbohydrate and energy metabolism	47	Glycolysis proteins, TCA cycle proteins, electron transport chain proteins, mitochondrial importers, ATP synthases
Protein folding/chaperones	43	Heat shock proteins, SEC63, DnaK family proteins
Protein degradation	30	Cysteine proteases, metalloproteases, proteasome subunits, peptidases
Trafficking	24	Vesicular transport proteins, Rab proteins, SNARE-like proteins, ER lumen proteins, translocation proteins
DNA/RNA metabolism	23	DNA synthesis, replication, degradation, and repair proteins. RNA synthesis, transcription, modification, processing, and degradation proteins. Includes histones.
Signal transduction	23	Kinases, phosphatases, GTP-binding proteins
Protein translation and modification	19	Aminoacyl tRNA synthetases, elongation factors, ubiquitin protein ligases, glycosyltransferases
Lipid metabolism	18	Proteins involved in lipid transport, biosynthesis, and degradation
Surface/secreted antigens	15	BMN family proteins, BmSA1 surface antigen
Motility and invasion	15	Actin, profilin, myosin, microneme and rhoptry proteins
Redox homeostasis	11	Thioredoxins, oxidoreductases, and other proteins that maintain the redox environment of the cell
Nucleotide and amino acid metabolism	8	Proteins involved in biosynthesis and degradation of nucleic acids and amino acids
Others	5	Proteins involved in porphyrin biosynthesis, chloroquine resistance, ubiquinone biosynthesis, and peripheral plastid proteins
Total	522	

[a] ABC, ATP binding cassette; TCA, tricarboxylic acid; SNARE, soluble N-ethylmaleimide-sensitive factor activating protein receptor; ER, endoplasmic reticulum; BMN, *Babesia microti* sero-reactive antigen family; BmSA1, *Babesia microti* secreted antigen 1.

enzymes involved in the synthesis of dolichyl D-mannosyl phosphate, a substrate of the GPI anchor biosynthesis pathway derived from dolichol, presumably obtained from the host.

The identification of glycolytic enzymes (Supplementary Table S2, Fig. 2) are in line with the requirement of carbohydrate metabolism for energy production during intra-erythrocytic asexual stages of the parasite. We detected the single sugar transporter found in the *B. microti* genome (BMR1_03g01335) (Mahmud and Kissinger, 2017) and eight of the 10 enzymes involved in the breakdown of glucose into pyruvate (Fig. 2). These included hexokinase (BMR1_03g04600), phosphofructokinase (BMR1_02g02830), aldolase (BMR1_03g01800), glyceraldehyde 3-phosphate dehydrogenase (BMR1_01G00230), phosphoglycerate kinase (BMR1_03g00625), enolase (BmR1_04g05965), pyruvate kinase (BmR1_04g06405), and lactate dehydrogenase (BMR1_01G00020). Proteomic analysis also identified all eight enzymes of the tricarboxylic acid (TCA) cycle and several components of the four integral membrane protein complexes involved in the mitochondrial electron transport chain (Supplementary Table S2, Fig. 2). Subsequent MS-based studies should examine the utility of these metabolic proteins as potential diagnostic markers of active *B. microti* infection in the host. In addition, since glycolysis has been proposed as a potential pathway for therapeutic intervention in babesiosis (Bork et al., 2004) and other parasitic diseases (Bruno et al., 2014; Haanstra et al., 2017), it would be relevant to examine the proteomic profiles of components of the pathway in response to glycolytic enzyme inhibitors.

Nanoparticles were also useful in trapping surface or secreted *Babesia* antigens known to elicit host immune responses in vivo (Silva et al., 2016). These included GPI-anchored, secreted, and transmembrane proteins, some of which share homologies to *B. bovis*, *T. annulata*, and *P. falciparum* proteins (Table 2). Seven of the 11 identified seroreactive antigens in Table 2 were among the top 10% of most expressed genes in *B. microti* (Silva et al., 2016). Four of these proteins were correspondingly highly abundant in the proteome based on identifications by seven or more peptides. These included proteins reported to elicit strong IgM/IgG responses such as BmSA1 (BMR1_03g00785), S1/P1 nuclease



- Flavoprotein subunit
- Iron-sulfur protein

- Cyt C
- Cyt b
- Uq-Cyt C-Red-sub 6
- Uq-Cyt C-Red-sub 7
- Uq-Cyt C-Red-FeS
- Uq-Cyt C-Red-Cyt C1

- COX2
- COX2, putative
- COX5B

- Subunit alpha
- Subunit beta
- Subunit gamma
- Subunit delta
- Subunit epsilon, putative
- Subunit C

- Identified in this study

Fig. 2. Use of proteomic analysis to support models for carbohydrate and energy metabolism of *Babesia microti*. Similar to other apicomplexans, *Babesia* possess all 10 enzymes of glycolysis in the genome (Cornillot et al., 2012). Enzymes circled in red were detected in the present study and support the presence of a functional glycolytic pathway and tricarboxylic acid cycle (TCA) cycle in *B. microti*. The flow charts for glycolysis, TCA cycle, and electron transport chain were adapted from *Babesia bovis* as described in the Liverpool Library of Apicomplexan Metabolic Pathways (LAMP) (www.llamp.net/) (Shanmugasundram et al., 2013). PGI, phosphoglucose isomerase; PFK, phosphofructose kinase; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; PGM, phosphoglycerate mutase; PK, pyruvate kinase; LDH, lactate dehydrogenase; ISDH, isocitrate dehydrogenase; OGDH, oxoglutarate dehydrogenase; Suc-CoA, succinate-CoA; SDH, succinate dehydrogenase; MDH, malate dehydrogenase; UQ, ubiquinone; Cyt, cytochrome; COX, cytochrome c oxidase; IMS, intermembrane space; IMM, inner mitochondrial membrane; MCM, mitochondrial matrix.

Table 2
Identification of *Babesia microti* proteins with reported immune responses in vivo.[a]

Gene ID	Description	Class[b]	Ig Response[a]	AA[b] length	MW[b] (kDa)	Orthologues in other piroplasmids and *P. falciparum*[c]
BMR1_03g00785	BmGPI12, BMN1 family, BMN1-9, BmSA1 orthologue	GPI	IgM/IgG	328	35.4	
BMR1_02g04275	BmGPI10, BMN1 family, N1-21a orthologue	GPI	IgG	304	34.4	
BMR1_02g03140	S1/P1 Nuclease	SEC	IgM/IgG	373	42.8	PF3D7_1411900
BMR1_03g00947	Conserved *Plasmodium* protein, unknown function	SEC	IgM/IgG	438	48.7	PF3D7_1324300
BMR1_01G00985	Conserved Plasmodium protein, unknown function	SEC	IgM/IgG	1025	114.4	PF3D7_0822900
BMR1_02g04280	BMN2 family, possible orthologue of N1-10-2	TM	IgM	519	60.9	
BMR1_04g07535	N1-15 protein, maltese-cross seroactive antigen	SEC	IgG	2396	265.7	
BMR1_03g04695	Rhoptry neck protein 2		IgM/IgG	1483	165.7	BBOV_I001630 TA19390 PF3D7_1452000
BMR1_03g00020	BMN2 family	SEC	IgM	474	55.4	
BMR1_04g07556	Uncharacterized protein	TM	IgM	218	24.4	
BMR1_04g05531	MDN1, REA1, midasin		IgG	4337	493.1	BBOV_III002250 TA18495 PF3D7_1434500

[a] As reported in Silva et al., 2016.

[b] GPI, Glycosylphosphatidylinositol; SEC, secreted; TM, transmembrane; AA, amino acid; MW, molecular weight.

[c] BBOV, *Babesia bovis* T2Bo; TA, *Theileria annulata* Muguga; PF3D7, *Plasmodium falciparum* 3D7. *B. bovis* and *T. annulata* orthologues were found in PiroplasmaDB.org. *P. falciparum* orthologues were previously reported by Silva et al., 2016.

(BMR1_02g03140), and the conserved protein BMR1_03g00947, as well as the strong IgG inducer BmGPI10 (BMR1_02g04275) (Table 2). Other proteins known to trigger significant IgG responses were the N1-15 maltese-cross seroactive antigen (BmR1_04g07535) and MDN1 (BmR1_04g05531). The remaining group of identified proteins included two members of the BMN2 family (BMR1_02g04280 and BMR1_03g00020) and uncharacterized protein BmR1_04g07556, which were previously reported to elicit strong IgM responses in vivo (Silva et al., 2016).

As mass spectrometry instrumentation has advanced sufficiently to allow protein discovery directly in complex biological fluids, the present study examined the *B. microti* proteome in whole blood in vivo by performing a prior nanoparticle-based approach to sequester proteins undergoing analysis. From the technical point of view, there are advantages to directly analyzing proteins of interest trapped in specialized buoyant porous hydrogel nanoparticles added to *Babesia*-infected blood. This approach avoids the cumbersome separation of infected RBCs and/or free parasites, which, to our knowledge, has been rarely documented with success in *Babesia* (Alkhalil et al., 2007; Rossouw et al., 2015). The method also circumvents pre-fractionation strategies and removal of detergents and other reagents used in conventional protein extractions, is simple to perform, and prevents proteolysis (Longo et al., 2009). The specificity of the approach for identification of parasite proteins is not derived from the bait itself but from the downstream MS analytical system.

Despite the ability of our nanoparticles to efficiently capture *Babesia* proteins, certain targets may exist in exceedingly low concentrations below the limit of detection of the nanoparticle/MS workflow (1 pg/ml) (Kim et al., 2018) or may avoid detection by the mass analyzer due to poor ionization efficiency. Preliminary enrichment steps may still be required for subsets of proteins such as parasite cytoskeletal proteins absent from our analysis (i.e., tubulin, dynein, and inner membrane complex proteins). Importantly, the resulting MS data set presented herein is a snapshot of the *B. microti* proteome during acute infection rather than a representation of protein expression over time. We found overall agreement between previous transcriptomic analysis and our study, particularly among the top 10 most highly expressed genes, which included seroreactive antigens, proteins involved in carbohydrate and energy metabolism, chaperones, transporters, and proteins with functions in translation, redox homeostasis, and degradation. Combined with other components of lipid metabolism, nucleotide and amino acid synthesis, and certain proteins associated with apicoplast metabolism, the catalog of proteins presented in this study largely mirrors metabolically active *B. microti* trophozoite populations present in acutely infected animals.

In summary, ours is the first known report to present a preliminary proteome map of *B. microti* in vivo. In the short term, this information can serve as a blueprint for studies of the proteomic response of the parasite at different times and under different conditions, as proteomes are not stable, but rather highly dynamic entities, reflecting the biological environment of the cell at the time of analysis. Importantly, studies of protein expression in the context of persistent infection could be facilitated with our nanoparticle-MS approach and the animal model presented herein as hamsters infected with the GI strain of *B. microti* can survive for months despite parasitemias of ~30%. Lastly, as recent advances in MS technologies are currently being used for the rapid and reproducible identification of pathogens in clinical microbiological laboratories, we foresee adapting our methods to an MS-based diagnostic test for the multiplex identification of *B. microti* and other tick-borne pathogens in human samples.

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Note added in proof

The U.S. FDA approved the first tests to screen for *Babesia microti* in March 2018 (www.fda.gov/NewsEvents).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpara.2018.08.004.

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