Review

**Tick-borne diseases in cattle: Applications of proteomics to develop new generation vaccines**☆

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**ABSTRACT**

Tick-borne diseases (TBDs) affect 80% of the world’s cattle population, hampering livestock production throughout the world. Livestock industry is important to rural populations not only as food supply, but also as a source of income. Tick control is usually achieved by using acaricides which are expensive, deleterious to the environment and can induce chemical resistance of vectors; the development of more effective and sustainable control methods is therefore required.

Theileriosis, babesiosis, anaplasmosis and heartwater are the most important TBDs in cattle. Immunization strategies are currently available but with variable efficacy. To develop a new generation of vaccines which are more efficient, cheaper and safer, it is first necessary to better understand the mechanisms by which these parasites are transmitted, multiply and cause disease; this becomes especially difficult due to their complex life cycles, in vitro culture conditions and the lack of genetic tools to manipulate them.

Proteomics and other complementary post-genomic tools such as transcriptomics and metabolomics in a systems biology context are becoming key tools to increase knowledge on the biology of infectious diseases. Herein, we present an overview of the so called "Omics" studies currently available on these tick-borne pathogens, giving emphasis to proteomics and how it may help to discover new vaccine candidates to control TBDs.

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1. **Control of tick-borne diseases in cattle — current situation**

Tick-borne diseases (TBDs) pose a major constraint of livestock production and have considerable economic impact to rural people affecting not only their food supply, but also their daily income and other agricultural activities [1]. In 1997, the annual global losses associated to ticks and TBDs to rural people affecting not only their food supply, but also their daily income and other agricultural activities [1]. In 1997, the annual global losses associated to ticks and TBDs amounted to between US$ 13.9 billion and US$ 18.7 billion [2]. Four groups of TBDs are of importance to the livestock production: theileriosis, babesiosis, anaplasmosis and heartwater (also called cowdriosis), posing major health and management problems of cattle and small ruminants in affected areas. In cattle, East Coast fever (ECF), tropical theileriosis and babesiosis are caused by protozoan parasites (*Theileria parva*, *Theileria annulata* and *Babesia bigemina*, respectively), whereas heartwater and anaplasmosis are caused by the *Rickettsiales Ehrlichia ruminantium* and *Anaplasma marginale*, respectively. In endemic areas, indigenous cattle have developed resistance to ticks and to tick-borne pathogens (TBP)s [1]. However, susceptibility of exotic breeds presents a major obstacle to the improvement of cattle production.

Chemical tick control, treatment of animals, chemoprophylaxis and vaccination are among the measures available to limit losses incurred by TBDs. However, chemical control is limited owing to selection of acaricide-resistant ticks; in addition, residues in meat and milk have raised public health concerns. Intensive acaricide use also interferes with enzootic stability, rendering animals susceptible to the diseases. Chemoprophylaxis can be effective, but only for a short period. Therefore, more effective and sustainable integrated control methods such as vaccines should be developed to control TBDs [1].

Veterinary vaccines are the most economical and sustainable method to prevent and control infectious diseases in animals, to improve animal welfare and decrease the cost of animal production [3]. Additionally, the implementation of mass vaccination programs for animals can significantly contribute to reduce the consumption of different veterinary drugs, therefore preventing the emergence of resistance of microorganisms or parasites and reducing the burden of diseases, including zoonoses. Thus, besides improving the animal health sector itself, veterinary vaccines can enhance public health [4]. The advances in vaccine research for theileriosis, babesiosis, anaplasmosis and heartwater are presented below and summarized in Table 1.

Cattle are particularly vulnerable to *T. parva* and *T. annulata* infections, as they induce the transformation of T-lymphocytes and metastasis of these host cells [5]. *T. parva* occurs in Eastern and Southern Africa while *T. annulata* is found in North Africa, Southern Europe and Asia; these two parasites are not usually found in the same regions. The only commercially available vaccine against *T. parva* is an infection-and-treatment method which consists of a live sporozoite challenge together with simultaneous treatment of the resultant infection with oxytetracycline; this vaccine is produced in International Livestock Research Institute (ILRI)’s Nairobi laboratories. Either sporozoites or schizonts can be used to produce live vaccines for protection against tropical
Theileriosis [6]. However, the only commercialized T. annulata vaccine is based on attenuated shizonts produced in cell culture (Rakshavac-T®, National Dairy Development Board, India) [1]. Live sporozoites from infected ticks have been tested experimentally as a potential vaccine for tropical theileriosis but have not yet been applied in field vaccination campaigns.

Infection with the apicomplexan hemoproteozoon B. bovis and B. bigemina results in the destruction of erythrocytes (causing severe anemia) which poses serious limitations to cattle development in tropical and subtropical regions of the world. Although there has been some progress in the development of vaccines for babesiosis (Table 1), no effective and safe vaccine is currently commercially available [7]. So far the production of live anti-Babesia vaccines is available but it requires the supply of fresh bovine erythrocytes and serum from specific donors; these bovine donors are maintained tick-free and under highly controlled conditions to ensure that no other blood transmissible infectious agents are present.

A. marginale is the most prevalent TBP of cattle with a world-wide distribution. Acute disease manifests with anemia, weight loss, and death. In animals that survive the acute disease, A. marginale establishes life-long persistent infection [8]. In susceptible animals, the control of anaplasmosis using live vaccines was initiated in the early 1900s and these continue to be the vaccines of choice in many parts of the world. These vaccines are based on erythrocytes from spleenectomized calves experimentally inoculated with defined isolates of A. marginale or A. centrale. Although a single infection is enough to induce lifelong protective immunity in cattle, these blood-derived vaccines can be contaminated with bovine cells or other pathogens that frequently cause persistent infections in cattle [9]. To overcome this issue, inactivated vaccines were developed based on A. marginale antigen partially purified from bovine erythrocytes [9]. These blood-derived killed vaccines reduced clinical anaplasmosis but are expensive to produce, difficult to standardize, and often not cross-protective in widely separated geographic areas with different endemic A. marginale isolates. A. marginale harvested from cell culture are infective for both cattle and ticks and, when used as antigen, conferred partial protection to immunized cattle [10]. Amongst some candidate antigen as subunit vaccines, the major surface proteins 1 (MSP 1) known to be involved in adhesion to host and tick cell induced partial protective immunity in cattle [11]. No A. marginale live attenuated vaccine is available since attenuation of A. marginale has not been achieved. Despite all these efforts, there is currently no widely accepted vaccine for anaplasmosis [8].

Heartwater, caused by the Rickettsiales Ehrlichia (formerly known as Cowdria) ruminantium, is one of the most important diseases of livestock in Africa. It is particularly serious in non-indigenous livestock that are moved into affected areas causing mortality rates up to 90%. It has long been recognized that animals which recover from heartwater acquire immunity to the disease. Based on this, numerous attempts at producing a vaccine have been made [12]. Early trials were made prior to 1926 and included the inoculation of bile, the use of hyperimmune serum, the inoculation of sub-lethal doses, the attenuation of the heartwater agent or its inactivation with formalin [13]. Nevertheless all these attempts failed. Nowadays, four vaccine strategies against heartwater have been developed; the “infection and treatment” method using live bacteria, infection with in vitro attenuated bacteria [14], immunization with inactivated in vitro grown bacteria [15] and recombinant vaccines [16,17] (Table 1). At the moment, the only commercially available vaccine is based on live Ball3 organisms (which are less virulent than the other strains) derived from the blood of infected sheep, and its use is limited to South Africa [18]. The vaccine is administered intravenously and, following inoculation, body temperature is monitored and antibiotic treatment has to be applied when a rise in temperature occurs, to prevent a more serious course of the disease or animal death. One of the most promising alternatives is the inactivated vaccine; not only because it has proven to protect against homologous and heterologous challenge under controlled conditions but also a complete trial process is now readily available for the production, purification and formulation of large amounts of E. ruminantium at low cost [19,20]. Nevertheless, the problems caused by antigenic strain differences and high diversity shown in restricted areas still remain, hampering the development of a fully effective vaccine [21,22].

Globally, most of the vaccines currently available to overcome TBDs are live (blood-derived or attenuated) vaccines (Table 1). Nevertheless they have many drawbacks such as the requirements of a cold chain, a short shelf life and the potential for the transmission of other pathogens and for reversion to virulence [23]. Because of the shortcomings, there is still the need for additional research on the development of alternative safer, more cost-effective and better defined live or subunit vaccines.

Alternative approaches for TBDs control involve the development of anti-tick vaccines [24]. Control of ticks by vaccination has the advantages of being cost-effective, reducing environmental contamination and preventing the selection of drug-resistant ticks that result from repeated acaricide application. The feasibility of controlling tick infestations through immunization of hosts with selected tick antigens was previously demonstrated using recombinant antigens such as Bm86 [25], Bm95, Fer2 [26], Subolesin [27], and EF1a, UBQ chimeric antigens [28]. In some cases, these vaccines also proved to reduce the transmission of TBP [27,29]. At the moment, these vaccines are commercially available: TickGARD (in Australia) and GAVAC (in Cuba and parts of South America), but they are not fully efficacious [30]. As for vaccines against TBPs, the identification of tick antigens inducing host protective response remains the limiting step in the development of effective vaccines limiting tick infestation in hosts [24,31,32].

From the above, it is clear that the development of new generation vaccine is required. For this, it is imperative to improve knowledge of the complex host–vector–pathogen interactions involved in the pathology, immunopathology and protective immune mechanisms of the disease in order to block critical host-pathogen or vector–pathogen interactions. This review highlights how proteomics (associated to other complementary post-genomic tools, such as transcriptomics and metabolomics) can shape further investigation and discovery towards the understanding of such interactions, constituting valuable tools for enhanced rational design of vaccines against these TBDs.
<table>
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<td>Tick gut cells and salivary glands</td>
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</tbody>
</table>

\(^a\) Tick genera: H = Hyalomma, R = Rhipicephalus; "n.a." stands for "not available" or unknown.
2. Studying host–vector–pathogen interactions: key issues on biological samples

The relationships between TBP s, their tick vectors and diverse vertebrate hosts can be represented by a triangle of parasitic interactions (Fig. 1). In interaction (1) the pathogen interacts with its vector, infecting and replicating within tick cells or extracellular spaces (including those of the gut and salivary glands). In interaction (2), the pathogen interacts with its vertebrate host, infecting and replicating within targeted cells. The third component of the triangle is the interaction between the tick and its host (Fig. 1, interaction 3). Along this review, special emphasis will be given to interactions 1 and 2, as they involve key infection processes related to the adhesion, multiplication and release of the parasite from host and vector cells.

To study pathogen–tick and pathogen–host interactions (interaction 1 and 2 in Fig. 1), biological samples are required, either from in vivo or in vitro origin (Table 1). One of the restrictions for proteomics research in vivo is the limited amount of proteins that can be obtained, namely from the pathogen and tick. In 2010, Villar and co-workers recently showed that Trizol could be used to extract simultaneous DNA, RNA and proteins from naturally infected and uninfected Rhipicephalus spp. ticks to perform Differential In Gel Electrophoresis (DIGE) saturation labeling, but 300 ticks were still required to perform the several assays presented [33].

In vitro cell culture samples have been used, whenever possible, as important alternatives to in vivo sampling despite the differential protein expression between the two models [34]. Diverse intracellular pathogens such as Chlamydia pneumoniae [35], Plasmodium spp. [36] or Leishmania spp. [37] have been extensively analyzed in vitro using either their specific host or vector cells, to mimic key aspects of infectious diseases such as intoxication of host cells by pathogen virulence factors, or bactericidal innate host mechanisms [32].

One of the major constraints when working with obligate intracellular parasites is a large excess of proteins of host or vector origin that can interfere with pathogen protein detection [38]. To overcome this problem, various methods have been used to enrich pathogen proteins. In particular, biochemical fractionation based on differential density and size, harsh detergent treatments dissolving host cells but not pathogen cells, sorting by flow cytometry, or a combination of any of these methods...
3. **Proteomic studies on tick-borne pathogens of cattle**

Proteomics aims to the large scale analysis of such proteins. It is a powerful tool for the identification of protein and to study their localization, modifications, function and possible interactions or complexes they can form. Unlike "the genome," there is no single, static proteome in any organism; instead, there are dynamic collections of proteins in different cells and tissues that display moment-to-moment variations in response to several conditions such as stress or infectious processes. Furthermore, proteomics has recently proven its value to find new vaccine candidates against bacterial species such as uropathogenic *Escherichia coli* (UPEC), serogroup B *Neisseria meningitides*, *Pseudomonas aeruginosa*, *Coxiella burnetii*, *Bacillus anthracis*, *Helicobacter pylori*, *Salmonella enteritidis* and many others [39,40].

This progress could be associated to several improvements achieved at all steps of proteomic analysis: sample preparation protocols, peptide/protein separation methods, mass spectrometry (MS) data collection, data analysis and interpretation.

Classical proteomics combines a gel-based analysis to separate proteins and MS for protein identification. Thus, using one- or two-dimensional gel electrophoresis (1-DE or 2-DE), all proteins or ideally a subset of proteins (e.g., surface-exposed) are separated. The resolved proteins can then be excised from the gel and digested into discrete peptides. The proteins present in the original sample are identified by measuring their peptide masses and fragmentation patterns using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS and comparing the experimental data to the predicted masses and fragmentation patterns of known proteins subjected to the same enzymatic digestion. Proteins in the sample that are not identified by MALDI-TOF MS can be further analyzed by electrospray ionization (ESI). Although it is considered a labor-intensive, low throughput technique with poor reproducibility (specially dealing with membrane and small proteins), 2-DE remains the primary method of separating proteins, enabling the detection of intact proteins and distinguishes with high resolution variants that differ in posttranslational modification [32,41]. Moreover, when coupled to immunological assays, 2-DE may also be used to identify B-cell and T-cell antigens within complex protein mixtures. Nowadays, shotgun proteomics is also being used for protein identification. This proteomic approach involves the separation in liquid phase of digested peptides from a complex protein extract, thus avoiding some of the common problems associated with gel separation of hydrophobic proteins or proteins with extreme mass/pl and usually provides markedly better proteome coverage. All these aspects are highly relevant for the proteome characterization of pathogen material from infection models where sample quantity is usually limited. The relative strengths and weaknesses of these two complementary methodologies (2-DE versus shotgun proteomics) has been previously extensively discussed [37,42-44]. Another proteomic tool that has gain interest in vaccinology is the use of protein microarrays which contain proteins from the parasite printed on glass slides and offer an unbiased screen to detect antigens that react with sera from infected patients [39].

For an accurate protein identification, the knowledge of pathogen and possibly host and vector nucleotide and protein sequence databases are also important [45].

The annotated genome sequences of *T. parva* [5], *T. annulata* [46], *B. bovis* [47], *A. marginale* [48], and *E. ruminantium* are currently available [49,50], and the sequencing of the *B. bigemina* genome is nearing completion (http://www.sanger.ac.uk/Projects/B_bigemina/) [51]. Bovine genome is also currently available [52,53] and despite the rapid advances in molecular acarology, information on tick protein sequences is extremely restricted [54,55]. Indeed genomic information on ticks is so far available for two tick species: *bodes scapularis*, *Rhipicephalus* (Boophilus) microplus therefore limiting the application of proteomics. Therefore, there is an urgent need for more genomic information and knowledge on tick proteins expressed in a variety of tissues, life stages and species (and ideally from more than one species).

In the following sections we will present the major outcomes from proteomics studies so far performed for the six TBPs introduced above and also summarized in Table 2.

3.1. **Theileria parva and Theileria annulata**

As an example for *Theileria* spp., *T. parva* life cycle is described in Fig. 2, showing the different developmental stages by which the parasite undergoes during its transition in the ruminant host and the tick vector. Proteomic studies are mostly available for host cells.

3.1.1. **Proteomics studies in host cells**

Proteomic studies on *T. parva* were first published in 1989 [56,57]. Biosynthetically radiolabelled *T. parva* schizonts were purified from bovine lymphoblastoid cells and their proteins were analyzed by 2-DE and autoradiography [56,57]. The studies aimed first to compare *T. parva* stocks from geographically distinct regions (Mariakani and Muguca stocks from Kenya and Uganda stock, from Uganda) and then analyze schizont-protein spot patterns of the same *Theileria* stable cultivated in two different infected cell lines. While no significant difference in protein expression pattern was observed between strains, the authors observed a differential protein expression pattern depending on host cells [56]. These results suggested the possibility that selection of phenotypically different parasites could occur in vivo or in vitro. Sugimoto et al. [57] also analyzed protein and glycoprotein changes induced in bovine lymphoblasts by infection with *T. parva*. The results showed that ten proteins were found in infected cells but not in uninfected cells, and seven of these were detected in preparations of purified schizonts. Four glycoproteins were also detected on the surface of infected cells labeled with [3H]borohydride while a major glycoprotein present on uninfected cells disappeared or was reduced in infected cells. In the 90s, 2-DE and Western blot (WB) analysis were used to identify *T. parva* immunodominant schizont surface antigen [58] and increased knowledge on humoral immune responses to *T. parva* in cattle [59].
As previously mentioned, *Theileria* spp. transform host cell (leucocytes) inducing uncontrolled proliferation [60,61]. However, the parasite factors responsible for the inhibition of host cell apoptosis or induction of host cell proliferation are unknown. Recently, Oura and co-workers performed studies on infected host cells using differential RNA display and proteomics to elucidate the interaction between *Theileria* spp. and host cells. The authors suggest that to transform host cells, *Theileria* spp. might modulate the ISGylation system, a key mechanism associated with resistance of host cells to intracellular infection by pathogens, stimulation of the immune response and terminal differentiation of leukemic cells [61].

### 3.1.2. Proteomics studies in tick cells

To our knowledge, only one report is currently available for *T. parva* proteins expressed in ticks, being mostly related to the validation of DIGE technology with limited amount of detected proteins in adult infected ticks [62].

### 3.2. *Babesia bovis* and *Babesia bigemina*

*B. bovis* and *B. bigemina* are apicomplexan parasites closely related to *Plasmodium* spp. (the causative agents of malaria transmitted by mosquitoes) and to *T. parva*, with similar life cycle in both cattle and ticks. The life cycle of *B. bovis* is presented in Fig. 3; as the developmental cycle in the tick is particularly complex, with the parasites undergoing many changes, these were not detailed in Fig. 3. Interestingly, and contrary to *Theileria* spp., most of the proteomics studies published for *Babesia* spp. have been conducted in infected ticks, with few reports available in host cells (Table 2).

### 3.2.1. Proteomic studies in host cells

One of the first attempts to perform a whole “proteomic” study on the surface antigens of *B. bigemina* infected erythrocytes was performed in 1994 [63]. Using gel electrophoresis and WB analysis, the authors showed that the surface of *B. bigemina* infected...
erythrocytes had parasite-surface exposed epitopes that were conserved among the seven strains examined.

So far, one of the most studied proteins in *B. bovis* is related to a region of synteny with *Theileria* spp. at the p67 and SPAG-1 loci, both highly studied vaccine candidates against ECF and tropical theileriosis, respectively [64]. Freeman and co-workers characterized the *B. bovis*, *bov57* gene in the tick transmissible strain T2Bo by testing for the presence of transcripts in tick and cultured blood stages, but also verifying expression in cultured blood stages using monoclonal antibodies raised against the recombinant protein [64].

### 3.2.2. Proteomic studies in tick cells

Interestingly, most of the studies on *Babesia* spp. are from tick cell models [65–67]. In 2007, proteomics studies were performed using both gel electrophoresis followed by MS or/and capillary-HPLC-electrospray tandem mass spectrometry (HPLC-ESI-MS/MS) to investigate differences in expression of soluble and membrane proteins from ovaries of adult female *Rhipicephalus* (Boophilus) microplus ticks — infected or not with *B. bovis* [66]. In 2008, Rachinsky and co-workers performed a similar study in the midgut tissue of uninfected and *B. bovis*-infected *R. microplus* [65]. Among the identified Babesia-affected tick midgut proteins six proteins are implicated in signaling processes, including three Ca²⁺-binding proteins, a guanine nucleotide-binding protein, a protein with signal peptide activity and a translocon-associated receptor protein. Up-regulation of five metabolic enzymes indicated parasite-induced changes in electron and proton transport, protein processing and retinoic acid metabolism. Among the down-regulated proteins were a molecular chaperone, a cytoskeletal protein and a multifunctional protein of the prohibitin family. Identification of these proteins may provide new insights...
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<td><em>Theileria annulata</em></td>
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</tr>
</tbody>
</table>

Table 2 – Transcriptomic and proteomic studies available for the tick-borne pathogens *T. parva*, *T. annulata*, *B. bovis*, *B. bigemina*, *A. marginale* and *E. ruminantium*. 
into the molecular interactions between *B. bovis* and its tick vector *Rhipicephalus* (*Boophilus*) microplus, and could lead to identification of anti-tick and transmission-blocking vaccine candidates [65]. In 2010, additional studies were performed using midgut from partially fed adult female cattle ticks which were analyzed using a combination of 2-DE and gel-free liquid chromatography (LC)–MS/MS [67]. The authors found novel proteins such as clathrin-adaptor protein (involved in the assembly of clathrin-coated vesicles) and membrane-associated trafficking proteins such as Syntaxin 6 and Surfeit 4.

3.3. **Anaplasma marginale**

In stained blood from infected cattle, *A. marginale* organisms viewed under an optical microscope are seen as black, irregular shaped dots, usually at the edge of infected red blood cells (as exemplified in Fig. 4). Nevertheless, little is known about the development cycle of *A. marginale* in ticks, although, it has been observed that ticks do become infected when they ingest infected red cells and they can retain the infection for at least several weeks.

3.3.1. **Proteomic studies in host cells**

Proteomic studies on *A. marginale* have been mainly targeted to identify surface proteins as these proteins are known to induce protective immune response in cattle and to understand the transition from the host to the tick vector. Outer membrane protein preparations have been demonstrated to elicit protective immunity for *A. marginale* [48]. In 2005, Lopez and co-workers identified some immunogenic proteins from the purified outer membrane protein complex using 2-DE to separate the proteins, performing WB analysis with sera from cattle immunized with the protective *A. marginale* outer membrane proteins. The immunoreactive proteins were then excised and subjected to LC–MS for definitive identification by mapping the annotated genome [68]. This approach facilitated the identification of 24 immunodominant proteins including the previously characterized major outer membrane proteins Msp2, Msp3, and Msp5 but also proteins...
from the type IV secretion system (TFSS), proteins VirB9, VirB10, and conjugal transfer protein (CTP). A complementary study was published later in 2008 to evaluate the effect of cross-linked surface protein complexes to induce protection against high-level bacteremia and anemia upon *A. marginale* challenge of cattle [69]. For this, intact *A. marginale* were isolated and treated with a membrane impermeable cross-linking reagent, which resulted in covalent linkage of a group of surface exposed outer membrane proteins. After purification, the protein complex was then used to immunize cattle, and was analyzed by MS [69]. This protein complex included only a subset of the complex outer membrane immunogen, comprising Omp1, Omp7–9, Msp1a, Msp2, Msp3, Msp4, OpgA2 and Am779. These results indicate that a surface protein subset of the outer membrane cross-linked using special molecules is capable of inducing protective immunity and serves to direct vaccine development. In 2007, Lopez et al. [70] demonstrate that in *A. marginale* outer membrane-vaccinated cattle, VirB9, VirB10, and CTP are recognized by serum immunoglobulin G2 (IgG2) and stimulate memory T-lymphocyte proliferation and gamma interferon (IFN-γ) secretion providing a strong support for the use of these protein as potential vaccine candidate against *A. marginale*.

### 3.3.2. Proteomic studies in tick cells

Several studies were performed to analyze differentially regulated proteins between *A. marginale*-infected and uninfected tick cells.

In 2007, the authors used DIGE and MALDI-TOF/TOF MS to compare the protein expression pattern of *A. marginale*-infected and uninfected IDE8 tick cells. Of the 17 differentially expressed proteins, 7 were from *A. marginale*, 7 could not be identified, and 3 were of tick origin and had homology to sequence databases [71]. Only one protein, homologous to translation elongation factor 1γ, was up-regulated in infected cells. This study was also complemented by gene expression analysis, as discussed afterwards.

In 2008, Noh and co-workers also analyzed the surface proteome of *A. marginale* isolated from ISE6 tick cells [69], showing that it is less complex than in host cells. Indeed, although, fifteen proteins were identified on *A. marginale* from bovine erythrocytes,
only five were found to be expressed in A. marginale from the tick cells. Four proteins, Msp2, Msp3, Msp4, and AmB54, a peptidoglycan-associated protein, were expressed in common, Am778 being the only protein to be expressed on the A. marginale isolated from tick cells. The authors therefore suggested that remodeling of the surface proteome accompanies the transition between mammalian and arthropod hosts and identify novel targets for blocking transmission [69].

More recently, Ramabu et al. [72] also analyzed A. marginale proteins specifically upregulated in ticks in contrast to the mammalian host. Proteins from A. marginale-infected and uninfected tick cells and infected bovine erythrocytes were separated using 2-DE [72]. Spots, which were unique to infected tick cells, were submitted for MS analysis. From those spots, 15 A. marginale proteins, all annotated as hypothetical were identified, including the previously identified Am778 as well as Am638, an ankyrin-repeat containing protein. Additionally, many metabolic and housekeeping genes were identified, including, but not limited to dnaK, groEL, and rpa. Two proteins from the TFSS were also identified, VirB10 and VirB11. Confirmation of the up regulation in tick cells of a subset of these proteins, including Am470, Am410, and AmB29 as compared to bovine erythrocytes was done using WB and densitometry.

3.4. Ehrlichia ruminantium

E. ruminantium life cycle has been well described in host endothelial cells [73,74]. Briefly, it has biphasic life cycle with two morphologically distinct forms: the elementary bodies (EB), the extracellular infectious forms of the bacterium and the reticulate bodies (RB), which are intracellular, non-infectious and metabolically active. RBs re-condense back into EBs towards the end of the cycle and are then released from the host cell (Fig. 5). It is known that Amblyomma spp. can become infected during the larval and nymphal stages when they feed on infected hosts [75] but E. ruminantium development cycle in the tick is poorly understood.

3.4.1. Proteomic studies in host and tick cells

Though several reports are available on gene expression studies in E. ruminantium (as discussed below), to our knowledge, only two proteomic studies on E. ruminantium are currently available.

In 2008, Postigo and co-workers used 2-DE/MS to analyze the differential expression of the immunodominant E. ruminantium MAP1 family proteins in infected bovine endothelial and tick cell cultures. The authors showed that the major proteins detected were MAP1 in E. ruminantium-infected endothelial cells, and MAP1-1 in infected tick cells. The authors therefore suggest that this difference in protein expression might indicate that the map1 multigene family is involved in the adaptation of E. ruminantium to the mammalian host and vector tick [76].

In 2011, our group used 2-DE and MALDI-TOF-TOF MS to establish the first 2-DE proteome map of E. ruminantium cultivated in endothelial cells [Fig. 6] [77]. Interestingly, amongst the sixty-four spots identified, only four proteins belonging to the MAP1-family were identified [Fig. 6]; the other proteins detected were mainly related to energy, amino acid and general metabolism (26%), protein turnover, chaperones and survival (21%) and to information processes (14%) or classified as hypothetical proteins (23%). Additionally, 25% of the detected proteins were found to be isoforms suggesting that post-translational modifications might be important in EB for the regulation of cellular processes such as host cell recognition, signaling, metabolism and in determining antigenicity as previously observed in other Rickettsiales. Additional studies on MAP1-family protein using immunochemical labeling also revealed that these proteins are differentially expressed along the bacterium life cycle, presenting different structural organization. Interestingly, when infectious EBs are released from host cells, MAP1 appears to be organized in SDS and heat-resistant dimers and trimers stabilized by disulfide bridges [77].

4. Complementing proteomics with transcriptomics analyses

Despite significant advances in protein separation and detection techniques and in the accuracy and sensitivity of MS, proteomics...
still presents bottlenecks with the critical disadvantage that, unlike DNA, proteins cannot be amplified to increase the sensitivity of detection techniques. To cope with the difficulties associated to global analysis of protein expression, some researchers still rely on mRNA expression levels as an indicator of the presence of active proteins and use microarrays, real time PCR (qPCR) and reverse transcriptase PCR (RT-PCR) for mRNA detection. Nevertheless, the relationship between mRNA and protein is a complex one and several studies have revealed a relatively weak correlation between mRNA expression levels and protein abundance, with many genes being uniquely detected either by transcriptome or proteome [45,78,79]. This could be due to at least three reasons: i) there are many complicated and varied post-transcriptional mechanisms involved in turning mRNA into protein that are not yet sufficiently well defined to be able to compute protein concentrations from mRNA; ii) proteins may differ substantially in their in vivo half lives; and/or iii) there is a significant amount of error and noise in both protein and mRNA experiments that limit the ability to get an accurate picture [80].

As for some TBPs reports on transcriptomics analysis are found in higher number than proteomics ones, we found of interest to also include them in this Review, as complementary information.

4.1. Theileria spp.

An interesting review on the T. parva available genome information was published in 2000 [81]. The authors briefly present the by then known characteristics of T. parva genome and previewed significant insights on vaccine development and disease control through the combination of genome annotation, microarray technology and comparative genomics. T. parva transcriptome was analyzed using massively parallel signature sequencing (MPSS) [82]. The authors have established that transcripts were widely distributed throughout the genome and that there was a significant concordance between transcription and protein expression for heat shock proteins, particularly over-expressed in the schizont stage. In 2004, Nene and co-workers analyzed the genes transcribed in the salivary glands of female Rhipicephalus appendiculatus ticks infected with T. parva [83]. The resulting EST data are particularly relevant since they can further be used for the construction of microarrays to probe vector biology, vector-host and vector-pathogen interactions and to underpin gene identification via proteomics approaches. In 2009, Schmuckli-Maurer et al. [84] analyzed subtelomeric variable secreted proteins (SVSPs) expression in T. parva-transformed cell lines established in vitro by infection of T or B lymphocytes. The authors defined that SVSP expression was largely influenced by the parasite genotype and not by the host background or cell type. Interestingly host genotype dramatically influences gene expression of cattle subjected to T. annulata infection. In fact, a transcriptomics profiling of Bos indicus and Bos taurus cultured macrophages infected with T. annulata revealed several transcripts with differential expression such as: Toll-like receptor 10 and signal-regulatory protein alpha (SIRPA) or MHC class II DQa, CD9 and prion protein (PRNP) [85,86]. These results clearly demonstrate the importance of the host genetic background in underpinning host response and in a successful disease control.

To identify both host and parasite genes that show altered expression during differentiation of T. annulata from the macroschizont to the merozoite stage of the life cycle, the RNA profiles of two T. annulata-infected clonal cell lines (D7 and D7B12) with the same genetic background have been compared by RNA display [87]. After cultivation of D7 and D7B12 macroschizont-infected cells either at 37 °C or at 41 °C to induce differentiation to the merozoite in the cloned cell line D7), RNA was extracted. From the experiment at 37 °C, 9 cDNA fragments showed altered levels between D7 and D7B12 cell lines, 8 being from host origin, while one was parasite derived. At 41 °C, 6 transcripts showed to be differentially expressed between D7B12 cells and differentiating D7 during a differentiation time course, 1 being of host origin and 5 of parasite origin. Globally, the authors identified a low number of parasite genes involved in T. annulata differentiation from macroschizont to the merozoite, but they still recommend the use of RNA display with additional methodologies for further isolation of subsets of differentially expressed genes to provide further insights on this complex differentiation process.

4.2. Babesia spp.

In B. bovis, Al-Khedery and Allred [88] have conducted a study on the heterodimeric variant erythrocyte surface antigen 1 (ves1) gene which is known to be involved in the virulence, persistence and antigenic variation of the parasite. The authors described the location of the genes and have provided evidence that variation of two transcriptionally active genes occurs through a mechanism of segmental gene conversion involving sequence donor genes of similar organization. The interest of this study to transcriptomics lay in the fact that it combined information from genomics, through the relevant ves1α gene and relate it to VESA1 protein and particularly with a key feature of TBPs which is antigenic variation, one of the constraints in vaccine development. In 2007, an expression oligonucleotide microarray was developed showing its potential to analyze B. bovis gene expression and hereby complete B. bovis infected erythrocyte expression profile [89]. A transcriptional analysis of rRNA gene unit expression in B. bovis was published recently [90]. In this study, the authors determine differential transcription of rRNA, depending on both the environment and the life stage of the parasite. Finally, in 2011, Mesplet and colleagues [91] used microarray analysis to the expression of Bovipain-2, a protease released into the host erythrocyte and an important virulence determinant that was found to show differential expression between virulent and attenuated strains.

The rap-1 gene family occurs in all babesial species examined, rhoptry proteins being considered as prime candidates for the development of improved vaccines against bovine babesiosis. In 2003, Suarez and co-workers studied the organization of the complete rap-1 locus, identifying the rap-1b and rap-1c genes and examined the expression of all rap-1 genes in the locus. To test whether all rap-1 genes in the locus were co-transcribed in merozoites, they used RT-PCR, Northern blots, and quantitative real-time PCR; the results showed that rap-1a genes produce the most abundant transcripts of the family, while rap-1b transcripts are the least abundant despite the large number of gene copies. Similar patterns of transcription were observed whether merozoites were obtained from in vitro cultures or in vivo infection. Western blot analysis of merozoites revealed the expected RAP-1a expression but failed to detect
expressed RAP-1b and RAP-1c, indicating that expression of the rap-1 genes is regulated both at the transcriptional and translational levels [92].

In 2008, the expression of rap-1a and hsp20 genes in sexual stages and kinetes of B. bigemina was studied using RT-PCR; a complementary expression analysis was carried out using an indirect immunofluorescence test with specific antibodies against HSP-20 and RAP-1a [93]. Globally, the results confirmed the hypothesis that these genes and corresponding proteins are expressed in sexual stages and kinetes, and stress the importance of these proteins in the cellular physiology of tick stages.

4.3. *Anaplasma marginale*

In *A. marginale*, transcriptomics approaches seem to be directed primarily to gene expression of the infected tick vector and infected host cells instead of the pathogen itself. In fact, a functional genomics approach was used to characterize tick genes regulated in response to *A. marginale* infection [71]. According to the authors, four genes of the tick vector, which encode for putative Gluthatione S Transferase (GST), salivary selenoprotein M, vATPase, and ubiquitin, are involved in *A. marginale* infection in different tick tissues/organisms. These results demonstrated that tick cell gene expression mediates the *A. marginale* developmental cycle and trafficking throughout its vector. More recently, Zivkovic et al. [94] compared tick gene expression in response to two *Anaplasmataceae* species (*A. marginale* and *A. phagocytophilum*) by microarray and qRT-PCR analyses. The results provided evidence of different gene expression responses in tick cells infected with *A. marginale* or *A. phagocytophilum* and the authors concluded that the differences in gene expression and *Anaplasmataceae*-tick interactions reflect differences in the pathogen life cycle at the level of the tick cells. The same research group has also studied differential gene expression at the level of the salivary glands in male *R. microplus* in response to feeding ticks, demonstrating that SCOTS has a key importance for gene expression and multiplication in ticks. More recently, Mercado-Curiel et al. analyzed the effect of *A. marginale* infection on the R. microplus tick midgut and salivary gland transcriptome during feeding and in response to infection confirming the existence of a massive organ-specific transcriptional response to tick feeding, as numerous R. microplus genes were regulated in response to feeding and were differentially regulated between the midgut and salivary gland [96]. Nevertheless, it was found that *A. marginale* exerts a minimal effect on the tick transcriptome.

Previous studies showed that the efficiency of *A. marginale* to develop inside the tick is markedly different for genetically distinct strains [97]. Thus, to confirm this hypothesis Agnes and co-workers analyzed the genes possibly involved in the transmission and infection of two *A. marginale* strains (St. Maries and Israel vaccine) in the tick [99]. The results indicated that although a small difference in gene expression was observed between tick salivary glands infected by St. Maries or Israel vaccine strains during feeding (20 versus 16 genes), the expression levels of certain genes were equal or lower than those observed in erythrocytes infected by the same stains, suggesting that these genes were not exclusively related to salivary gland colonization.

4.4. *Ehrlichia ruminantium*

In 2002, Bekker and co-workers performed a transcriptional analysis of the MAP-1 (major antigenic protein 1) multigene (map1-1, map1-2 and map1) family [99]. In this study, RT-PCR was used to study the transcriptional activity of these genes in different isolates of *E. ruminantium* grown in bovine endothelial cells, in two different tick cell lines, and in *A. variegatum* ticks. The authors concluded that, as for MAP1-1 proteins (mentioned above), the map1-1 gene transcript was detected in *A. variegatum* ticks, and also in bovine endothelial cells for virulent Gardel and attenuated Senegal. Still, map1-1 gene was not found in virulent *E. ruminantium* Senegal strain grown in bovine endothelial cells but, being found in different passages of the in vitro attenuated Senegal isolate grown in bovine endothelial cells, as well as in the Gardel isolate grown in two tick cell lines. Van Heerden and co-workers have characterized the major outer membrane multigene protein family [100]. Both studies contributed to improve the knowledge on the genetic structure and organization (mapping) of the map1-gene family, and the detection of their transcript in different forms of the bacteria either in host or vector cell cultures. In 2005, Bekker and co-workers continued the initial analyses on map1-1 gene expression and observed that in three *E. ruminantium* strains (Gardel, Senegal and Welgeworden) map1-1 gene was indeed predominantly detected in infected ticks [101]. The authors finally concluded that the map1 gene cluster is relatively conserved, but nevertheless subject to recombination. A study on the map1 multigene family transcription has been carried out in vivo in unfed and feeding *A. variegatum* ticks [102]. The results point out that in feeding ticks, map1-1 transcripts were more abundant in midguts whereas high levels of map1 transcripts were observed in salivary glands, indicating that the cluster occurs in different tissues before and during transmission, playing therefore a preponderant role in the life cycle of *E. ruminantium*. More recently Emboulé and co-workers optimized the Selective Capture of Transcribed Sequences (SCOTS) methodology to successfully capture *E. ruminantium* mRNAs, avoiding the contaminants of host cell origin and eliminating rRNA which accounts for 80% of total RNA encountered [103]. Overall, the authors conclude that SCOTS has a key importance for *E. ruminantium* transcriptomics analysis (namely for microarrays) and is of potential use in other Rickettsiales species, but also in other obligate intracellular bacteria. Pruneau and colleagues [104] have just determined the genome-wide transcriptional profile of *E. ruminantium* replicating inside bovine aortic endothelial cells using cDNA microarrays. Interestingly, over 50 genes were found to have differential expression levels between RBs and EBs. A number of genes involved in metabolism, nutrient exchange and defense mechanisms, including those involved in resistance to oxidative stress, were significantly induced in *E. ruminantium* RBs, a result likely due to the oxidative stress and nutrient starvation occurring in bacteria located in vacuoles. Finally, the authors demonstrate that the transcription factor dksA, known to induce virulence in other
microorganisms is over-expressed in the infectious form of *E. ruminantium*.

### 5. Metabolomics — providing additional insights in pathogen virulence and survival

Metabolomics is the systematic study of all metabolites in a biological cell, tissue, organ or organism, which are the end products of cellular processes. While mRNA gene expression data and proteomic analyses give partial indications of cell modifications, metabolic profiling provides an instant snapshot of its physiology. As with proteomes, metabolomes are dynamic and change in response to nutrition, stress, disease states, and even show diurnal variations. The chemical diversity of metabolome components makes its comprehensive analysis with single analytical technology difficult. The principal technology platforms for metabolomics are NMR spectroscopy and gas chromatography MS (GC–MS) or LC–MS [105].

The metabolome of infectious pathogens has gained a particular interest as researchers have found that the expression of virulence factors might be tightly controlled by their metabolism [106]. For instance, carbon metabolism was found to be crucial for the modulation of the virulence factor PrfA in *Listeria monocytogenes* [107] and certain metabolites involved in iron acquisition are crucial in infections caused by *E. coli* [108]. Additionally, the identification of key metabolites for pathogen development can contribute to develop improved culture medium and, in particular, new axenic medium as previously done for *C. burnetti* [109] and *Tropheryma whipplei* [110]. At the moment, the metabolism of TBPs is poorly known. The published reports are only related to the pyruvate metabolism by *A. marginale* in cell-free culture [111], the amino acid metabolism of *E. ruminantium*-infected endothelial cells [112–115] and the carbohydrate metabolism of Babesia spp. in basal salts media [116].

### 6. System biology: integrating “Omics” data to improve vaccine development

An extraordinary wealth of Omics data generated from genomic, proteomic and metabolomic methodologies provide unprecedented opportunities for biomarker and antigen identification. Accurate understanding of these data and rational prioritization of potential candidates require integrative approaches such as systems biology [117]. To take the full advantages of systems biology methodology, it is necessary to have (i) bioinformatics tools, but also (ii) large data libraries and robust datasets for accurate data mining [118].

Tools such as open reading frame, epitope prediction, and sequence conservation algorithms are commonly used but the implementation of more complex algorithms and software packages is required in order to meld heterogeneous data types coming from the different “Omics” [118]. For instance, modeling software such as BioSignatureDS™ have been used to compare data from both transcriptomic and proteomics approaches to create an overall dynamic of the host–pathogen interaction in order to better understand the host dynamic responses to *Brucella melitensis*, *Mycobacterium avium paratuberculosis*, *Salmonella enterica Typhimurium* (STM), and a *Salmonella* mutant [117] providing a basis for rational development of vaccines against diseases such as brucellosis and salmonellosis.

Data repositories are also critical to perform an efficient literature mining. As an example is the Vaccine Ontology based approach VO-SciMiner that generated a comprehensive vaccine–gene interaction network, using *Brucella* as a case-study [119]. Information regarding this pathogen as well as many others is also compiled in a web-based vaccine database and analysis system, Vaccine Investigation and Online Information Network (VIOLIN) (http://www.violinet.org) [120]. This database compiles both general information regarding pathogens, host range, host protective immunity and vaccine specific information such as analysis of vaccines of commercial use describing type and methods, or vaccine candidates in clinical trials or early stages of development. There are several bioinformatic tools available for data query and analysis, allowing users to search curated data and analyze vaccine-related genes in order to simplify and improve vaccine research. Within VIOLIN, researcher can also use Vaxpresso and Vaxmesh, to search for all possible vaccine-related papers as well as sentences that match specific keywords and ontology-based categories, giving a specific pathogen. Other data repositories include the Gene Expression Omnibus, the Open Proteomics Database, or the Biomolecular Network Database.

Nevertheless, systems biology research is still in its infancy and the maturation of the field will proceed as the many challenges that it faces are addressed and successfully solved. Indeed, despite the increasing availability of data generated by large-scale microarray, proteomic, or sequencing technologies, these must be carefully analyzed and cataloged due to the inherent technical and biological noise in the data set before they can be used for integrative approaches. Furthermore, new predictive computational models that incorporate sufficient molecular details and make effective predictions of pathogen–host or vector–pathogen–host interactions for experimental in silico testing need to be developed. This not only involves ample investment cost but also require researchers to be fully engaged in translational research, especially bridging bench and bedside scientists during the development of its own infrastructures and collaborations [121].

Post-genomics tools such as transcriptomics and proteomics and, particularly, metabolomics were only recently being used to study theileriosis, babesiosis, anaplasmosis and heartwater and, of course, the research output is therefore extremely limited for systems biology to be used at the moment. The challenges in using systems biology to TBDs will be related to the integration of “Omics” data from host–vector–pathogen interaction, the different developmental phases of pathogens in both the host and the tick, the parasite attack, host response and parasite counter-response, and the interactions between transmitted pathogen and the vector tick. Another challenge in developing vaccines against these TBDs is related to the wide spectrum of animal hosts in which vaccines must perform effectively; indeed these pathogens can cause diseases in a series of hosts other than cattle such as Theileria spp. (infecting African buffalo, Indian water buffalo, waterbuck, and yak) and *E. ruminantium* (which can affect cattle, sheep, goats, and water buffalo).
7. Conclusion

Despite their veterinary importance, no fully effective vaccine is currently available for any of the TBDs theileriosis, babesiosis, anaplasmosis and heartwater.

Until now, vaccines have been mainly developed by following the basic principles established by Louis Pasteur over a century ago: isolation, inactivation, and injection of the causative agent. However, this conventional form of vaccine production is limited in the ability to target pathogens for which no suitable animal model exists and/or bacteria that are antigenically diverse [118].

Nowadays, new breakthroughs in vaccine research are increasingly reliant on novel “Omics” approaches that incorporate high throughput cutting edge technologies such as genomics, proteomics, transcriptomics, and other less known “Omics” such as metabolomics, immunomics, and vaccinomics [122] to deepen our understanding of the key biological processes that lead to protective immunity, observe vaccine responses on a global, systems level, and directly apply the new knowledge gained to the development of next-generation vaccines with improved safety profiles, enhanced efficacy.

Although these strategies have already proven to be useful to develop vaccines against other fastidious pathogens, the use of “Omics” are still in their infancy regarding to TBDs. With the recent availability of TBPs genomes, these high-throughput technologies will now significantly contribute to overcome knowledge gaps on the role of key parasite molecules involved in cell invasion, adhesion, asexual and sexual reproduction, tick transmission and, surely revolutionize the length of time and capacity for discovering potential candidate vaccines (such as proteins involved in protective immune response, tick feeding, or parasite development). The recent development of transient and stable in vitro transfection systems for these parasites (such as B. bovis [123] and A. marginale [124,125]) also pave the way for future exciting developments as they will facilitate the functional analysis of the bacteria genes and will improve our understanding of the biology of and immunological response to these parasites.

By joining all these efforts using an integrative systems biology approach, researchers might then be able to develop a new vaccine against TBDs that would ideally contain antigens from both pathogen and the tick vector to simultaneously avoid the transmission of the pathogens and control tick infestations. Such vaccine would enhance animal productivity, reduce tick control costs, and ultimately improve the standard of living of local populations in susceptible regions.

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