Vaccine Technology in Bovine Theileriosis

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Contributor: Onyinyechukwu Ada Agina , Mohd Rosly Shaari , Nur Mahiza Md Isa , Mokrish Ajat , Mohd Zamri-Saad , Hazilawati Hamzah

Theileriosis is a blood piroplasmic disease that adversely affects the livestock industry, especially in tropical and sub-tropical countries. It is caused by haemoprotozoan of the *Theileria* genus, transmitted by hard ticks and which possesses a complex life cycle. The infection and treatment method (ITM) is currently used in the control and prevention of *T. parva* infection, and recombinant vaccines are still under evaluation. The use of gene gun immunization against *T. parva* infection has been recently evaluated.

Bovine theileriosis DNA vaccine

1. Introduction

Bovine theileriosis is an important tick-borne disease of domesticated cattle in tropical and subtropical countries, caused by several *Theileria* species belonging to the phylum Apicomplexa [1]. Some species cause serious economic losses through bovine mortalities and morbidities in many countries [2][3][4][5].

Theileria species that infect bovines include *T. annulata*, *T. parva*, *T. mutans, T. orientalis* complex (*orientalis/sergenti/buffeli*), *T. tarurotragi*, *T. velifera*, *T. sinensis* and *Theileria* sp. Yokoyama, a newly discovered *Theileria* species closely related to *T. annulata* [6][7][8][9][10][11].

2. Pathophysiological Mechanisms of Anaemia in Bovine Theileriosis

2.1. Anaemia in Oriental Theileriosis

The pathophysiologic mechanism of anaemia in oriental theileriosis is multifarious [12]. Anaemia is the primary clinicopathological finding in oriental theileriosis and usually occurs due to intravascular haemolysis caused by the intra-erythrocytic stage. *Theileria orientalis* is also known to induce immune-mediated haemolytic anaemia [13]. The life span of erythrocytes in oriental theileriosis caused by *T. sergenti* is usually shortened as the immune system produces antibodies directed against the parasites as well as against its own erythrocytes [14][15]. Autoantibody production against RBCs is due to the altered RBC membrane, as phosphatidylserine molecules, which are normally localized on the inner leaflets of cell membranes, translocate to the external surface of RBCs in *Theileria*-infected cattle. Exposure of the phosphatidylserine on the cell surface can induce an antibody response and function as a marker of the phagocytic clearance of RBCs by macrophages [15][16]. Hagiwara et al. [17] evidenced in

an experimental model with immunodeficient mice that haemolysis of *T. sergenti* infected RBCs occur without the involvement of antibodies or complement. Shiono et al. [15] demonstrated that elevations in methaemoglobin concentration contribute to the progression of anaemia, as an increase in methaemoglobin can alter the oxidant–antioxidant balance and cause oxidative damage of RBC membranes and their removal from circulation by phagocytes [18].

2.2. Anaemia in East Coast Fever

Mbassa et al. [19] reported, in 1994, unusual cases of East Coast fever in zebu and taurine–zebu crosses cattle in Tanzania, where the infection of the haematopoietic precursor cells resulted in severe pancytopaenia and the severe anaemia was not associated with reticulocytosis, haemoglobinuria or jaundice. Additionally, this *T. parva* strain caused lymphocytolysis in lymph nodes where lymphoproliferation was low and only few schizonts were found. Conversely, anaemia was mild and regenerative in cattle and buffaloes with East Coast fever, and numerous macrophages were present in the lymphoid organs [19]. However, non-regenerative anaemia and pancytopenia were observed in chronic forms of the disease, because *T. parva* merozoites infect erythroid and other haematopoietic precursor cells, resulting in the extensive destruction of haematopoietic cells in bone marrow [19].

3. Diagnosis of Bovine Theileriosis

Tentative diagnosis of theileriosis is made based on suggestive clinical signs, such as enlarged lymph nodes, pyrexia, anorexia, a loss of condition and pale mucous membranes. Confirmatory diagnosis is obtained with the microscopic examination of Giemsa-stained blood smears and lymph node fine needle aspirate smears, serological and molecular techniques. The use of the optical light microscopy method has been, in the past, the only available diagnostic tool that provided the morphological identification of blood parasites in ruminants. However, diagnosis solely based on the blood or lymph node smear method has low accuracy and is associated with technical problems [20]. The microscopic examination of thin blood smears and lymph node fine needle aspirate smears from cattle showing the acute disease are best and routinely performed to detect piroplasms in erythrocytes and macro schizonts (Koch's blue bodies) in leukocytes, respectively [21][22]. This method is time-consuming and has low sensitivity in cases of low levels of parasitaemia or in asymptomatic carriers [23]. Thus, it is not reliable for the large-scale monitoring and screening of cattle populations. Specificity is also low, as morphologically similar blood parasites and parasites within the same genus cannot be differentiated [24]. Additionally, artefacts (e.g., stain precipitates) and Howell–Jolly bodies can be confused with intra-erythrocytic piroplasms by inexperienced microscopists.

Serological methods measure *Theileria*-specific antibodies by employing ELISA assays such as the *T. annulata* surface protein (TaSP)-ELISA [25], and the recombinant polymorphic immunodominant molecule (PIM)-ELISA [26]. The indirect fluorescent antibody technique (IFAT) has limitations due to the cross-reactivity between different *Theileria* species [27]. Mohamed et al. [25] demonstrated the high sensitivity of TaSP-ELISA when compared to the standard microscopic method and suggested its suitability for the diagnosis of *T. annulata* infection in cattle under

field conditions. A recombinant antigen ELISA based on MPSP has been developed for detection of *T. orientalis* [28].

Molecular diagnostic techniques, such as PCR based on the 18S ribosomal RNA gene, MPSP gene, 28S ribosomal RNA genes and the sequencing of PCR amplicons [29][30][31] and Taqman® quantitative real-time PCR (qRT-PCR) assay [32], are regarded as the most accurate because of their high sensitivity and specificity and ability to differentiate between *Theileria* species and strains. Moreover, PCR molecular techniques can detect newly emerging and mutant strains [11] and can distinguish between acute and chronic infections by the quantitation of the gene copy numbers using qRT-PCR [32]. Serological techniques and PCR were found to be more sensitive and specific than the blood or lymph node smear observation in diagnosing carrier cattle in which parasitaemia has dropped to microscopically undetectable levels [20][33] and are therefore highly recommended and utilized for epidemiological studies. Other molecular biology techniques employed for the rapid detection of *Theileria* species include the loop-mediated isothermal amplification (LAMP) assay for the detection of *T. annulata* [34], bead-based luminex xMAP technology [35] and random amplified polymorphic DNA (RAPD) [36][37]. A low-density DNA microarray kit has been designed for the detection of 12 species of tick-borne pathogens, including *Theileria* [38].

4. Immunization against Bovine Theileriosis and Advanced Vaccine Technology

Immunization is one of the most successful strategies for the prevention of infectious diseases and vaccines against bovine theileriosis are among the few vaccines available for protozoal diseases of animals [39].

The infection and treatment method (ITM) is currently the only immunization protocol available for *T. parva* infection [40][24]. The ITM involves the inoculation of live *T. parva* parasites, alongside the treatment with expensive depot formulation of antibiotics. The ITM is not cost-effective and has a cumbersome production process as it requires large numbers of cattle for vaccine production. It is also difficult to standardize, store and distribute [41]. Live attenuated organisms are available in some countries to prevent bovine tropical theileriosis [40].

4.1. Theileria Vaccines under Evaluation

The aims of an ideal vaccine is to produce the same immune protection that usually follows natural infection but without causing disease to generate long-lasting immunity, to prevent clinical disease and mortality after natural challenge and to interrupt the spread of infection to susceptible animals. Therefore, to achieve successful immunization, several factors have to be considered and they include the choice of appropriate antigen and adjuvant, dosing or immunization schedule and delivery platform. The choice of antigen is highly dependent on the ability of the antigen to express immunodominant epitopes and whether it possesses the ability to induce the production of fully neutralizing antibodies and activate cytotoxic T cell response. Adjuvants enhance the immunogenic properties of vaccines by prolonging antigen persistence, enhancing co-stimulatory signals, increasing local inflammation and stimulating lymphocytes via induced cytokines. Proinflammatory cytokines such as IL-12 and IL-2 stimulate both an innate and adaptive immune response and promote T-lymphocyte proliferation.

These two cytokines could act as immunopotentiators if added to a *Theileria* subunit vaccine (Reviewed in [42]). The route of vaccine administration—e.g., intramuscular, subcutaneous, intranasal, ocular, oral or *in ovo* immunization—depends on the type of pathogen, cell tropism and the stage of infection (acute, chronic or latent). Controlled release and needle-free (transdermal/topical) approaches are new delivery methods that are still in the research and development stage.

Several vaccine trials utilizing various antigens and delivery routes have been performed against *T. parva* infections. Challenges encountered facing the production of a global *T. parva* subunit vaccine include genetic complexities of *T. parva* strains [43], the high polymorphic nature of bovine MHC loci [44], the biodiversity of *T. parva* strains [45], and the dominant cellular immune response following *T. parva* subunit vaccination [46].

In order to produce new *T. parva* vaccine antigens, Bastos et al. [41] investigated molecular and antigenic properties of Tp9 as a candidate vaccine antigen expressed by sporozoite and schizont parasite stages. They replaced a weakly functional signal peptide contained in Tp9 with a human tissue plasminogen activator signal peptide (tPA) and in this way they increased secretion of Tp9 from mammalian cells. Interestingly, they demonstrated that *T. parva*-immune cattle develop both humoral and cellular immune response to this antigen and significant amounts of IFN-γ were produced by $CD4^+$ T cells following ex vivo exposure to recombinant, mammalian-expressed Tp9. Therefore, recombinant Tp9 can be further evaluated as a component of a *T. parva* subunit vaccine.

Mucosal and/or systemic antibodies—and most especially the $CD8^+$ T cell response—are stimulated by antigens such as *T. parva* schizont antigens (Tp1-Tp12) [41][46][47][48], *T. parva* sporozoite p67 antigen [49] and *T. annulata* sporozoite antigen SPAG1 [50]. Specific immune responses to these antigens are required for protozoa clearance from the host. These antigens recognized by MHC class I-restricted $CD8^+$ T cells have been tested for their ability to induce immune responses and have been found to be vaccine candidates. These antigens also play a role in preventing or reducing the entry of sporozoites into host lymphocytes [46]. The polymorphic immunodominant molecule (PIM) is a structurally complex protozoal protein with immunogenic properties, expressed by both sporozoite and schizont stages of *T. parva* [51], and it plays a role in sporozoite entry into lymphocytes [52]. The antigen is rich in glutamine and proline and challenged cattle mount, a powerful humoral and cellular immune response, but there is no evidence yet that it can confer or sustain long-term immunity [53]. Antigenic proteins similar to PIM—*Theileria lestoquardi* surface protein (TlSP) and *Theileria annulata* surface protein (TaSP)—are expressed in *T. lestoquardi* [54] and *T. annulata* [55], respectively. Both have been demonstrated as possible components of a subunit vaccine [55]. The development of a subunit vaccine against one parasite species can protect against the other. Nene and Morrison [56] extensively reviewed several approaches to vaccination against *T. parva* and *T. annulata* and suggested that a *T. annulata* subunit vaccine is likely to protect against *T. parva* infections. This is because p67 and SPAG1 antigens can confer cross-species immunity [57], even though their protein sequence similarity is only 47% [58]. The ability to confer cross-immunity was attributed to the highly conserved epitope sequences between them, meaning that anti-p67 serum recognizes SPAG1 and neutralizes *T. annulata* sporozoites, and vice versa.

The development of an effective subunit vaccine or live vaccine against *T. orientalis* complex may not be feasible, although the development of a live vaccine based on one or two of the *T. orientalis* benign genotypes may be considered. Even though a certain immunological and genetic diversity exists among them, they are clustered together on one clade of the phylogenetic tree. It was proposed that, since they are grouped together, a cross-immunity between genotypes may exist [59]. The use of variable piroplasm surface proteins to develop a subunit vaccine against *T. orientalis* has had no success to date, and progress for this approach has been negligible over the years. Globally, there is no suitable vaccine against *T. orientalis* complex infection in cattle to date [59][60]. This is because of the difficulty of extracting pure isolates for studies, as the benign form of the disease is caused by more than one genotype of *T. orientalis* and there is a low parasitaemia [61]. The *buffeli/chitose* genotypes are more closely related to each other than to the *ikeda* genotype; therefore, this genetic diversity may have implications for vaccine design [62]. It is not yet certain if the *buffeli/chitose* genotypes can stimulate heterologous immunity against the *ikeda* genotype. This lack of suitable vaccines further complicates *T. orientalis* management. Despite the development of a live vaccine being much more feasible than the subunit vaccine, Jenkins and Bogema [28] opined that MPSP still represents a promising target for a subunit vaccine against *T. orientalis* complex.

4.2. DNA Vaccines

A possible solution to challenges facing the use of a *T. parva* subunit vaccine is next-generation vaccine technology based on DNA vaccines. A DNA vaccine is particularly attractive for the prophylaxis of intracellular pathogens such as herpes simplex virus and mycobacteria, and since the *Theileria* parasites are intracellular pathogens, a DNA vaccine that expresses cytokines should be appropriate [63][64]. In fact, DNA vaccines have a strong capacity to induce cell-mediated immune responses characterized by the production of T helper 1 cytokines (IL-12, IFN-γ, TNF-α, IL-21). These cytokines are a critical component in the host defense against chronic/persistent pathogenic infections and facilitate the adjuvant activity of DNA-based vaccines [65]. For instance, IL-21 can be used as a molecular adjuvant because of its involvement in T cell and NK cell activation, and its effect on $CD4^+$ T cells can aid in the response to chronic or latent infection. An increased IFN-γ level enhances the activities of cytotoxic T lymphocytes and NK cells. The use of DNA constructs encoding molecular adjuvants such as IL-6 and TNF-α has proven useful in several cattle diseases, such as foot and mouth disease. The addition of a molecular adjuvant enhanced antigen-specific cell mediated responses elicited by the DNA vaccine [66]. DNA vaccination with chitosan nanoparticles has also been used in vaccination against *Staphylococcus aureus* in dairy cows [67]. Recombinant DNA vaccine constructs encoding the Tp1, Tp2, Tp4, Tp5 and Tp8 antigens have been previously described [68]. The adjuvant activity of DNA constructs expressing bovine foetal liver tyrosine kinase 3 ligand (Flt3L) and granulocyte macrophage-colony stimulating factor (GM-CSF) has been proven in vivo by Mwangi et al. [69]. These cytokines induced the recruitment of an increased number of dendritic cells to the site of inoculation and enhanced antigen-specific $CD4^+$ T cell responses [69]. However, the administration of the bovine Flt3L and GM-CSF vaccine prior to DNA vaccination in *T. parva*-challenged cattle induced $CD4^+$ and $CD8^+$ T cell IFN-γ responses but not the antigen-specific cytotoxic T -ymphocyte (CTL) response [70].

By delivering DNA via different routes, DNA vaccines can generate a different type of immune response (cellular and/or humoral). For instance, intradermal injection of DNA vaccine elicits a predominate Th1 response, while the so called biological ballistic or biolistic DNA injections mainly stimulate a Th2 or a balanced Th1/Th2 response [71]. Fry et al. [72] demonstrated for the first time the particle-mediated epidermal delivery of a DNA vaccine whereby a DNA-encoded *T. parva* codon-optimized and native sequence PIM antigen was delivered through the intra-dermal route in Holstein steers. This method is also known as gene gun immunization, whereby DNA-encoded antigens are delivered directly into the nucleus of epidermal and dermal professional and non-professional antigen-presenting cells. These antigens are then expressed and processed to elicit an immune response. The gene gun has been used for the delivery of influenza vaccine in ferrets [73]. The advantage that gene gun immunization has over traditional intramuscular DNA immunization is that it requires 10 to 100-fold less DNA and yet elicits a strong humoral and cellular immune response . The use of gene gun immunization against *T. parva* infection elicited a robust protective immune response characterized by significant antibody and cell-mediated responses (Th1/IgG2 and INF-γ responses). Although the antibody response mounted was not enough to prevent East Coast fever in the *T. parva*-challenged calves, gene gun immunization may serve in the future as a suitable vaccine platform against *T. parva* and other bovine blood pathogens in cattle that sufficiently express the MHC class I molecules, with the role of binding and presenting PIM epitopes to cytotoxic T cells [72].

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