Review

Ehrlichia Canis Vaccine Development: Challenges and Advances

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Review

Ehrlichia canis Vaccine Development: Challenges and Advances

Bruna Samara Alves-Ribeiro [1,2], Raiany Borges Duarte [1], Zara Mariana de Assis-Silva [1], Ana Paula Carvalho Gomes [1], Yasodaja Assis Silva [2], Lizandra Fernandes-Silva [1], Alice Caroline da Silva Rocha [3], Iago de Sá Moraes [1], Klaus Casaro Saturnino [2], Dirceu Guilherme de Souza Ramos [1], Isis Indaiara Gonçalves Granjeiro Taques [4] and Ísis Assis Braga [1,*]

[1] Laboratory of Veterinary Parasitology and Clinical Analysis, Academic Unit of Agricultural Sciences, Federal University of Jataí, Jataí 75801-615, Goiás, Brazil
[2] Laboratory of Veterinary Anatomical Pathology, Academic Unit of Agricultural Sciences, Federal University of Jataí, Jataí 75801-615, Goiás, Brazil
[3] Academic Unit of Agricultural Sciences, Federal University of Jataí, Jataí 75801-615, Goiás, Brazil
[4] EDUVALE College, Caiçara Street, 2.114, Jaciara 78820-000, Mato Grosso, Brazil
* Correspondence: isisbraga@ufj.edu.br

Simple Summary: Over the years, inactivated, live attenuated, and recombinant vaccines against *Ehrlichia canis* have been evaluated to mitigate the impact of canine monocytic ehrlichiosis. However, these results are not sufficient to produce an effective immunizing agent. With the advancement of bioinformatics, immunoinformatics, and structural modeling tools, new investigations can be conducted, potentially ending a long-standing gap in research related to the development of vaccines against *E. canis*. Based on this technological advance, we believe that we are increasingly closer to developing a vaccine composed of multiple epitopes capable of eliciting robust humoral and cellular immune responses.

Abstract: Canine monocytic ehrlichiosis (CME) is an infectious disease caused by *Ehrlichia canis*, a globally recognized obligate intracellular bacterium. In addition to dogs, other animals, including humans, may be affected. Despite its epidemiological importance and impact on public health, there is currently no commercial vaccine against *E. canis*. This study aimed to present relevant aspects of the challenges and advances encountered in the development of vaccines for CME and highlight perspectives for future investigations. High genetic variability, along with the various evasion mechanisms employed by *E. canis*, has hindered the identification of an antigen that targets Th1 cells and is immunogenic to most *E. canis* isolates, considering their distinct antigenic characteristics. To achieve robust immunity, the vaccine must predominantly confer cellular and humoral immunity. Early production efforts have been challenging owing to low immunogenicity, difficulties in establishing long-term protection, and limitations of the techniques used. However, with the refinement of bioinformatic tools, research in this area will be facilitated, thereby accelerating the development of effective vaccines for CME. According to these authors, a vaccine should consist of multiple epitopes.

Keywords: bioinformatics; canine monocytic ehrlichiosis; immunizing; immunoreactive proteins; tick-borne disease

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1. Introduction

Canine monocytic ehrlichiosis (CME) is an infectious disease caused by *Ehrlichia canis*, an obligate intracellular bacterium transmitted by ticks. It mainly affects domestic dogs but can also infect other domestic and wild animal. Moreover, there have been reports humans parasitized by *E. canis* [1–4].

Despite the epidemiological impact of *E. canis* on public and veterinary health, a vaccine against this pathogen is not commercially available. The absence of lipopolysaccharides in this bacterium invalidates strategies against glycoconjugates and requires the identification of protein antigens, which has been the greatest challenge for researchers because of the mechanisms of immunological subversion and the high antigenic variability of the species [5,6].

In vitro research and murine experimental models are significant obstacles to vaccine development, as they do not reflect the reality of natural infection and therefore interfere with the practical assessment of the immune response [7]. However, after three decades of investigation, advances have been made in identifying the immunoreactive proteins of *E. canis*, such as p28, which acts as an outer membrane protein, and the tandem repeat proteins (TRPs) TRP19, TRP36, TRP140, and ankyrin repeat protein (Ank200), some of which are targets for vaccine development [8].

Recent research has focused on the development of immunogens for intracellular bacteria such as *E. chaffeensis*. Bioinformatics has provided agility, eliminating the need for *in vitro* and *in vivo* testing during screening [9–11]. A team of researchers successfully identified new hypothetical immunoreactive proteins in *E. canis* [12–14]. This discovery holds great promise for understanding the antigenic and immunogenic potential of these proteins.

The objective of this review is to present relevant aspects regarding the challenges and advances faced in the development of vaccines for *E. canis* and highlight perspectives for future investigations.

2. Challenges for Production a *E. canis* Vaccine

The challenges faced in formulating vaccines for CME are related to the complexity of the immunopathogenesis and significant antigenic variability of *E. canis*, which can make it difficult to select specific epitopes for effector cells. However, the limitations of *E. canis* culture, *in vitro* techniques, and models used for experimental testing have been significantly reduced owing to the potential of bioinformatics to address these challenges.

The effective host immune response to combat infection by bacteria of *Ehrlichia* genus occurs through antigen presentation by conventional dendritic cells (CDC), followed by an acquired cellular immune response with activation of CD4+ cells in Th1 associated with humoral (Th2) responses [15,16]. Some vaccines stimulate the production and transformation of CD4+ helper T cells into Th1 cells. Then, it stimulates the production of CD8+ cells that secrete IFN-γ, TNF-α, IL-2, IL-3, and GM-CSF [17]. The potential of vaccines targeting antigens for dendritic cells is a promising approach that may be effective against obligate intracellular bacteria.

At the beginning of infection, the bacteria reduce the expression of primary histocompatibility complex type I (MHC-I), contributing to the maintenance of intracellular survival. During infection, there is a reduced expression of MHC-II, which is essential for the maturation of CD4+ T lymphocytes, resulting in an adequate cellular and humoral immune response. Meanwhile, the saliva of *R. sanguineus* complex ticks has an immunosuppressive effect by inhibiting the Th1 immune response [18].

Several mechanisms used by *Ehrlichia* spp. to escape the host immune system and survive intracellularly have been elucidated. Pioneering studies have suggested that this bacterium can inhibit phagolysosomal fusion in infected cells, allowing survival within phagosomes [19]. This inhibition results from the suppression of proteins that form a complex that juxtaposes the vesicular membranes, which makes fusion difficult, and also the reduction in IFN-γ levels, which is inversely proportional to the frequency of cells infected by *Ehrlichia* [16].

With the advancement of proteomics, several immunogenic proteins have been described and characterized as essential for the maintenance of infection. Outer membrane proteins (OMPs; P30/P28) [20], ankyrin repeat proteins (Anks) [21], and a group of proteins with amino acid repeat

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sequences (TRPs) have been identified in different species of *Ehrlichia* [22–24]. Luo et al. [25] reported that TRPs are involved in essential functions for the entry of *Ehrlichia* spp. into the host cell (phagocytosis, cytoskeletal reorganization, and intracellular transport) and replication mechanisms (cell signaling, metabolism, post-translational modification, and transcriptional regulation), in addition to interfering with the exit of the infected cell (apoptosis and exocytosis). Bui et al. [26] reported that TRPs are substrates of the type 1 secretion system (T1SS), which plays an important role in maintaining bacterial infection by secreting proteins that interact with a diverse network of targets in the host associated with essential cellular processes [27].

Two major TRPs, TRP19 and TRP36, have been identified in *E. canis*. McBride et al. [22] highlighted the importance of these proteins in immunodiagnosis and considered them essential targets for vaccine development [28]. TRP 19 and TRP 36 are species-specific. However, only TRP 19 is conserved among different *E. canis* isolates [23,24,28].

TRP36, despite its early expression in the infective form of *Ehrlichia* (dense core), has shown significant genetic diversity among different *E. canis* isolates worldwide. This global distribution of diversity, with six genotypes identified from TRP36 in different regions, poses a challenge to vaccine development. The genotypes include American (USTRP36), Brazilian (BrTRP36) [29,30], Costa Rican (CRTRP36) [3], Taiwanese [31], Cuban (CUBTRP36) [32], and the most recent, YZ-1, isolated in China [33].

The USTRP36 genotype has been reported in *E. canis* isolates from North America, Brazil, Nigeria, Cameroon, Spain, Turkey, Israel; and Taiwan, South Africa, Thailand, Turkey, and Colombia [8,29,32,34–38]. BrTRP36 is widespread in Brazil [36] but has also been reported in Turkey [35] and Colombia [38]. CRTRP36 was first described by Bouza-Mora et al. [3] in human blood donors, which reinforces the zoonotic potential of this genotype and has also been reported in Brazil, Turkey, and Colombia [35–38].

Aguiar et al. [29] described an *E. canis* isolate, #Cba16, which presented a unique combination of the American TR region and the N region of the Brazilian genotype. This finding suggests the possibility of genetic recombination owing to co-infection. This finding, a result of collaborative research, reinforces the hypothesis established by Doyle et al. [28], who stated that TRP36 was under high level of selective pressure.

Recent studies have reported clinical differences among dogs positive for different genotypes. Navarrete et al. [32] described a greater hemorrhagic tendency in dogs of the Cuban genotype. These authors suggested that genetic diversity in Cuba supports the emergence of more virulent strains. In Brazil, Borges et al. [37] highlighted the high prevalence of the CRTRP36 genotype, which has been previously described as potentially zoonotic. Furthermore, these authors described USTRP36 as the most pathogenic genotype in this population of dogs because it is associated with inflammatory responses. Melo et al. [39] reported, in the Pantanal region of Brazil, the first serological evidence of dogs seropositive for *E. minasensis*, as they presented higher titers in IFAT than in *E. canis*. Thus, it is believed that the effective immune response of a vaccine may be altered owing to differences in the pattern of the immune response in dogs exposed to different genotypes of *E. canis*, different species of *Ehrlichia*, and co-infection between them because of the diversity of virulence.

Another critical aspect to consider is the immune response produced by the murine experimental model to antigenic stimuli. The capacity for infection by *Ehrlichia* species in this model is variable, and the route of infection interferes with the immune response, which can generate unreliable results [40,41].

3. History on Vaccine Production for *E. canis*

Research on *E. canis* began in 1970, with a focus on the characterization of its pathogenesis, clinical description, and immunological response after infection. This research is fundamental for the development of vaccines against ehrlichiosis [42–45]. Notably, Ristic and Holland [46] played a pivotal role as pioneers in the evaluation of an immunizer despite the limited publicity of their study.

In 1998, Breitschwerdt [47] conducted a study on 16 dogs experimentally infected with *E. canis* to verify the efficacy of doxycycline in the treatment of CME. In addition to the therapeutic results,

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they provide the first insights into the essential implications for the development of a vaccine after considering the roles of innate and adaptive immune responses. Mahan et al. [48] investigated the response of German Shepherds after immunization with an inactivated *E. canis* containing the Quil A adjuvant. A bacteremia-suppressive effect was observed after the challenge. However, western blot analysis revealed a short-lived response, with a drop in antibody titer, exposing the animals to reinfection.

Or et al. [49] inoculated two dogs intravenously with a suspension of attenuated *E. canis* (Israeli strain) through multiple passages in culture media. The analysis consisted of verifying the transmission to naïve *R. sanguineus* and challenging the dogs after 119 days with a blood sample from a known infected dog. However, the transmission of bacteria to ticks has not yet been verified. The "immunized" dogs developed bacteremia on day seven after the challenge, and one of them showed mild petechiae and splenomegaly. However, no hematological changes were observed in either dog. This study suggests the possibility of using *E. canis* attenuated by multiple passages in cell culture as a vaccine candidate. In another study using the same methodology, an additional study was conducted on 12 dogs, reinforcing the results described previously [50]; however, the immunity period of was not evaluated in either study.

The use of primitive inactivated and live attenuated vaccines has led to undesirable effects and has not met expectations, and improvements in research using modern techniques have become evident. Advancements in reverse vaccinology have allowed the identification of antigenic proteins, and the idealization of a peptide-based vaccine design has been employed [7,8,51,52]. In this context, research using murine models has been initiated to evaluate the dynamics of the immune response stimulated by immunizers containing synthetic peptides. The recombinant protein p29 from *E. muris* and the OMPs from *E. chaffeensis* p28 were the first targets of these investigations, and a significant reduction in bacterial load was observed, as well as the induction of a protective immune response in mice mediated by antibodies and T cells [53,54].

The identification of immunoreactive proteins of *E. canis*, followed by evidence of significant genetic and antigenic differences in antibody epitopes between the same *E. canis* strain [8,24], has stagnated the search for immunogens because of efforts to understand the immunopathogenic mechanism of these synthesized proteins. Moreover, the p19 protein of *E. canis* is the most preserved among these strains [31,55].

In one study, the *in vitro* neutralization capacity of hyperimmune serum synthesized from GP19 was evaluated, and promising data were revealed [56]. Then, a prototype of a recombinant *E. canis* vaccine (rGP19) was tested in mice, which showed significantly higher mean antibody levels than the control group, followed by a lower ehrlichial load in the blood. The authors assumed that the immunizer could eliminate *E. canis* by stimulating CD4+ T cells, which produce IFN-γ, in addition to the production of antibodies [57].

The preliminary production of a vaccine against *E. canis* is challenging because of its low immunogenicity, difficulty in long-term protection, genetic variability, and undesirable effects. Since the Sars-CoV-2 pandemic, researchers have developed new technologies that combine immunoinformatics and cell-free protein expression, directly leading to the resumption of targeted research for immunoreactive antigens in the development of vaccines against *E. canis* [12]. The same team of researchers identified reactive immunogens of *E. canis* using bioinformatics, revealing 18 TRP19 immunoreactive proteins, some of which were immunodominant and had conformational epitopes. They hypothesized that some proteins were type I secreted effectors (T1SS) [13,14].

4. Prospects for Vaccine Development Against *E. canis*

After decades of research, we may be able to develop a vaccine against CME, as several studies have focused on identifying immunoreactive antigens of *E. canis* combined with reverse vaccinology, as well as improving bioinformatics and structural modeling, which are essential for the development of vaccines against intracellular pathogens.

Bioinformatics is fundamental for identifying essential protein targets and non-host homologs in the pathogen proteome that can be used as potential vaccine candidate targets [9,58]. This protein

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is generally involved in metabolic pathways critical for bacterial infectivity. Luo et al. [12,14] identified previously undiscovered hypothetical immunoreactive proteins in *E. canis* and provided additional options for immunogenic antigens. This discovery underscores the need for further studies to determine the T cell epitopes, secretion mechanisms, and functions of these proteins in ehrlichia pathobiology and immunity.

The next crucial step is to select new proteins and evaluate them through immunoinformatics, focusing on those that are immunodominant and effectors of the T1SS [14], as well as the proteins identified as TRP19, TRP36, TRP140, and Ank200 [40,51,57], to predict immune responses, particularly the interaction between epitopes and the host immune system, using tools that predict the antigenicity, allergenicity, and druggability of epitopes [60]. Research seeking to identify and evaluate pathogen epitopes unrelated CME, transmitted by ticks, has also been conducted with good results [11,59].

Structural modeling is an essential component of computational vaccine design. Three-dimensional models of pathogenic proteins allow the prediction of which protein conformations effectively trigger an immune response depending on the host [60,61]. Computational tools, molecular docking simulations, and molecular dynamics are frequently employed to model the binding of epitopes to immune receptors and predict the stability and efficacy of potential vaccine candidates [62]. A multi-epitope vaccine against *E. chaffeensis* was designed, and immunological simulation analysis showed strong interactions with toll-like receptors and acceptable immunoreactivity, which induced high levels of cytokine (IL2 and IFN-γ), B cells, and T cell populations [11].

Antigenic epitopes can be identified through *in silico* experimental models, preferably conformational or mixed, and can be expressed by the MHC-II of conventional dendritic cells (cDC) that activate Th1 and Th17 cell receptors. This activation is crucial as it leads to intense stimulation of the CD4+ T cell immune response and consequently secretion of IFN-γ. Importantly, CD4+ helper T cells play a pivotal role in driving the development of B cells, which differentiate into antibody-secreting plasma cells, memory cells, and long-lived plasma cells (LLPCs) that provide long-term and sustained antibody production. Epitopes for Th2 cell receptors are also desired, as a vaccine that produces a robust humoral, LLPC-stimulated, and T cell-mediated immune response will have a better chance of providing protection [6].

A strong cytotoxic cellular response to antigens is desirable. Although Th1 cells discretely stimulate the CD8 + T cell response, additional stimulation may be essential. Therefore, peptide presentation is required in a manner restricted to MHC-I, usually induced by antigen-presenting cells that secrete appropriate cytokines and co-stimulate cDCs to induce T cell differentiation [63]. Therefore, subunit vaccines against *E. canis* would require new formulations of adjuvants specialized in cross-antigen presentation [64].

After identifying the epitopes and understanding the essential characteristics of a robust immune response against *E. canis*, the next step is to define the correlates of protection (CoPs) for licensing an effective vaccine. This phase depends on humoral responses, well-defined CoPs, and cell-mediated responses that are predominantly tissue-resident memory T cells (TRM), as predicted by Schaik et al. [6].

After the *in silico* approach, prototypes of vaccines structured with multiple *E. canis* should be tested *in vivo* in the preferred host or in experimental models that mimic natural infections as much as possible. Budachetri et al. [65,66] investigated the vaccine potential of proteins synthesized by *E. chaffeensis* and found a high production of antibodies and INF-γ in immunized dogs, as well as rapid elimination of the bacteria. Vaccines against CME using immunoreactive proteins comprise two different delivery platforms: protein subunits and mRNA vaccines. The mRNA vaccines are more accessible and have a lower risk of adverse reactions [67].

Live attenuated vaccines (LAV) have been less explored in CME because of the difficulty in maintaining bacteria in cell culture. This methodology has been investigated using new attenuation approaches for the production of vaccines against other *Ehrlichia* species [68–70]. In one study, dogs immunized with an attenuated mutant strain of *E. chaffeensis* produced antibodies and activated

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CD4+ T cells, which consequently induced protection for up to 12 months [70]. Similar to LAV, DNA vaccines against *E. canis* have not been investigated. However, preliminary results indicate positive effects on the protection of *E. ruminantium* in animals [71,72].

5. Conclusions

New methodologies and machine-learning algorithms developed during the SARS-CoV-2 pandemic have sparked curiosity among researchers, renewing expectations for the formulation of a vaccine against *E. canis*. In just a few years, we have accelerated the process of discovering potential antigens, and we believe that the next phases of investigation will proceed rapidly and safely, aided by bioinformatics, immunoinformatics, structural modeling, and *in vivo* validation experiments.

The challenges remain, as *E. canis* is an intracellular bacterium with high genetic variability and poorly understood evasion mechanisms. However, we believe that we are close to developing a vaccine against CME. From the authors' perspective, the vaccine should be structurally composed of multiple conformational and linear epitopes, which, in addition to activating B cells, will have a strong binding affinity to CD4+ and CD8+ T cells, promoting the production of cytokines such as IFN-γ, which are essential for pathogen destruction.

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