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Advances in tick vaccinology in Brazil: from gene expression to immunoprotection

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1. ABSTRACT

Rhipicephalus (Boophilus) microplus has substantial economic impact on the cattle breeding industry and, chemical control and tick resistance development are the major concern. There is a worldwide search for new options, and control using vaccines has been the main focus nowadays. Studies performed in Brazil found that Bm86-based immunization of bovines reduced the infestation of *R. (B.) microplus* of vaccinated bovines by 45% to 60%. Native *Boophilus microplus* tripsin inhibitors (BmTIs) with trypsin-, kallikrein-, and elastase-inhibiting activities have been used as immunogens in bovines reaching 72.8.% of efficacy. The reverse vaccinology approach has also been used for antigen search using transcriptome analysis to identify and characterize potential antigens. Study has generated more than 600 million sequences using RNA-seq of larvae, nymphs, salivary glands, intestines, and ovaries of the tick *R. (B) microplus*. Based on the set of transcripts obtained using this strategy, a total of 20,326 protein sequences have been identified. A pipeline analysis built in house identified the protein sequences that were most likely to be immunogenic based on the overall structural characteristic analysis.

2. INTRODUCTION

2.1. Vaccine and vaccination

Rhipicephalus (*Boophilus*) *microplus* is an ectoparasite that has substantial economic impact on the cattle breeding industry, both directly (through the effects of bites, irritability, blood loss, weight loss, reduction in milk production, predisposition to myiasis, leather damage and transmission of parasitic and infectious vectors) and indirectly (through the increased costs of chemical control and residue left in products and in the environment) (1–2).

Dairy herds in Brazil are most commonly established with European breeds or crossbreeds; however, despite the more limited presence of European breeds in beef cattle herds, there has been an increase in the use of European cattle for industrial breeding over time (4). Because these breeds are more susceptible to tick infestation, a systematic control method must be established (3–4). To achieve this, the search for new control tools is necessary.

There is a worldwide search for new tick control options, and control through the use of vaccines has been the focus of various research groups. In Brazil, the use of vaccines for tick control associated with chemical control and pasture management could create possibilities for more effective and integrated control, thus diminishing the residues in the environment and the development of resistance.

Bm86 is a protein found on the apical surface of the *R. (B.) microplus* intestinal cells (5). Though it is not abundant, it plays an important physiological role: when ticks ingest blood from bovines immunized with this antigen, endocytosis inhibition occurs in the cells of the digestive tract through the fixation of the host's antibodies. Since endocytosis is responsible for the energy production required for egg formation, its inhibition

causes a reduction in the weight and number of engorged females. Therefore, after vaccination, the reproductive efficiency of engorged females decreases (6–7).

In Brazil, tests on bovines subjected to natural *R. (B.) microplus* infestations found that Bm86-based vaccination with the Gavac® vaccine formula reduced the infestation rate by approximately 47% (8). However, different calculations have been used to estimate the efficacy of commercially available vaccines, which has been found to range from 51% to 91% depending on the tick population and the nutritional condition of the bovines used in the tests (9).

In an experiment with GavacTM conducted in Cuba, Brazil, Argentina and Mexico to control *R. (B.) microplus* infestations, an efficacy rate of 55%-100% was obtained in bovines in open fields after 12–36 weeks from the first vaccination (10). In studies performed in Brazil, Bm86-based immunization of bovines subjected to natural *R. (B.) microplus* infestation reduced the infestation index of vaccinated bovines by 45% to 60% (8).

In the state of MatoGrosso do Sul, Brazil, a study with animals vaccinated using the GavacTM Bm86-based vaccine formula (Heber Biotec, Havana, Cuba) (11) and the TickGARD ^{PLUS} formulation (Intervet, Bendigo, East Vic, Australia) (12) showed that they presented an immune response. IgG levels were considered, and protection efficacy rates of 49.2.% and 46.4.% were found for Gavac^{TM} and TickGARD^{PLUS}, respectively (13).

It has been suggested that the variations in efficacy observed in different regions around the world is due to a variation in Bm86 amino acid sequences among different tick populations (14). In fact, an analysis of a tick population from Argentina found a polymorphism of the equivalent Bm86 gene that resulted in a soluble protein rather than a protein attached to the membrane, as has typically been detected in ticks from Australia and Cuba. This difference would explain why Argentine ticks were erroneously considered resistant to Bm86-based vaccination. To overcome this resistance, a new recombinant vaccine was produced from the Bm95 gene(an allele of the Bm86 gene). This new antigen was found to be effective at protecting bovines against infestation in Argentina and Cuba (14).

Amino acid sequence variations above 2.8.% would be sufficient for diminishing vaccination efficacy when recombinant antigens are used (15). Samples from different regions in Brazil, Argentina, Uruguay, Venezuela, and Colombia were analyzed, and the Bm86 and Bm95 genes were found to exhibit variations in amino acid sequences from 3.4.% to 6.8.%, and from 1.1.4% to 4.5.6%, respectively (16). A study of the Bm86-Campo Grande (Bm86-CG) variety, a Bm86 homologous protein isolated from a strain from Campo Grande, Mato Grosso do Sul, was found to have variations of 3.5.% and 3.7.% in amino acid sequences when compared to Bm86 and Bm95, respectively (17).

A Bm86-CG (Accesion number EU352677) gene sequence analysis comparing it to the Bm86 (Accesion number AF150895) and Bm95 (Accesion number AF150891) pairwise alignment showed that the identity between Bm86-CG and Bm86 is 0.2.% higher than the identity between Bm86-CG and Bm95, which were found to have identities of 96.5.% and 96.3.%, respectively. In terms of hydrophobicity, the Hoop and Woods calculation method suggests that the most significant differences between sequences are in two regions. The first is around Asp20 residues, where Bm86 samples are hydrophilic relative to Bm95 and Bm86-CG. The second hydrophilic behavior occurs close to the Asn85 residue, where Bm86 also exhibits an exclusive pattern. Bm86-CG has a hydrophobic profile closer to that of Bm95 in the N-terminal region, but it exhibits a behavior similar to that of Bm86 in the C-terminal region (17).

In addition, bovines vaccinated with Bm86 exhibited varying protection levels against species that are phylogenetically close to *R. (B.) microplus* (18–20). These protection levels reflect not only the variation among *R. (B.) microplus* isolates, but also the phylogenetic relationships among different tick species. Based on the degree of efficacy of protection across species, the levels also indicate that immunologically important epitopes are partially conserved (16, 17, 20).

In addition to Bm86, which is part of existing vaccines, other proteins also offer some degree of immune protection or induce antibody production that interferes with the reproductive success of ticks. These proteins include *R. (B.) microplus* trypsin inhibitors (BmTIs) obtained from tick larvae (21).

Initial studies with BmTI isolation were performed in Australia in the 1970s. A trypsin inhibitor was purified in ionic exchange columns and filtration gel with approximately 18.5. kDa. Its activity was found to include trypsin and chymotrypsin inhibition. It was also shown that this BmTI had different binding sites for the two enzymes (an inhibitor with “two heads”). Moreover, this inhibitor was also found to provoke an immediate hypersensitivity reaction after intradermal injection in bovines, and this reaction was found to be a specific immune response (22). At that time, it was believed that this immune reaction could be responsible of the varying levels of protection among bovines.

The quantity of inhibitors present in larvae is rapidly reduced after the beginning of the parasitic

stage, a factor which suggests that inhibitors could be secreted inside the host (22). These findings corroborate the idea that these inhibitors are important to start successful *R. (B.) microplus* larval feeding.

The Beef Cattle Unit of the Brazilian Agriculture Research Corporation (EMBRAPA Gado de Corte) has been working for years to select BmTIs that may become antigens from the *R. (B.) microplus* larval phase (21, 23–26). In previous studies, native BmTIs with trypsin-, kallikrein-, and elastase-inhibiting activities have been used as immunogens in bovines in an attempt to revert the anticoagulant and immunosuppressant activities of the inhibitors secreted and released by *R. (B) microplus* larvae at the attachment site. After infestation, immunized bovines exhibited a reduction in the total number of ticks (69.7.%), total egg weight (71.3.%), and engorged female weight (69.5.%) when compared to those fed on non-immunized bovines. The efficacy reached with this formulation was 72.8.% (21). Years later, a BmTI N-terminal fragment was synthesized and used in a bovine vaccination assay. Vaccination with this synthetic antigen compared to BmTI did not produce differences in the antibody-mediated immune response, and protection was not effective against subsequent *R. microplus* tick infestations, since it was only 18.4.% (25).

Another study found that a recombinant chimeric peptide designed from BmTI and carrapatin sequences induced immune response in Balb/C mice, but when used in bovines as a complete adjuvant formulation, did not induce a protective response (27).

In partnership with the Federal University of São Paulo School of Medicine (FM-UNIFESP), EMBRAPA Gado de Corte described one of these BmTIs, referred to as BmTI-A (28), which was cloned into an expression plasmid and expressed in *Pichia pastoris*. The recombinant protein expressed by this system was named *R. microplus* larvae trypsin inhibitor, or rRmLTI. When used for vaccination, this protein provided a 32% protection rate after infestation with *R. (B.) microplus* larvae (26). At this time, a similar recombinant BmTI (29), named BmTI-6, had been found to present inhibitory activity toward trypsin (Ki 1.7. nM) and plasmin (Ki 20 nM), but did not inhibit HuPK, HNE, chymotrypsin, FXa, FXIIa, or thrombin.

Bovines acquire partial immunity to *R. (B.) microplus* after extensive natural exposure to infestations, an immunity which was largely due to an immediate hypersensitivity reaction to the tick (30). It is known that mast cells and histamine contained within their cytoplasmic granules are of fundamental importance to bovines' grooming behavior, which is a critical part of the animal's resistance to *R. (B.) microplus* ticks (31). Furthermore, a study conducted by Veríssimo *et al*. (2008) (31) on bovine resistance mechanisms to *R. (B.) microplus* found the immediate hypersensitivity mechanism to be highly important, since it determines host resistance to this tick species in particular. We believe that, when selecting an antigen from the large number of potential immunogens on the basis of the type of immune response it elicits in bovines, it is important to consider where this effect must act. For proteins such as Bm86 which are situated in the midgut of the tick, its speculated that a higher antibody title is necessary. However, when working with proteins contained in the tick saliva, we believe that an immediate response, combined with the participation of the innate immune response and cellular acquired response, is essential for the successful resistance of cattle infestation.

Given the immunogenic potential of BmTIs, which are capable of reacting with the immune system and creating an immediate hypersensitivity reaction, it is probable that the production of a BmTI-based recombinant vaccine and that cattle breeding will reduce production costs, even in a system in which the vaccine's effect is partial and combined with chemical control and other management practices; the additional costs of vaccinating the herd would be lower than the reduction in costs with chemical acaricides.

One study described ATAQ, a protein considered homologous to Bm86, with high similarity in primary and secondary structures. Despite RNAi experiments that found it to be expressed in few quantities, showing to be a weak phenotype (low number of genetic copies) for both Bm86 and ATAQ, Bm86 is capable of providing strong protection when used as antigen (32). Considering the immunogenic potential of Bm86, Aguirre *et al*. (2016) (33) raised the hypothesis that ATAQ could be a potential antigen for the development of a vaccine, since it is present in the digestive tract and Malpighian tubule system of all instars of the tick species *R. (B.) microplus*, *Rhipicephalus (Boophilus) annulatus*, *Rhipicephalus (Boophilus) decoloratus* and *Rhipicephalus evertsi evertsi* (34). With the objective of finding an antigenic peptide of the ATAQ protein in *R. (B.) microplus*, a reverse vaccinology approach was used. It resulted in the identification and synthesis of a peptide with immunogenic potential (33). With different adjuvants and tests with different host species, vaccine formulations including this peptide were tested against *Rhipicephalus sanguineus* (in the case of rabbits) and against *R. (B.) microplus* (in the case of bovines) (33). The synthetic peptide presented protection rates of 29% and 47% among rabbits infested with *R. sanguineus* when inoculated with the adjuvant Montanide ISA 61 VG (Seppic, Paris) alone or conjugated with KLH, respectively, and thus depends on the formulation of the vaccine. Among bovines infested with *R. (B.) microplus,* the same peptide presented protection rates of 35% and 98%, depending on the immune response,

when the animals were and were not considered responders, respectively. These results confirm the initial hypothesis and allow for the conclusion that this synthetic peptide, designed using bioinformatics tools, is capable of stimulating specific immune protection against ticks used in the experiment.

Studies to calculate efficacy rates of Bm86-based vaccines in bovines in Brazil (35) have found efficacy rates below those found in other countries. Similar results were found in a sequence analysis of Bm86-homologous proteins, and these results reflect differences in amino acid sequences, thus providing support for the study of regional tick populations (36). Csordas *et al*. (2016) (36) collected *R. (B.) microplus* specimens from Brazil's five geopolitical regions in order to study the genetic variation of this tick population. The study used molecular markers (COX-1 and ITS-2) to infer phylogenetic relations. Based on the COX-1 marker, the authors suggested that the Brazilian *R. (B.) microplus* population is composed of at least two different subpopulations, and that ticks from the Brazilian states of Roraima and Pernambuco belong to a different subpopulation than ticks from other locations in the country (36). While the study argues for the existence of two different populations, most Brazilian land is infested by one subpopulation, a factor which calls into question the efficacy percentage variations found in different vaccine studies performed with ticks from the country, thus reinforcing the hypothesis that a subunit vaccine could be used for *R. (B.) microplus* population control in Brazil.

2.2. Transcriptome

The reverse vaccinology approach, first published by Rappuoli (2000) (37), is a strategy involving the search for antigens that got its start in genome analysis, transcriptome analysis, or analyses of organisms' other biological sequences. With the help of tools from the field of bioinformatics, the genomes of pathogens are interrogated *in silico* in order to search for potential vaccine targets. The proteins predicted are selected based on desirable attributes, such as the characteristics associated with the immunity induced by vaccines. Lab experiments are performed, and the targets are then tested (38).

The efficacy of the technique has been demonstrated through the development of vaccines for a series of pathogens, particularly ones that are bacterial or viral in nature (37, 39). The first vaccine developed using this method, which was developed to be a vaccine against invasive meningococcal disease caused by the serogroup B of *Neisseria meningitidis*, was recently patented (40; 41).

The delay in applying reverse vaccinology to the development of vaccines against parasites (relative to the advances seen in bacterial and viral vaccines) resides in the fact that few parasite genomes have been sequenced. This limitation is due to their complexity and size, which is often many times greater than that of bacterial genomes (42). In studies on ticks from the family Ixodidae, the genome of the species *Amblyomma americanum* has been estimated to be 3.3.Gb (43). The repetitive content and size of the *Rhipicephalus (Boophilus) microplus* genome were estimated to be 70% and 7.1.Gb, respectively (44). The first assembly for a genome of a vector tick, *Ixodes scapularis* was obtained using Sanger sequencing and revealed a size of 2.6.Gb with approximately 70% repetitive content and 20,486 protein-coding genes (45).

Even, with the advent of new sequencing technologies, which have dramatically reduced the cost and time required for obtaining sequences and which have thus increased the number of genomes sequenced (46), the issue of the ambiguity generated by the repetitive sequence within the alignment and genome assembly remains a challenge; it introduces biases and errors and leads to mistaken interpretations in the final assembly (47). However, in attempts to overcome this limitation, studies involving functional genomics combined with reverse vaccinology has led to the identification of many potential vaccine candidates (48).

Functional genomics uses genomic data obtained from high-throughput technologies in order to study the function of genes and proteins. Sequencing the portion of the genome that is effectively transcribed (the transcriptome) significantly reduces the investment required to sequence a given genome; despite new sequencing technologies, this process is still very expensive. Though it does not allow for access to the organism's complete genetic repertoire, transcriptome sequencing is a highly versatile tool that has been used in a series of applications, from the identification and quantification of transcripts, to genome annotation, to rearrangement detection, to the discovery and quantification of non-coding RNA's (49).

In the search for vaccine candidates, the transcriptome of a pathogen can be analyzed in order to identify and characterize potential antigens, which are important to pathogenesis and survival within the host (50). Candidates identified using this methodology may therefore be expressed as recombinant proteins and tested *in vitro* and *in vivo* to determine immunogenicity and protection (51). Furthermore, the study of the transcriptome does not only aid in the identification of new candidates; it also contributes to the understanding of gene function and the biological mechanisms of the organism in question.

One of the first high-throughput technologies available for use in the study of transcriptomes

is the technology involving expressed sequence tags (ESTs), sequences of approximately 200–800 nucleotides obtained through the sequencing of the 5' and/or 3' ends of clones obtained from cDNA libraries created using mRNA from any tissue from an organism (52). Largely used in the first genome projects, this method has aided in the discovery of new genes, the characterization of genetic structures, and genome annotation. Over time, EST sequencing has provided an exponential amount of information on many different organisms, which has been published in public databases. This information has aided substantially in genome analysis, even with the arrival of new data generation techniques and additional methods of analysis. The public sequence EST database from the National Center for Biotechnology Information (NCBI), known as dbEST (http://www.ncbi.nlm.nih.gov/dbEST/), currently contains more than 74 million ESTs. Of these, 52,901 correspond to *R. microplus* sequences, which represent a vast source of data for studies that use genomic information to understand aspects of the species' biology and to search for candidates for parasite control strategies.

One of the first studies involving the identification of ESTs from different *R. microplus* tissues was performed by Crampton *et al*. (1998) (53), who identified 234 ESTs uniquely expressed by the parasite. With the sequencing of 20,417 ESTs using cDNA library larval clones exposed to different treatments, as well as eggs, nymphs, adults, and various *R. microplus* organs by Guerrero *et al*. (2005) (54), the first *R. microplus* index was established with 8,270 unique sequences – the BmiGI. Later, the sequencing of another 22,095 ESTs and their clusterization with existing sequences in the BmiGI produced a new version of the gene index, BmiGI Version 2, with a total of 13,643 ESTs (55). Searches and analyses involving these databases may aid in many different studies, including those involving the characterization of genes or studies searching for vaccine candidates against *R. microplus*. Another very important database that includes genomic and transcribed *R. microplus* sequences is CattleTickbase, which provides not only the sequences, but also certain bioinformatics tools to aid researchers in their analyses (56).

In addition to these rich databases, other studies that have identified *R. microplus* ESTs serve as a source of information that can advance both our knowledge on parasite biology and new control strategies. One of these studies includes that of Santos *et al*. (2006) (57), who used mRNA isolated from ovaries, hemocytes, and salivary glands of *R. microplus* to identify 1,344 ESTs.

A total of 4,045 ESTs were identified using a subtracted library of ovaries from *R. microplus* females feeding upon hosts, some of which had been infected by *Babesia bovis* (58). A differential expression analysis was also performed on *R. microplus* using salivary gland ESTs in response to infection by *Anaplasma marginale* (59). In both cases, the authors' objective was to identify genes involved in the host-pathogen interaction between *B. bovis* and *R. microplus*, and a series of new *R. microplus* larval transcripts were identified and made available.

Using the suppressive subtractive hybridization technique, Lew-Tabor *et al*. (2010) (60) identified differentially expressed transcripts associated with *R. microplus* attachment and feeding in response to host stimuli, and, in doing so, these researchers contributed to the understanding of the mechanisms used by the tick to adapt to a blood-feeding environment, as well as to the discovery of new control methods genes.

With the goal to identify candidate antigens for a cattle tick vaccine development, a research group from the state of São Paulo, Brazil, began an expressed sequence tag sequencing project on *R. microplus* nymphs and larvae in 2002. The first study led to the identification of 8,487 valid ESTs, 4,137 of which were generated from infesting larvae, and 4,350 of which were obtained from tick nymph libraries (61). A second study resulted in the identification of candidate antigens through the immunization of animals with soluble protein extracts from the intestine, ovary, and salivary gland of the tick, including a vitellogenin and a glutathione S-transferase (62). In the latter study, the cDNA library sequencing of the intestine, ovary, and salivary gland of *R. microplus* enabled the generation of 11,965 ESTs, 4,256 of which were from the intestine, 4,390 of which were from the ovary, and 3,319 of which were from the salivary gland of the parasite, leading to the identification of 6,165 unique tick genes.

The information provided by Costa (2004) (61) and Costa (2008) (62) was added to an EST database that is now a very important source in the search for potential candidate antigens, both through knowledge on their function and as a gateway to other analytical tools.

Researchers' obtaining and depositing of EST sequences in public and private databases has allowed for the development of a new data generation technique and gene expression analysis based on hybridization – cDNA microarrays. This technique consists of the immobilization of short cDNA fragments (probes) and a solid support (nylon membrane or glass slide). These fragments are then hybridized with a cDNA population stained with fluorescent dyes. When hybridization by the corresponding cDNA occurs, the signal is detected. As a tool for the study of gene expression that has been available since 1995, when Schena *et al*. (1995) (63) demonstrated the use of cDNA microarrays

in the quantitative analysis of the transcriptome of various lineages and organs of the *Arabidopsis thaliana* plant model, the technology has come to be widely used in many different approaches involving gene transcriptome levels. Unlike the methodologies available before, This technique has made possible the simultaneous investigation of thousands of transcripts, which revolutionized many fields of biology through the substantial increased capacity of molecular process analyses. Because it enables the simultaneous analyses of gene expression in different tissues and under different conditions, microarray technology generates data that can be used generates, can be used in downstream applications such as network analyses, which investigate the biological pathways involved in pathogen and host responses during their interaction, examples of which can be found in Jensen *et al*. (2007) (64). As in the case of ESTs, the data generated by the use of microarray technology may be deposited in databanks, thus making them available for reference and use by the scientific community.

The Gene Expression Omnibus, or GEO (https://www.ncbi.nlm.nih.gov/geo/), is a repository of data generated from experiments with microarrays on different platforms (both commercial and custom-made). Data deposited into this repository must be added in a standardized way that follows the minimum information about a microarray experiment (MIAME) standard (65) and which respects the terminology to be used in the experiments (MGED Ontology). These norms allow for experiment reproduction, result validation, and comparisons between similar experiments. Today, the GEO contains more than 122 *R. microplus* samples from experiments involving different tissues and states of development, as well as different types of hosts (resistant hosts and sensitive hosts) and with the application of different acaricides.

The understanding of the mechanisms used in tick feeding and attachment (which involve the bypass of host defenses) and the identification of *R. microplus* salivary gland components may both lead to the identification of the genes involved in the regulation of the host's immune system; these genes represent intriguing targets for effective *R. microplus* control strategies. Other potential targets reside in the molecules involved in digestive and reproductive processes, since the inhibition of these vital functions may affect the parasite's survival. The genes expressed during tick embryogenesis may also represent an important target for parasite control, since they directly affect oviposition, which would thus reduce tick populations in the environment (66–67).

Therefore, in order to identify the genes expressed by *R. microplus* during its initial feeding stages, Rodriguez-Valle *et al*. (2010) (68) used a microarray created using the 13,000 transcripts available in the BmiGI databank (54). They identified differentially expressed genes in the parasites feeding on tick-resistant cattle and tick-susceptible cattle and in the larval, pre-attachment, and early adult stages. Their study provided evidence of differences in gene expression patterns that were influenced by host breed.

Long-term alterations to *R. microplus* gut and salivary gland transcriptomes, which occurred during feeding, were evaluated using microarray technology, and a dramatic response was seen in two tissues, with thousands of genes being differentially regulated in response to feeding (69). Another study found differential gene expression in the salivary gland to be dynamic, since it changed over the course of the study period (2 and 9 days of feeding). Examples included a metalloprotease, which was upregulated only on Day 9, and a metalloprotease inhibitor, the upregulation of which began on day 2 and which lasted throughout the rest of the study period.

Maritz-Olivier *et al*. (2012) (48) constructed a microarray containing 13,456 probes based on sequences deposited in public databases and using a strategy that included VaxiJen, an antigen prediction software (70). VaxiJen predicts antigens using an alignment-independent sequence similarity method based on the physical and chemical properties of proteins. Using this tool, the authors identified 791 vaccine candidates based on samples of larvae, nymphs, intestines, ovaries, and the salivary gland. Among the targets, some proteins associated with the membrane exhibited a better binding capacity to IgGs than Bm86 epitopes" .

The same microarray detailed by Maritz-Olivier *et al*. (2012) (48) was used to identify and quantify the genes expressed in the intestine, ovaries, and salivary gland of adult *R. microplus* females obtained 20 days post-infestation (71). These genes were added to a catalog containing tissue-specific transcripts with anti-hemostatic and immunomodulatory functions (salivary gland), genes involved in digestion (intestine), and genes involved in reproduction. In another study using the same microarray hybridization, a total of 2,476 genes expressed in the midgut were identified as being shared between *R. microplus* and *R. decoloratus*. Genes have also been found to be involved in lipid transport and metabolism, and have been suggested by authors as representing a class of potential targets for parasite control (72).

Microarray technology has proven to be very promising in transcriptome studies and gene expression analysis due to its higher throughput and cost-effectiveness relative to EST sequencing. However, the technology has some limitations, including the need for previous knowledge on the

sequences that make up the microarray, higher background levels, and cross-hybridization (73). Furthermore, while the comparison of expression levels between different treatments is possible, it requires the use of complicated normalization methods that hinder its application (74).

All of the limitations to the study of transcriptomes have been overcome with the advent of new sequencing technologies, namely next-generation sequencing, or NGS. Characterized by a dramatic increase in the data generated, as well as by substantial decreases in costs of production, new sequencing technologies have generated very short sequences compared to both Sanger sequencing and traditional sequencing (46–47, 75). Traditional sequencing generates substantial challenges in terms of storage capacity and data processing, as well as the need to develop new tools for analysis. NGS offers characteristics that create challenges for bioinformatics, particularly in genome assembly: the short sequence length makes it difficult to distinguish between repetitive regions, thus resulting in fragmented assemblies and requiring optimized tools to resolve the issue; these tools differ from those used with data generated by Sanger sequencing.

The application of NGS has revolutionized transcriptome study through the use of RNA-seq technology. This technology offers many advantages over microarrays; there is no longer a need for previous knowledge on the genome of the organism being studied, and much smaller sample sizes can be used. These are almost always the conditions in attempts to obtain the transcriptomes of specific tick organs, such as the salivary gland. Furthermore, RNA-seq is an extremely sensitive technique, that allows the discovery of new and rare transcripts. The clear benefits of this method suggest that RNA-seq will revolutionize the study of gene expression, including research that seeks to understand the changes that occur during host-pathogen interactions. This research may lead to tremendous advances in knowledge on the molecular foundations of immune response (76).

In RNA-seq technology, a population of total or fractioned RNA molecules (poly(A)+, for example) is converted into a collection of cDNA fragments (a library) with adaptors connected to their ends. Each molecule is then sequenced in a high-throughput manner in order to obtain sequences using one end (single-end sequencing) or both ends (pair-end sequencing) (74).

There are no recent reports of *R. microplus* transcriptomes obtained using RNA-seq technology, but a series of studies performed using mRNA from the salivary glands and intestines of different tick species have been performed. Tick blood feeding is maintained by the parasite's saliva, which is injected regularly into the host. This saliva contains hundreds or even thousands of bioactive compounds that enable the tick to feed by inhibiting blood clotting, platelet aggregation, vasoconstriction, pain, and itching; the saliva also contains antimicrobial peptides (77), which allow for the identification of antigens that may offer immunity to the host. In light of this important factor, the genes expressed in the salivary glands (sialotranscriptomes) of other tick species have been analyzed. Examples include *Amblyomma maculatum* (78), *Ixodes ricinus* (79–81), *Dermacentor andersoni* (82), *Amblyomma triste*, *A. parvum*, and *A. cajennense* (83), *A. americanum* (77), *Haemaphysalis flava* (84), *Rhipicephalus pulchellus* (85), and *Rhipicephalus appendiculatus* (86). Together, these studies have identified thousands of transcripts and have described a series of mechanisms that occur in the salivary gland; in doing so, these studies have contributed to the understanding of the processes involved in the tick-host relationship. Indeed, the uncovering and detailed characterization of the processes involved in the tick-host relationship is certainly the beginning of successful target identification. Usually, the number of significant differentially expressed transcripts identified in transcriptome analyses with the use of NGS is very large and merely represents a "gene list." Downstream functional analyses such as co-expression, gene set enrichment analysis (GSEA), and pathway analysis are necessary for providing the greatest amount of biological information on the role of these genes. However, because functional analysis requires the availability of sufficient functional annotation data for the transcriptome under study, which is rarely possible, there is a large gap between targets identified using NGS technology and an effective discovery.

Ticks feed on blood, and the midgut is the first region where the ingested blood enters on contact with the parasite's internal tissues (87). The antigenic potential of intestinal proteins has already been determined in the case of *Bm86*. *R. microplus* midgut transcriptomes have yet to be obtained using RNA-seq, and the few milanomes obtained with this technology thus far have ben from the tick *Ixodes ricinus* (80–81, 88).

3. FINAL CONSIDERATIONS

When TickGARD was released to the market in 1994, this vaccine against *Boophilus microplus* was the first commercially available anti-parasite using a recombinant antigen. It was, therefore, highly innovative, with a unique mechanism of action and use recommendations that were different from conventional acaricides.

The last two decades have seen an escalation in chemical resistance problems. Since the original release of the vaccine, resistance to three different

chemical acaricides (macrocyclic lactone, fipronil, and insect growth regulators) have been reported in Australia and South America (89). There is a growing perception that any sustainable and successful parasite control program must consider an integrated approach in order to meet the need for "green" alternatives. All of these factors make an improved anti-tick vaccine a desirable product.

The vaccines developed from Bm86 provide partial protection for bovines against future infestations of *R. (B.) microplus* by diminishing tick number, egg production and tick fertility. These results, however, do not guarantee the needed protection level in cattle production and reflect the need to incorporate an additional protective antigen.

Though hidden antigens are the basis of commercial vaccines and have been studied in other ectoparasites, such as *Lucilia cuprina* and *Pediculus humanus*, the association of antigens that are naturally exposed to the host's immune system, with hidden antigens may potentialize immune protection, especially if they act upon the ectoparasite's different phases or upon different organs. This is the case for using vaccines formulated with Bm86-CG, which act by destroying adult intestinal cells, associated withRmLTI, which is secreted by the larvae and is also present in ovaries.

The serological evaluation of immunized animals with different antigens for tick control showed that antibody levels tend to decrease within a few months if the title of specific antibodies against Bm86 becomes less than 1:640, a change which likely means that protection decreases (90). This change reveals the necessity of booster doses with the different antigens available. Therefore, the use of adequate adjuvants is an important aspect in the development of the vaccine against bovine ticks.

In a vaccine, the specificity of the immune response is given by the antigen; however, adjuvants are substances that amplify and modulate the immunogenicity of the vaccine antigen. An aspect that highly influences in the development of a vaccine is the interaction that occurs between the pathogen and the host. This interaction determines the type of immune response that the vaccine needs to be able to protect the bovine effectively against parasitic infections. However, there is an extra challenge for tick vaccines when compared to those used against other pathogens, because ticks present different life cycles stage when parasites the host and while in the environment. Furthermore, both immune response and the parasites themselves are highly complex, and the immunological aspects of the parasite-host interaction are not well known.

Despite this complexity, immunology optimization may be nothing more than an assessment of a variety of appropriate commercially available adjuvant formulations, but the criteria used to evaluate them are critical.

The development of a vaccine is a complex process with various stages. Investment in research and development could accelerate many stages of this long development process. Currently, the greatest challenge is the lack of recombinant antigens proven to be highly effective against *R. (B.) microplus*.

Because a vaccine superior to the commercially available vaccines is likely to need more than one antigen, the efficacy of combining currently known antigens must be explored, as should the identification of new antigens and the combination of these antigens with immune-system-stimulating molecules. The resulting rate of efficacy of the vaccines may be dependent not only upon immunogen selection, but also upon the vaccine's combination with adjuvant and/or immune stimulant/modulator molecules.

In Brazil, an aspect that is critical to the development of vaccines against cattle ticks is trying to revert the constant loss of experienced professionals in the fields of tick biology and vaccine development. This loss has been considerable, especially among education professionals. Another important challenge is the limited availability of adequate experimental infrastructure, the conditions of which have been having declining due to lack of investment.

A study beginning in 2015 and financed by the Brazilian Agricultural Research Corporation (EMBRAPA) and led by researcher of our group has generated more than 600 million sequences using RNA-seq of larvae, nymphs, salivary glands, intestines, and ovaries of the tick species *R. (B.) microplus*. In addition to the extensive analysis of the tissue-specific transcriptome of the parasite obtained through *de novo* assembly of the sequences, the study has also sought to identify differentially expressed genes in each of the tissues. The ticks have been fed on resistant bovines (Nelore cattle), susceptible bovines (Holstein cattle), and bovines with intermediate resistance to the parasite (a Nelore x Holstein crossbreed). The *de novo* transcriptome assembly used the ESTs identified by Costa (2004) (61) and Costa (2008) (62), as well as those available in the dbEST databank, in an attempt to obtain the best possible transcriptome assembly for *R. microplus* available to date, given the lack of the parasite's genome sequence. Based on the set of transcripts obtained using this strategy, a total of 20,326 protein sequences have been identified. A pipeline analysis built in house was used to identify the protein sequences within this set that were most likely to be immunogenic based on the overall structural characteristic analysis. These characteristics included the presence of signal peptides, GPI anchors,

transmembrane helices, the presence of B-lymphocyte epitopes, and intrinsically unstructured proteins. The peptides that were identified and ranked as part of our reverse vaccinology strategy are now being tested according to the strategy described by Aguirre *et al*. (2016) (33). In summary, after peptides are selected, they are tested to stimulate mouse immune systems, with or without any carriers and/or adjuvants. After the confirmation of their immunogenicity, the immunogenic peptides, in their respective formulations, are inoculated in bovines and tested in challenges against tick infestation in stall tests.

A common belief is that multi-antigen formulations could increase efficacy, but experimental evidence is extremely limited. In addition, vaccines and acaricides may be used in a combined program in order to achieve better tick control in a profitable manner and minimizing the use of chemical products. However, the perspectives for these changes are not in the short term.

The first commercial vaccine, TickGARD, was launched in 1994 and had a recombinant antigen produced in *E. coli*, in a standard adjuvant formulation. A year later, TickGARD^{PLUS} was launched. In the second vaccine, the *E. coli* recombinant antigen was replaced by a *P. pastoris* expression product, and the adjuvant formulation was modified. As a result, the average antibody title in vaccinated herds was approximately twice as high TickGARD. Part of the increase in antibody titles was due to the decrease compared to animals that had low antibody titles, which means that the phenomenon of non-response was removed by the change in adjuvant. The minimization of the phenomenon was highly important for the development of TickGARD^{PLUS}. As in other parasite populations, the distribution of ticks in hosts is highly variable; therefore, a high proportion of ticks infest a low proportion of animals.

This data is particularly important on the efficacy of vaccines based on rBm86-CG and rRmLTI, since there is a strong correlation between specific antibody titers and vaccine efficacy. Associating these immunogens among themselves with an auxiliary T-lymphocyte-stimulating epitope can increase their immunogenicity, resulting in a higher production of antibodies in vaccinated bovines and, as a consequence, greater protection against tick infestationn.

Brazil has specific conditions in the bovine production system that include specialization and intensification of production systems, a predominantly tropical climate that directly influences tick reproduction, a tendency of improper management of tick control due to a lack of national policies to address the problem, and increasing complaints of inefficiency among those employing the anti-tick products available on the market. These factors reinforce the importance of implementing integrated control measures.

The development of an effective vaccine against bovine ticks would provide many benefits – not only to the beef cattle industry, but also to the general population, which would have ecologically correct products available due to the decrease in environmental contamination. The producer would have lower costs for tick control, which would impact the price of the final product for the consumer. Another considerable aspect would be the increased quality of Brazilian leather; despite the country's widespread cattle production, only a small fraction of leather from Brazilian cattle may currently be used for high-quality leather products.

It is important to note, however, that even if a vaccine is developed with all of the desired characteristics, its contribution as a tool within a multi-faceted bovine tick control system will only be effective if it is used adequately. For this, Brazil requires a policy to establish a control program. This public policy should include recommendations and technical orientations that are adequate for the different tools available, as well as with vaccine distribution logistics, a national tick management program, and, perhaps most importantly, vaccine prices that are affordable for the country's cattle ranchers.

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