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Genome-Based Vaccinology Applied to Bovine Anaplasmosis

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Abstract

Bovine anaplasmosis is an infectious non-contagious disease transmitted mainly by ticks or fomites contaminated with *Anaplasma marginale*. Once cattle have developed the disease it can be treated with antibiotics or chemotherapy, although with partial success. Still, there is no effective and global prophylactic method available, mainly because of variability and diversity showed by different *A. marginale* strains distributed worldwide. In this regard, several proteins have been proposed as immunogens, MSPs, OMPs, Type IV Secretion System Proteins and some other hypothetical proteins, which have been chosen either by experimental evidence or more recently by genome-based analysis. So far, the results suggest that a single molecule will not be enough to trigger a protective immune response in the host, so it is necessary to identify other proteins or epitopes with adequate immunological properties, a process in which omics tools have potential. In order to develop a vaccine against bovine anaplasmosis, it has been proposed by the use of combinations of molecules, exposure formats and application protocols to provide an effective control of the disease.

Keywords: *Anaplasma marginale*, genomics, vaccinology, bovine anaplasmosis, OMP, MSP

1. Introduction

Tick-borne diseases are considered a major obstacle and the cause of great economic impact for livestock production [1]. Control measures currently available for tick-borne diseases include the use of acaricides for reduction or tick populations, specific chemotherapy, chemoprophylaxis, controlled exposure and vaccination. These measures limit losses caused by ticks and the

diseases they transmit [2]. Globally, the most important rickettsial disease in cattle is bovine anaplasmosis caused by *Anaplasma marginale* [3].

Vaccination is the method of choice for preventing infectious diseases. In the case of bovine anaplasmosis, while there are live vaccines, these pose many risks, including: (*i*) spread of other blood-borne pathogens, such as *Babesia* spp. and virus-like bovine leukemia virus to mention a few [4]; (*ii*) standardization of vaccine dose; (*iii*) maintenance of carrier animals; (*iv*) quality control and production; (*v*) maintenance and transportation of vaccines to the end user, including the need of a cold chain [5, 6].

Inactivated vaccines based on the use of the extracted bacteria while effective, are restricted due to: (*i*) potential contamination with erythrocyte membrane antigens; (*ii*) wide antigenic variation between *Anaplasma* strains [7]; (*iii*) possible short-term immunity; and (iv) amelioration of clinical signs while not preventing infection, so the animals remain carriers for the rest of their lives [8].

Vaccine design is compounded by the large antigenic and genetic diversity found in strains from a region to another, within the same herd and even within the same animal [9, 10]. Current investigations focus on the search for new alternatives for designing vaccines and diagnostic assays [11–13]. In this review, besides discussing some fundamental aspects of anaplasmosis, we focus on the molecular characteristics that make *A. marginale* capable to persist in nature including: (*i*) the mechanisms of evasion of the host's immune response; (*ii*) diversity; (*iii*) hypervariability of some of its components; or (*iv*) replication.

2. The causal agent

Anaplasma marginale is a tick-borne pathogen and the causative agent of bovine anaplasmosis [14]. *A. marginale* is classified in the Rickettsial order, reorganized into two families such as Anaplasmataceae and Rickettsiaceae [15]. *Anaplasma* organisms are obligate intracellular Gram-negative rickettsia, found exclusively within vacuoles derived from the erythrocyte membrane, and are membrane-bound within the cytoplasm of the host cell. *A. marginale* persist in nature in mammalian and ticks hosts, which serve as reservoirs of infection [16]. In the bovine, *A. marginale* infect erythrocytes and endothelial cells [17]. The infection process in endothelial cell has not been described and it is considered as of no relevance within the persistence mechanisms for the rickettsia [18]. Ticks transmit rickettsia from the salivary glands during feeding (**Figure 1**), and within the erythrocyte, the rickettsia replicates by binary fission to form 8–12 initial bodies and exit from the erythrocyte does not involve destruction of the host's cell [19]. Once out of the host cell, the initial bodies invade new erythrocytes in endless cycles. Ticks acquires the rickettsia while feeding on carrier hosts. In the tick, the rickettsia infects midgut cells, where there is a first cycle of replication and from here dense forms move to other tissues. After several rounds of replication, dense forms travel to the salivary glands where the rickettsia is transmitted to a new mammalian host [16, 20].
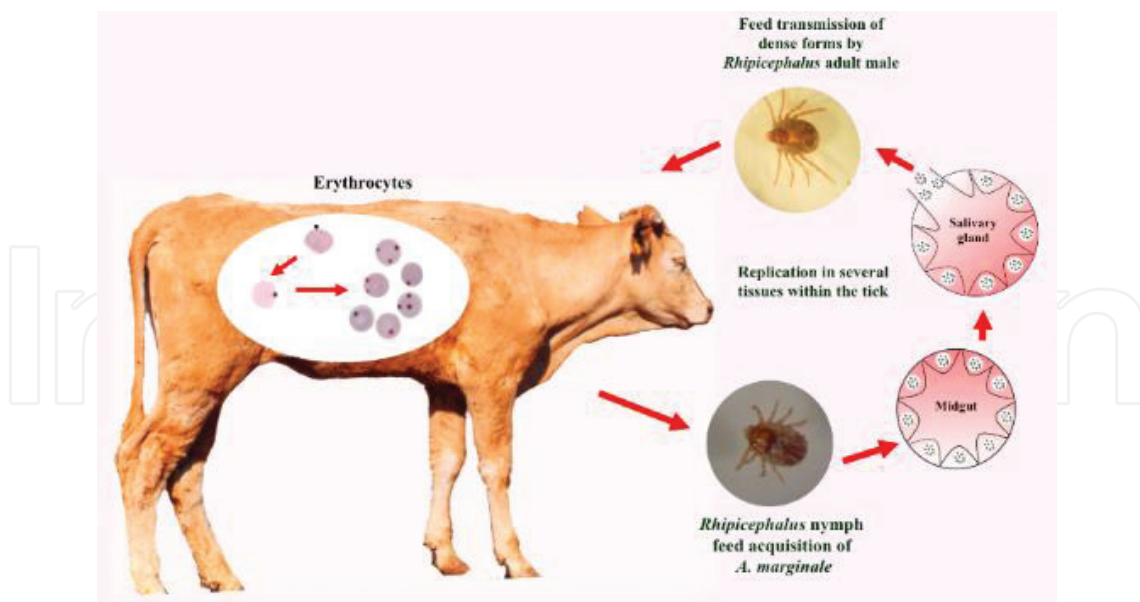
Figure 1. Proposed life cycle of *A. marginale* in bovine. Modified from [20].

Many species of ticks have been implicated in the transmission of *A. marginale*, although *Dermacentor andersoni* is the most studied of all [16]. Recent studies have focused on the role of ticks *Rhipicephalus* (*Boophilus*) *microplus* as vectors of bovine anaplasmosis, an issue discussed below [21, 22].

The first *A. marginale* sequenced genome published [23] present a very complete description of the known features to that date. Up to now, there are at least 2 full genome sequences published (St. Maries and Florida), other 2 almost complete sequences, and 10 more partially annotated sequences (NCBI/Genome). Comparative studies with the available genome sequences have been carried out with very interesting results as far as the study of hypervariable genes/antigens [24], while other genome/transcriptome analysis have focused on the transmission phenotype genes involved [25].

In this chapter, we will review the information available, since the publication of the St. Maries genome to the specific genes that have been studied as vaccine candidates. Vaccination against bovine Anaplasmosis traditionally relied on attenuated [5, 26] and inactivated organisms [6, 27]. Both of these types of vaccines will, in most cases, induce a degree of immunity, which do not prevent infection. There are a number of examples of experimental vaccines, but in this review only those cases where there has been modification of the organism or recombinant antigens are included as vaccine candidates will be discussed.

2.1. Major surface proteins as vaccine candidates

Over the last 30 years, six neutralization-sensitive membrane-exposed proteins were originally reported in 1984 [28, 29] and later named **M**ajor **S**urface **P**roteins [30]. This group of proteins has been the subject of a great number of studies aimed at developing a vaccine.

Msp1 is a heterodimer composed by Msp1a (100 kDa) and Msp1b (105 kDa) joined in a non-covalent manner and are exposed on the surface of *A. marginale* [31]. Details of the genetics, structure, and composition of these two peptides have been described elsewhere [24]. Msp1a is coded by a single gene and its product is composed of a variable number of tandem-repeat units of 28–32 aa in length at the amino terminus. The carboxyl end is conserved and extends mostly as an intracellular domain [32]. Msp1b is coded by a multigene family that expresses several variants during the acute and chronic phases of the infection [33]. Msp1 is an adhesin toward erythrocytes and tick-gut cells whereas Msp1b only toward bovine erythrocytes [34, 35]. It is now known that the adhesion function in Msp1a is located in the variable region which is composed of several short amino acid sequences (repeats). Analysis of Msp1a repeats from different isolates has shown no association between tick-transmission capabilities and the type or number of repeats present within this variable region [36]. Repeat sequences though have been used for defining genotypes associated to other markers such as the pseudogenes present in Msp2 [37] or distinguishing genotypes in cattle superinfected with two or more different *Anaplasma* strains [10, 38, 39].

Bioinformatic analysis carried out with Msp1a amino acid sequence have shown that Msp1a variable region is rich in highly immunogenic B cell epitopes, yet these sequences are considered distracters to the immune system of the host, despite the fact specific monoclonal antibodies neutralize infection to both the erythrocytes and tick cells [40]. Msp1a also contains Th1 cell epitopes in the carboxyl conserved region, which may be involved in immunoprotection [41]. Initial immunization experiments with recombinant Msp1a showed that autologous immunity was afforded; yet heterologous immunity was poor [42]. In a semi-controlled experiment where Msp1a was used as marker for matching the vaccine strain and the local strain in an inactivated vaccine trial in the field, which resulted in partial immunity except when the challenge was carried with the autologous strain but not when challenge was carried with heterologous Msp1a-matched strains [7].

More recently, two epitopes, STSSQL (Am1), located within the consensus sequence of the repeat and SEASTSSQLGA (Am2), which is located in C-terminal end of the 28-aa repetitive motif of the MSP1a protein, were identified using phage display technology for identification of immunodominant epitopes recognized by a neutralizing monoclonal antibody against MSP1a; these peptides were recognized by many but not all healthy infected animals tested by ELISA assay [43]. These synthetic peptides were conjugated to bovine serum albumin and used for immunization of mice, which the authors claim were infected with *A. marginale* was achieved. Details of the challenge strain used in this experiment are absent [44] and there have been no follow-up articles using the same peptides in cattle.

The inclusion of Msp1a or its "conserved epitopes" should be carried with caution as the diversity of this marker is wider in regions where the tick vector is *R. microplus* than in its absence [10, 45]. Vaccination based on Msp1a epitopes is further confounded by the fact that the Msp1a variable region may contain one or several repeats which may include the same or different repeats and that there are more than 300 repeats reported [45, 46] and this number is bound to increase as the number of epidemiology studies is published. Furthermore, the

number of Msp1a-distinct strains (up to nine) within the same herd or even within the same animal [10, 47, 48] complicates even more the design of an effective vaccine.

While the adhesion function of Msp1a protein is located on the variable region, number or type of different Msp1a repeat sequences has not been associated with tick-transmission phenotype [49, 50]. Repeat sequences though have been used for defining genotypes associated to other markers such as the pseudogenes present in Msp2 [37] or distinguishing genotypes in cattle superinfected with two or more different *Anaplasma* strains [10, 38, 39].

As for Msp1b, initial experiments indicated that it was a poor immunogen [51, 52]. Further evidence indicates that immunization of naive calves with a recombinant fraction containing the Msp1a-T cell epitopes linked to recombinant Msp1b1, induced a much greater antibody titer to Msp1b than what was previously observed [52, 53].

DNA vaccines based on either one of Msp1a or Msp1b have given disappointing results. In an early effort using a construction pVCL/MSP1a for the immunization of mice and cattle [54], a predominant IgG1 antibody pattern was observed in the two immunized calves. No challenge was performed, however, as it has been proven, an IgG2 response is necessary in order to achieve protection [11, 55]. In a similar study with an Msp1b DNA construct, immunization not only did not induce immunity but immunized animals developed more severe clinical disease than controls [56]. These latter authors did not test for the type of immunoglobulin induced by vaccination.

Further studies with DNA vaccines constructs which included bovine herpes virus 1 tegument protein, BVP22 domain and an invariant-chain major histocompatibility complex class II-targeting motif capable of enhancing dendritic cell antigen uptake and presentation were fused to a sequence encoding a B and T cell antigen from the *A. marginale* Msp1a [57, 58]. This approach included the intradermal inoculation with a mixture of 2 mg of DNA encoding the molecular adjuvants bovine FLT3L and GM-CSF to recruit DCs to the intradermal immunization site, the results of this experiment were very encouraging as they stimulated the desired type of immune response with rapid recall of antibody production over a reasonable period after a single immunization. This study, however, suffers of several flaws (i) the inoculation in different points of the dendritic cells stimulant and the vaccine itself, (ii) while the responses as measured, were in all senses the appropriate ones for resistance, there was no challenge of vaccinated animals and second, the age of the vaccines would not allow for distinction between a solid immune response induced by vaccination or natural resistance commonly observed in animals under 1 year of age [3].

Msp2 is a highly immunodominant 36 kDa protein coded by a multigene family consisting of a functional gene that codes for the amino and carboxyl ends and a variable number of pseudogenes (5 in the St. Maries strain) [59] which recombine with the main gene, through gene conversion, in a single expression site such that the protein is expressed as a new variant in each cycle of rickettsemia, every 6–8 weeks [60]. *msp2* pseudogenes code for hypervariable, hydrophilic sequences containing highly immunogenic B cell epitopes which induce a new immune response consequent to a new Msp2 variant [61]. The amino and carboxyl ends of the

protein are hydrophobic conserved segments inserted into the membrane of the rickettsia [62] (and contain Th1 cell type epitopes that are preserved along different geographic strains) [29, 55].

Just like Msp1 and Msp1b, Msp2 was discovered through the neutralization of infection by specific monoclonal antibodies and it was considered a vaccine candidate [28], this has not been the case. While the hypervariable region of the protein contains a number of highly immunogenic type B cell epitopes, it has been recognized that antibody directed to these epitopes are distractors of the immune response during the periodical appearance of Msp2 variants [60, 63].

Msp3 is a very immunogenic 86 k Da protein located on the surface of the rickettsia [28]. *msp3* is also composed by a central hypervariable region coded by several pseudogenes which recombine with the conserved amino and carboxyl ends [64, 65]. Based on previous studies [64] and analysis of the published genome sequence, it is speculated that MSP2 and MSP3 originated from a common ancestor [66], and have diverged since that event. Sequence identity between *msp3* and *msp2* pseudogenes is reduced, an average of 38%, identity within *msp3* pseudogenes 68% and, within *msp2* pseudogenes 78% [66, 67]. Immunologically, Msp2 and Msp3 share epitopes recognized in vitro by CD4$^+$ cells clones from vaccinated cattle [55]. Recombination of pseudogenes in a mosaic pattern also adds to the presentation of polymorphic antigens that, when resolved through 2D electrophoresis, are observed as a series of antigens with the same molecular weight and different but very close isoelectric points [68]. Appearance of Msp2 and Msp3 variants in the persistently infected bovine gives rise to a more complex situation with negative implications for immune protection. Though Msp3 induces production of large amounts of antibodies [69], protection afforded is very limited [70]. Msp3 is known to cross-react with other rickettsiae such as *A. ovis, Ehrlichia risticii, E. wengii, E. equi* and *E. ruminantium*, which make it unsuitable for specific *A. marginale* sero-diagnosis [71].

Msp4 is a 31 kDa protein, encoded by single highly conserved gene, *msp4* [72]. Msp4 is also present in *A. marginale* subsp. *centrale* with 83% identity in the nucleotide sequences and 91.7% in the amino acid level [73]. To date, there is no solid evidence that Msp4 may be involved in protection as, initial studies showed lack of recognition by sera of animals immunized with an initial body membrane fraction [42], however when animals are immunized with a recombinant Msp4 adjuvated with Iscometrix as adjuvant, there seems to induce an antibody response. *msp4* has been used as a base for phylogenetic studies which have shown that there are variations in 168 bp and, of these, 39 bp show utility in parsimony analysis such that isolates from several countries in the Americas can be grouped according to their geographic location [50]. Msp4 is highly conserved over several Mexican isolates [9].

Msp5 is a highly conserved 19 kDa MW protein in *A. marginale, A. marginale* subsp. *centrale* and *A. phagocytophilum* [23, 73–75]. Immunization with Msp5 induces the production of large quantities of non-protective antibodies [69] so it is no suitable for vaccination. Animals naturally infected with the rickettsia produce specific antibodies that can be found in recent and old infections so the protein has been used successfully in a diagnostic competitive-ELISA test [76]. Despite the cross-reaction of antibody between *A. marginale* and *A. marginale* subsp. *centrale* at the competitive-ELISA [74, 77], the test has been adopted as the standard for serologic diagnostic of bovine anaplasmosis.

2.2. Type IV secretion system proteins

Secretion systems in bacteria are complex structures by which they communicate with its environment. There are several secretion systems some which span both the inner membrane (IM) and the outer membrane (OM), and those that span the OM [78]. Among several secretion systems described in nature, type 4 secretion systems (T4SSs) have the unique ability to mediate translocation of DNA (in addition to proteins) into bacterial or eukaryotic target cells. T4SSs are found in both Gram-negative and Gram-positive bacteria and also in some archaea [79]. Their most common role is to mediate the conjugation of plasmid DNA; thus, these systems contribute to the spread of plasmid-borne antibiotic resistance genes. As the ability to conjugate is a common bacterial trait, T4SSs are the most ubiquitous secretion systems in nature. In addition, T4SSs are involved in bacterial pathogenesis in a few organisms, and they mediate the secretion of transforming proteins in *Helicobacter pylori*, toxins in *Bordetella pertussis* and other effector proteins required to support an intracellular lifestyle in bacteria such as *Legionella pneumophila* [79].

Along with the publication of the first complete genome of *A. marginale*, and its annotation, some real or putative homologous genes of T4SS were described in St. Maries genome [23]. Although many studies have been done about MSPs, so far, we still require other approaches to find better vaccine candidates. An approach was shotgun sequencing of the proteins of a membrane-enriched fraction of *A. marginale*, which induced an antibody response in naive calves [80]. In this study, 25 immunoblot positive spots were sequenced and identified through their annotation in the genome. Among the proteins identified VirB9, VirB10 and conjugal transfer protein (CTP), were shown to stimulate an antibody response. Further studies using the same membrane-enriched fractions for the immunization of young cattle showed that their antibodies (IgG2) and Th cells reacted with the recombinant versions of CTP, VirB9 and VirB10 proteins [12]. In a more sophisticated study using far-Western blotting to identify protein linkage between possible antigenic proteins, it was shown that VirB proteins, VirB9-1, VirB9-2 and Virb10 when physically linked, could stimulate a more specific and stronger immune response than when used individually [81]. While presence of B cell epitopes is important in any protein to be used as vaccine candidate, Th cell epitopes are also important as their presence might determine the actual potential use of any antigen in a vaccine. An interesting study, takes synthetic overlapping peptides from VirB9-1, VirB9-2 and VirB10 to test for the presence of such epitopes [82]. T cells from six different MHC Class-II phenotypes outer membrane fraction immunized animals were tested and as expected, it was observed that not all animals reacted with peptides from all three TFSS proteins. While all six animals reacted to the membrane fraction which contained all three VirB9-1, VirB9-2 and VirB10, some animals did not react against rVirB9–1, others reacted poorly against rVirB9-2 or rVirB10 or against only one or two of the overlapping synthetic peptides [82]. The differences in response of T cells from these animals are explained in the context of the Class-II MHC molecules involved in presentation of the epitopes. Interestingly, these authors restricted themselves to Holstein cattle as the subjects to their studies yet, at least in Mexico and other Latin American countries, Holstein cattle are used for milk production and most of them are reared under conditions which preclude the contact with ticks.

These studies clearly show that an immune response that fills the criteria for protection as described is induced [11, 55], these authors though, fall short of proving that the induced immunity is protective as there was no actual confrontation with the virulent live agent. In a different study recombinant VirB9, Virb10 and Elongation Factor Tu (EF-Tu) were tested against the sera of immunoprotected animals naturally infected with two *A. marginale* isolates [83]. These works showed that while all experimentally infected cattle with the autologous isolate had relevant antibodies (IgG2) against VirB9, VirB10 and EF-Tu, only 87% of the animals naturally infected with a heterologous isolate reacted with the recombinant protein by ELISA.

2.3. Outer membrane proteins

The outer membrane of bacteria delimits its structure and is the interface with the host cell. Outer membrane proteins (OMPs) are key components of Gram-negative bacteria and because are involved in adhesion and infection processes they are targeted on vaccine development.

Some effective attempts have been achieved using whole membrane fractions as immunogens against bacterial diseases and due to their relevance OMPs from several pathogens have been extensively studied and proposed as vaccine component. It is known that in addition to composition, OMPs show diversity in function too, but they share structural patterns. Usually, regions with antigenic properties are located on the extracellular loops and show variable composition, meanwhile residues in the transmembrane β-barrel show the highest conservation [61, 84–86]. However, the use of individual components has only been partially successful [86–92], although, it appears that the most relevant OMP antigens have not yet been identified.

High-throughput sequencing technologies are currently available and allow the identification of several genes with potential important functions in the metabolism of the pathogen or in the interaction with its surroundings. For example, from the complete genome of *Anaplasma marginale* [23], the existence of additional outer membrane protein has been elucidated. Additionally, to Msp2 and Msp3, new members of pfam01617 family have been identified, and designated as Omp 1-14. *omp2*, *omp3* and *omp6* genes are not transcribed in *A. marginale*-infected erythrocytes, tick midgut and salivary glands, and the IDE8 tick cell line, while OMPs 1, 4, 7, 8, 9 and 11 were confirmed to be differentially expressed as proteins in those cell types [93]. Unlike Msp2 and Msp3, these OMPs exhibit high conservation at sequence level as seen in the follow-up of the infection and in comparative analyses with the St. Maries and Florida strain genomes, which increases the possibility of choosing molecules capable of inducing a protective immune response against bovine Anaplasmosis. Omp7 to Omp9 appear as tandem repeats with almost 75% amino acid identity, Omp10 is related to Omp7 to Omp9 with ~ 30% residues identity and Omp6 is a truncated and it is not expressed version of Omp10. Omp7 to Omp9 are part of protective outer membrane fraction and are highly expressed than Omp10 [23, 25, 93, 94].

In spite of the fact that none of these molecules has induced protection when is applied as an individual protein, the importance of OMPs in protective immune response induction has been revealed above all as complexes or associated with a membrane environment.

For example, protection against *Leptospira* was reached using OmpL1 and LipL41 expressed simultaneously in the context of the *E. coli* membrane but, immunization with either membrane-

associated protein or as part of a mixture of non-membrane-associated proteins was not protective [53, 95].

These results confirm the importance of OMPs in the infection process and the generation of a protective immune response against pathogens and also reveal the interactions between OMPs and other proteins as well as with their environment. However, production, solubilization and purification of membrane-associated recombinant proteins is not easily achieved [96].

2.4. Hypothetical proteins

Genomic analyses of *A. marginale* have allowed identification of novel annotated proteins whose function has not yet been determined, however, *in silico* analysis and predictions may provide unrevealed information about immunogenic potential.

Some hypothetical proteins have been identified by structure prediction of β-barrel outer membrane and orthology and bioinformatic analysis, such as Am1108, Am127, Am216, Am202, Am936, Am854, Am368, Am854, Am1041, Am109 and Am1096. Some of these proteins have been evaluated as recombinant molecules and recognized by IgG from immunized animals with outer membranes protein, in this case, Am1108 and Am216 elicited specific T cell response proliferation [13, 97]. On the other hand, cattle immunized with recombinant Am854 or Am936 developed higher bacteremia as compared to adjuvant-only controls and outer membrane vaccinates after challenge [13].

The absence of a protective immune response after application of recombinant proteins presumably exposed to *A. marginale,* and therefore, with antigenic characteristics still seems to be insufficient to develop prophylactic methods against bovine anaplasmosis.

Although genomic analyses have revealed valuable information about the composition of *A. marginale*, it will be necessary to complement this knowledge with experimental evidence based in other methods, such as proteomic and transcriptomic tools.

Acknowledgements

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