

Article

Immunization with Recombinant TRP19 Reduces Clinical Severity of Experimental *Ehrlichia canis* Infection in Dogs

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Simple Summary

Canine monocytotropic ehrlichiosis (CME), a tick-borne disease caused by the bacterium *Ehrlichia canis*, is a major global health concern for dogs. Currently, there is no effective commercial vaccine available. This study aimed to evaluate a new vaccine candidate, the recombinant TRP19 protein (rTRP19), for its ability to protect dogs against experimental *E. canis* infection. We vaccinated beagles with two different doses of rTRP19 and then exposed them to the infectious agent. The results showed that vaccination with rTRP19 elicited a strong and sustained antibody response in the dogs. Crucially, the vaccine led to a significantly lower rickettsial load (bacterial numbers), particularly at the higher 100-μg dose, compared to the unvaccinated control group. Fourteen days after exposure, both vaccinated groups showed a complete absence of the bacteria. The higher dose of the vaccine also helped reduce fever, a key clinical sign of acute ehrlichiosis. These findings are important because they demonstrate that rTRP19 is a promising vaccine prototype that can induce a measurable immune response against CME in dogs. This is a crucial step toward developing a much-needed tool that can mitigate the impact of monocytotropic ehrlichiosis on canine health worldwide.

Abstract

Canine monocytotropic ehrlichiosis (CME), caused by the intracellular bacterium *Ehrlichia canis*, is a significant tick-borne disease in dogs that requires effective vaccination strategies. This study aimed to evaluate recombinant TRP19 (rTRP19), a highly conserved immunodominant antigen, as a promising vaccine candidate against experimental *E. canis* infection in dogs, following its success in a mouse model. Fifteen *E. canis*-negative beagles were immunized intramuscularly with either 50-μg or 100-μg of rTRP19 in alum adjuvant or a PBS control, on days 0, 30, and 60. All dogs were then exposed intravenously to *E. canis* on day 90 and monitored for 120 days for clinical signs, hematological changes (platelet count, hematocrit, and body temperature), and antibody titers. The rTRP19 vaccine prototypes induced strong antigen-specific humoral responses. They caused a significant reduction in rickettsial load, with complete elimination observed in the 100-μg group by day 7 and in



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both vaccinated groups by day 14 of exposure. Furthermore, the mean body temperature in the 100- μ g rTRP19 group was significantly lower than that of the control group, suggesting that the higher-dose vaccine mitigated febrile response. Collectively, these results suggest that rTRP19 vaccine prototypes hold promise in overcoming the clinical signs and hematological abnormalities of *E. canis* infection by inducing a strong antigen-specific antibody response.

Keywords: canine monocytotropic ehrlichiosis; dog; *Ehrlichia canis*; recombinant TRP19; vaccine prototype

1. Introduction

Canine monocytotropic ehrlichiosis (CME) is a tick-borne disease affecting dogs, caused by *Ehrlichia canis*, a small, Gram-negative, obligate intracellular bacterium belonging to the family *Anaplasmataceae* [1]. CME can manifest in acute, subclinical, or chronic phases and presents with a wide range of clinical and hematological symptoms. The acute phase is characterized by high fever, depression, lethargy, anorexia, lymphadenomegaly, and splenomegaly, and may include clinical signs such as dermal petechiae, ecchymosis, and epistaxis [2].

Early vaccine efforts using inactivated or live-attenuated preparations yielded unsatisfactory results. They provided only short-lived immunity and consistently failed to prevent the transmission of disease; consequently, vaccinology has shifted toward subunit- and peptide-based approaches, which involve leveraging reverse vaccinology to identify stable, immunogenic antigens such as the tandem repeat protein 19 (TRP19) and various TRPs [3]. However, major challenges remain, notably the genetic diversity of *E. canis* and the need for vaccines that elicit both robust humoral responses and strong Th1 cell-mediated immunity to treat intracellular infection [4,5].

Several *E. canis* antigenic proteins have been identified, including major immunodominant antigens of 19, 37, 75, and 140 kDa proteins [3,6,7]. Among these, the TRP19s are highly conserved, exhibiting 98.8–100% identity among *E. canis* strains [4,7,8]. Furthermore, recombinant TRP19 (rTRP19) has been developed and demonstrated to be a potential vaccine prototype. In a mouse model, rTRP19 elicited both humoral and cell-mediated immune responses capable of eliminating *E. canis* [9,10]. Since rTRP19 induced protective immune responses in a laboratory mouse model, this study aimed to evaluate rTRP19 as a promising vaccine candidate against experimental *E. canis* infection in dogs.

2. Materials and Methods

2.1. *E. canis* Cultivation and Preparation for Challenge

The *E. canis* BF W053712 X + 5 strain (PTA-5811TM, ATCC[®], Manassas, VA, USA) was propagated in DH82 canine macrophage cells (CRL-10389TM, ATCC[®]) and maintained in DMEM supplemented with 10% heat-inactivated FBS under standard conditions [10]. Upon reaching 80% infection, the DH82 cells were harvested and cryopreserved at -80°C for subsequent experimental use. While the BF W053712 X + 5 strain was used for recombinant protein expression, the *E. canis* Ebony X + 3 T75 strain was utilized for the experimental dog challenge.

The viability of the -80°C stored *E. canis* was confirmed biologically [10]. The post-thaw viability and infectivity of *E. canis* stored at -80°C can be definitively assessed using an in vitro infectivity assay, which remains the gold standard for verifying the biological activity of frozen stocks. This method involves inoculating a fresh culture of naive DH82 cells

with a thawed sample and monitoring the progression of the infection daily. By employing Diff-Quik or Giemsa staining to visualize the formation of intracytoplasmic morulae within the macrophages, viability is confirmed if the percentage of infected cells increases over a 3-to-5-day period. Furthermore, the infectious dose 50% (ID₅₀) can be calculated based on the rate at which the culture reaches 80% infection, providing a precise quantification of the stock's pathogenic potential before experimental use. Despite these differing laboratory designations, the TRP19 protein remains highly conserved, exhibiting 98.8–100% amino acid identity across diverse *E. canis* strains, thereby ensuring the immunological relevance of the vaccine to the challenge inoculum.

2.2. Production of rTRP19 and Vaccine Formulation

The rTRP19 protein was produced by cloning the *E. canis* TRP19 open reading frame (ORF) into an *Escherichia coli* pET30a expression vector, which was then transformed into *E. coli* BL21 Star™ (DE3) cells [10]. Following induction, the cultures were lysed, and the rTRP19 protein was purified via Ni-NTA affinity chromatography (GE Healthcare Life Sciences, Piscataway, NJ, USA). The purity and identity of the protein were verified using SDS-PAGE and Western blotting before quantification for immunogen formulation [10]. Two treatment groups were immunized with rTRP19 50-μg or 100-μg, in alum adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Aluminum hydroxide (Alum) was selected as the adjuvant for this study due to its well-established safety profile and its efficiency in enhancing humoral immune responses in canine models [11]. Alum functions by creating a depot effect at the injection site, ensuring sustained antigen exposure and promoting the production of antigen-specific IgG.

2.3. Experiment and Monitoring of Dogs

2.3.1. Experiment in Dogs

A total of fifteen *E. canis*-negative beagles were divided into three groups ($n = 5$ per group). The dogs were vaccinated intramuscularly with either 50-μg or 100-μg of rTRP19 in alum adjuvant or a phosphate-buffered saline (PBS) control on days 0, 30, and 60. On day 90, all dogs were exposed intravenously to *E. canis* (6×10^7 *E. canis* Ebony X + 3 T75) and monitored for 120 days post-initial inoculation for clinical signs, changes in hematological parameters, and antibody titers using ELISAs. Complete blood counts (CBC) were performed using the Dymind DF56Vet automated hematology analyzer (Shenzhen Dymind Biotechnology Co. Ltd., Shenzhen, China). Serum biochemistry was analyzed using the BX-3010 automated chemistry analyzer (Sysmex Asia Pacific Pte Ltd., Tampines Grande, Singapore).

All dogs tested seronegative for antibodies against *Anaplasma* spp., *Borrelia burgdorferi*, *Ehrlichia* spp., and *Dirofilaria immitis* antigen, as determined by the IDEXX SNAP 4Dx Plus Test kit (IDEXX Laboratories Inc., Westbrook, ME, USA). All dogs previously received core vaccinations in accordance with the World Small Animal Veterinary Association (WSAVA) guidelines [12]. Prior to the commencement of this study, the dogs were housed in an Animal Biosafety Level 2 (ABSL-2) facility for 2–3 weeks to ensure proper acclimatization.

2.3.2. Determination of Antibody Responses

The dogs were vaccinated on days 0, 30, and 60, with blood samples collected at each of these time points and subsequently on Day 90 (pre-challenge). Post-challenge, samples were collected at regular intervals to monitor antibody kinetics. Serological screening of all sera was conducted with a commercial ELISA test kit (ImmunoComb, Biogal, Galed, Israel) to detect total antibodies (or specifically IgG) to determine if an animal has been exposed to the pathogen, according to the manufacturer's instructions. Antibody levels specific to rTRP19 were measured to quantify the immune response directly induced by the

vaccine prototypes rather than a general infection, using an in-house ELISA as previously described [10].

For the in-house ELISA, serum samples were diluted 1:100 in blocking buffer to quantify rTRP19-specific IgG levels. Serum of all dog groups was collected pre- and post-*E. canis* challenging to determine antibody titers. An in-house indirect ELISA was performed, and a concentration of 10 µg/mL rTRP19 was coated on the immunoplates. The plates were incubated with individual dog sera at a dilution of 1:1000. Horseradish peroxidase-conjugated rabbit anti-dog IgG (Bioss antibodies, Woburn, MA, USA) was used as the secondary antibody at a dilution of 1:5000. Uncoated wells were used as blanks. The data were expressed as means with standard errors measured at the optical density (OD) of 450 nm. The optical density (OD) was measured at 450 nm using an AccuReader automatic ELISA plate reader (Metertech Inc., Taipei, Taiwan).

2.3.3. Quantification of *E. canis*

Ehrlichial load was quantified via quantitative real-time PCR (qPCR) targeting the 16S rRNA gene of *E. canis*, following established protocols [13,14]. Genomic DNA of *E. canis* was extracted from peripheral whole blood samples according to the manufacturer's instructions, with 100 ng of DNA utilized for each reaction. The assays were performed using the SensiFAST™ SYBR® Lo-ROX Kit (Meridian Bioscience, London, UK) on a CFX96 Touch™ Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA, USA). Absolute copy numbers were determined using a standard curve generated from 10-fold serial dilutions of an *E. canis*-16S plasmid, ranging from 10⁹ to 10 copies. Samples were considered negative if the cycle threshold (Ct) values exceeded 40 cycles.

2.4. Treatment

Doxycycline (10 mg/kg, orally, once daily) was administered for 28 days to dogs exhibiting clinical signs of canine ehrlichiosis, including fever, anorexia, lethargy, and thrombocytopenia. The normal platelet count range for dogs is 117,000–460,000/µL; a critical level is defined as below 40,000/µL. A dog's normal body temperature ranges from 101 to 102.5 °F, and a temperature exceeding 103 °F is considered a fever. The normal hematocrit (HCT) range for dogs is 36–56%, with levels below 35% typically indicating anemia.

In cases of fever, dogs were treated intramuscularly with Tolfedine CS® (1 mg/20 kg body weight; VETOQUINOL, Lure Cedex, France). Additionally, they were supplemented with vitamin B complex (Thompson's Vitamin B Complex®, Verrierdale, QLD, Australia) at a dose of one tablet a day, administered orally.

Throughout the 14-day post-challenge period, dogs were monitored daily for clinical manifestations of canine monocytic ehrlichiosis, including lethargy, anorexia, and pyrexia (rectal temperature >103 °F; normal range: 101–102.5 °F). To ensure an unbiased assessment of vaccine efficacy and natural rickettsial clearance, no therapeutic interventions or antibiotics were administered to any experimental groups during this observation window. This strict protocol was maintained to exclude any drug-induced confounding effects on rickettsial load or hematological parameters, such as hematocrit (normal: 36–56%) and platelet counts (normal: 117,000–460,000/µL).

Following the conclusion of the 14-day study, dogs exhibiting clinical signs—particularly those reaching critical thresholds, such as severe thrombocytopenia (<40,000/µL) or anemia (HCT <35%)—initiated a remedial treatment regimen. This included a 28-day course of doxycycline (10 mg/kg, orally, SID) and supportive care consisting of Tolfedine CS® for fever management (1 mg/20 kg, IM) and daily oral vitamin B complex supplementation.

All procedures were conducted under the strict oversight and approval of the Institutional Animal Care and Use Committee (IACUC).

2.5. Ethical Approval

The experimental procedures involving dogs were approved by the Institutional Biosafety Committee (IBC) of the Faculty of Veterinary Medicine, Chiang Mai University, Chiang Mai, Thailand (IBC Approval No. CMUIBC-A-0764015). All animal experiments were conducted in an animal biosafety level 2 facility at the Faculty of Veterinary Medicine, Chiang Mai University, with approval under the Animal Use Protocol (AUP) for Permission of Animal Care and Use (Approval No. Code: R10/2564).

2.6. Statistical Analysis

Descriptive statistics for body temperature, platelet count, and antibody levels are reported as the mean $\pm$ standard deviation. Differences in mean body temperature and platelet count were assessed among groups administered 50- μ g and 100- μ g rTRP19 doses and the control group using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for pairwise comparisons.

For whole-protein levels, a generalized linear mixed model (GLMM) was used to evaluate the effects based on group (50- μ g rTRP19 dose, 100- μ g rTRP19 dose, and control) and time (pre-vaccination and post-exposure). Group and time were included as fixed effects, while individual animals were treated as random effects to account for repeated measures. Additionally, the interaction between group and time was incorporated into the model to assess potential differential responses over time. Statistical significance was determined at $p = 0.05$, $p = 0.01$, and $p = 0.001$.

3. Results

3.1. Clinical Signs

Notable differences were revealed when monitoring body temperature, hematocrit, and platelet count post-*E. canis* inoculation.

3.1.1. Platelets

Mean platelet counts and standard deviations for the three experimental cohorts (50- μ g rTRP19, 100- μ g rTRP19, and control) over a 14-day observation period are presented in Figure 1. Throughout the study, platelet levels across all groups remained within the physiological range ($250 \times 10^3/\mu\text{L}$ to $450 \times 10^3/\mu\text{L}$). While biological fluctuations were observed in all cohorts, the control group exhibited a steady decline starting at day 4, reaching a minimum of $280.2 \times 10^3/\mu\text{L}$ by day 13.

In contrast, both rTRP19-treated groups effectively mitigated this depletion. The 100- μ g group maintained significantly higher levels during the early phase (Day 1) and the late-recovery phase (Days 10, 11, and 13) compared to the 50- μ g rTRP19 group. However, for the majority of the mid-study period (Days 2–9), the two dosages were statistically indistinguishable. Overall, while the higher dose offered more robust recovery in the final stages, both concentrations significantly outperformed the control group ($p < 0.05$), demonstrating a protective effect against platelet loss.

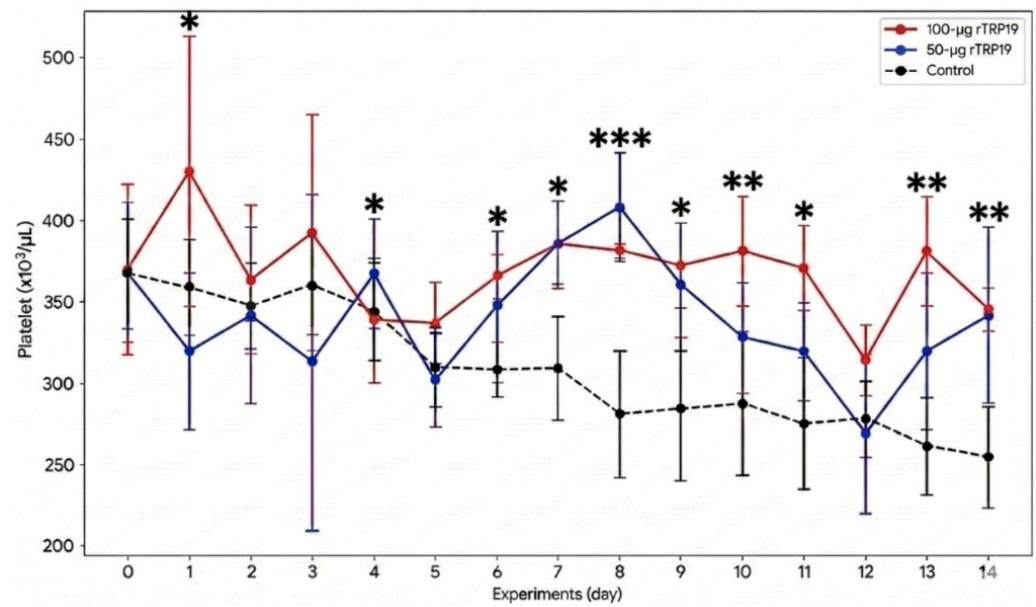


Figure 1. Platelet counts. Data points represent mean platelet counts ($10^3/\mu\text{L}$) and error bars represent mean $\pm$ SD. The 50- μg rTRP19 and 100- μg rTRP19 groups both effectively mitigated the platelet loss seen in the control group. One-way ANOVA revealed that both rTRP19-treated groups maintained significantly higher platelet levels than the control group (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$). Although both rTRP19-treated groups showed significant platelet counts compared to the control ($p < 0.01$), no significant difference was observed between the 50- μg rTRP19 and 100- μg rTRP19 dosage groups.

3.1.2. Hematocrits

Over a 14-day observation period, hematocrit levels in the 50- μg rTRP19, 100- μg rTRP19, and control groups remained comparable until day 7, fluctuating within a narrow range of 43.0% to 48.0% (Figure 2). A distinct divergence occurred beginning on day 8; while the control group underwent a steady decline from an initial 45.4% to a study low of 36.7%, both rTRP19-treated groups maintained stable levels between 44.7% and 49.5%. Statistical analysis via one-way ANOVA confirmed that both experimental dosages sustained significantly higher hematocrit levels than the control group from day 9 through day 14 ($p < 0.05$). Although the 100 μg dose trended slightly higher toward the end of the study—peaking at 49.5% on day 11—there was no sustained statistical difference between the two treatment concentrations. Ultimately, both dosages were equally effective at reducing the severe attrition seen in the control, suggesting that rTRP19 provides an efficient reduction in disease-induced anemia that is not strictly dose-dependent within the 50- μg –100- μg range.

3.1.3. Body Temperature

Over the 14-day observation period, body temperature profiles were recorded for the 50- μg rTRP19, 100- μg rTRP19, and control groups (Figure 3), with values generally ranging between 100 °F and 104 °F across all cohorts. All groups initially exhibited an upward trend peaking around day 3 or 4; the 100- μg rTRP19 group showed a more pronounced and significant reduction in body temperature during the latter half of the study. Statistical analysis revealed that the 100- μg rTRP19 group maintained significantly lower body temperatures compared to both the 50- μg rTRP19 and control groups on days 6, 7, 8, 9, 10, 13, and 14 ($p < 0.05$). In contrast, the 50- μg rTRP19 and control groups followed a more similar downward trajectory from day 5 onward, though they remained elevated

relative to the high-dose group during the marked intervals. These findings suggest that the 100- μ g dosage of rTRP19 is more effective at modulating or reducing fever-like temperature elevations associated with the experimental conditions than the 50- μ g rTRP dosage.

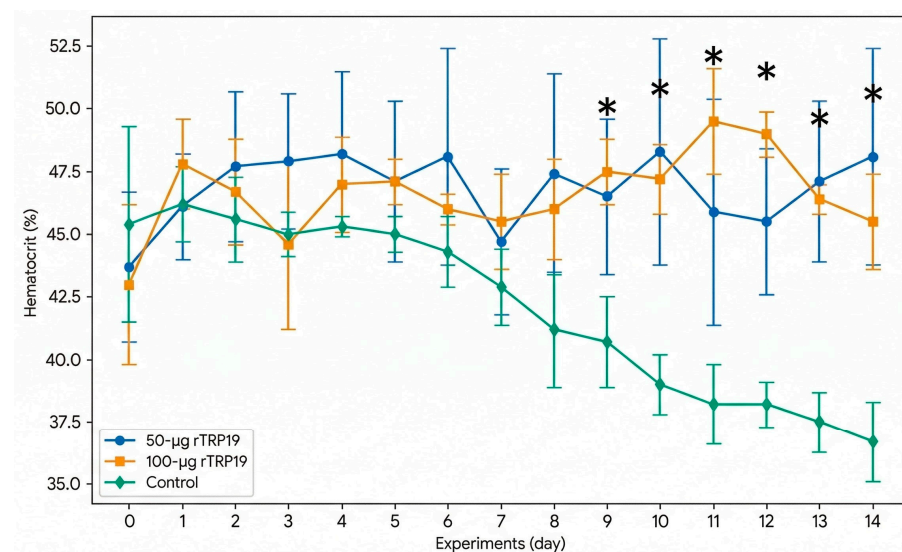


Figure 2. Hematocrits. The graph illustrates the mean hematocrit (%) $\pm$ SD for control, 50- μ g rTRP19, and 100- μ g rTRP19 groups. One-way ANOVA indicates that both rTRP19-treated groups maintained significantly higher hematocrit levels than the control group from day 9 through day 14 (* $p < 0.05$). No statistically significant difference was observed between the 50- μ g rTRP19 and 100- μ g rTRP19 treatment dosages.

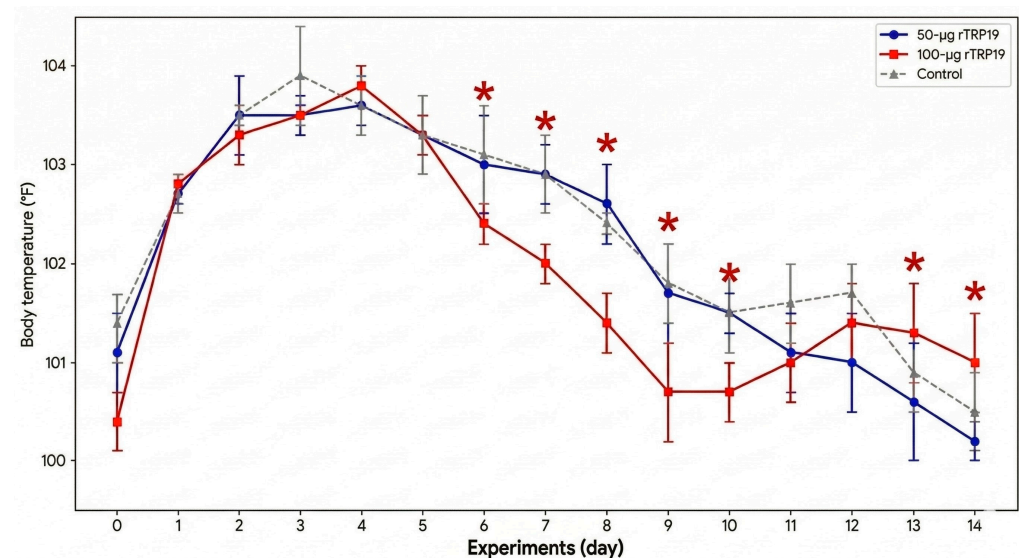


Figure 3. Body temperature trends across the experimental groups. The graph depicts the mean body temperature (°F) $\pm$ SD for the control, 50- μ g rTRP19, and 100- μ g rTRP19 groups over 14 days. Asterisks (*) indicate days where the 100- μ g rTRP19 group was significantly different from both the control and the 50- μ g rTRP19 groups ($p < 0.05$).

3.2. Rickettsial Load

Data is provided for the measurement of rickettsial load across three experimental conditions (control, 50- μ g rTRP19, and 100- μ g rTRP19 groups) and time points (day 3, day 7, and day 14) are shown in Figure 4. Immunization with rTRP19 demonstrated a potent, dose-dependent reduction in *Ehrlichia* copy numbers compared to the control group across all sampled time points. On day 3, the control group exhibited a high rickettsial load of

4899.24, whereas the 50- μ g and 100- μ g rTRP19 doses significantly reduced these levels to 1459.75 and 459.75, respectively. This inhibitory effect became more pronounced by day 7; while the control load remained elevated at 3735.26, the 50- μ g rTRP19 group dropped to a minimal 10.71, and the 100- μ g rTRP19 group achieved complete clearance with a value of 0. By day 14, both treatment groups successfully eliminated the rickettsial load, whereas the control group continued to maintain a significantly higher level of 1835.26. Statistical analysis confirmed that these reductions in the rTRP19-treated groups were significant compared to the control group throughout the study ($p < 0.05$). These findings demonstrate that rTRP19 reduces the clinical severity of experimental *E. canis* infection, with higher doses accelerating rickettsial clearance. The data suggest that rTRP19 exerts a potent, dose-dependent suppressive effect on the pathogen, resulting in total elimination at higher concentrations over time.

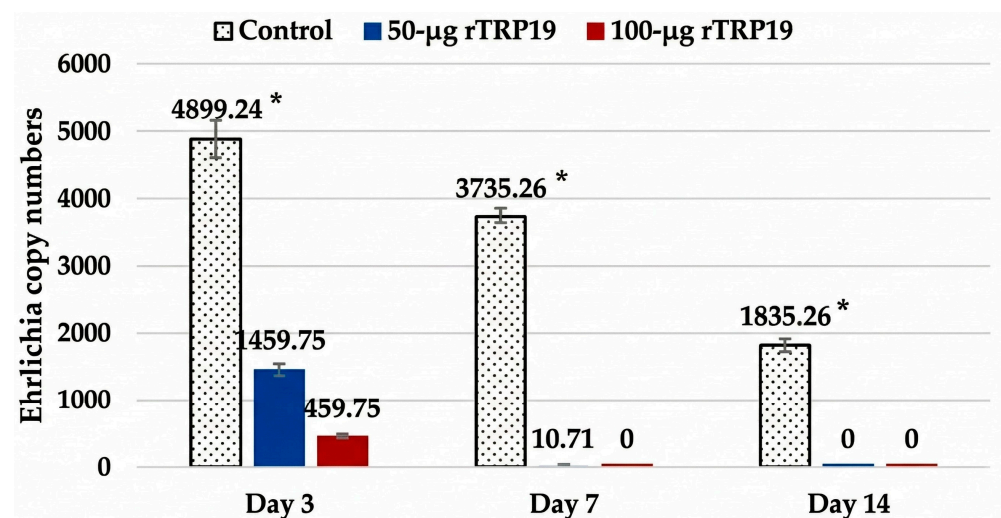


Figure 4. Rickettsial load. The chart illustrates the mean *Ehrlichia* copy numbers $\pm$ SD for the control, 50- μ g rTRP19, and 100- μ g rTRP19 groups at days 3, 7, and 14. Asterisks (*) denote that the 50- μ g rTRP19 and 100- μ g rTRP19 groups show a statistically significant difference compared to the control group ($p < 0.05$).

3.3. Antibody Responses

The serological results presented in Figure 5 demonstrate that the rTRP19 vaccine prototypes are highly immunogenic, successfully eliciting robust, antigen-specific antibody responses as measured by both an in-house indirect ELISA (Figure 5A) and a commercial *E. canis* ELISA kit (Figure 5B). Prior to the challenge, both the 50- μ g and 100- μ g immunized groups exhibited significantly higher mean anti-rTRP19 antibody levels compared to the naive control dogs, confirming effective priming of the humoral immune system. Following exposure to the disease, the vaccinated groups showed a significant increase in antibody titers from pre-vaccination levels, indicating a strong anamnestic (booster) response to *E. canis*. In contrast, the control group's antibody response remained significantly lower and showed no comparable change over the same period. These findings suggest that the heightened and sustained antibody production in vaccinated animals—statistically distinct from the control group ($p < 0.05$)—is essential for neutralizing extracellular *E. canis* and limiting bacterial spread, establishing rTRP19 as a suitable antigen for eliciting a humoral response.

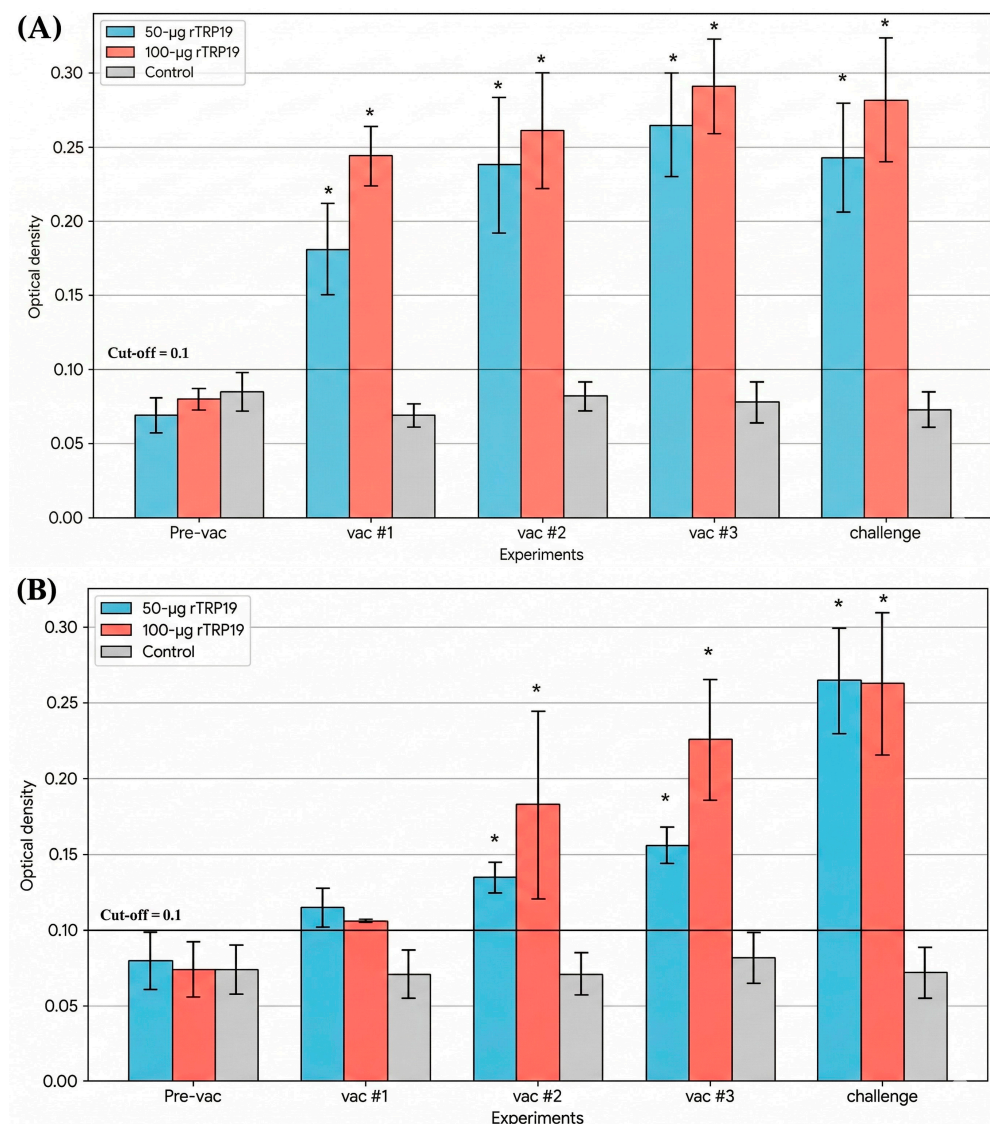


Figure 5. Antibody responses. The bar graphs represent the mean optical density (OD) $\pm$ SD for dog antibody responses to (A) whole-protein rTRP19 via in-house ELISA and (B) *E. canis* via a commercial ELISA kit. Horizontal lines indicate the diagnostic cut-off. Asterisks (*) denote that the 50-µg rTRP19 and 100-µg rTRP19 groups show a statistically significant difference compared to the control group: * $p < 0.05$.

4. Discussion

The TRP19 protein of *E. canis* has emerged as a crucial immunogenic target with considerable potential for both serological diagnosis and vaccine development [15,16]. TRP19's diagnostic ability is primarily attributed to its high degree of conservation across diverse *E. canis* strains and geographical regions [17]. This stability contrasts sharply with the substantial antigenic variability observed in other *E. canis* proteins, such as TRP36. As such, TRP19-based assays would be reliable for global pathogen detection [16,17]. Furthermore, TRP19 consistently elicits a robust humoral immune response, resulting in the production of high titers of specific antibodies in infected canines, making it an excellent candidate for ELISA and other serological tests [16,18].

Efforts to develop an *E. canis* vaccine have evolved significantly since initial research characterized its pathogenesis and immune response [19,20]. Early attempts were conducted with inactivated and live-attenuated vaccines. While offering initial insights into immune responses and some bacteremia suppression, these experiments often resulted in

short-lived protection or unverified prevention of transmission, highlighting their limitations [21,22]. Modern vaccinology has shifted toward subunit- and peptide-based designs, utilizing advancements in reverse vaccinology and immunoinformatics to identify highly immunoreactive and conserved antigens such as recombinant GP19 (rGP19) and novel TRPs [17,23–25]. Promising studies in murine models and in vitro neutralization assays demonstrate that these newer candidates can induce significant antibody production and stimulate protective cell-mediated immunity, including CD4+ T-cell responses and IFN- γ production, leading to reduced bacterial loads [9,10,24]. To reinforce the validity of rTRP19, our further research priority involves evaluating cell-mediated immune responses through cytokine profiling. Systematic measurements of IFN- γ , IL-12, and TNF- α are planned to delineate the Th1-mediated pathways essential for the clearance of intracellular *E. canis* infection. These assays will provide critical evidence regarding the vaccine's ability to stimulate a multifaceted immune response in the natural canine host. Despite the ongoing challenges posed by *E. canis*'s genetic variability and low immunogenicity, the identification of conserved epitopes, such as those found in TRP19 and certain newly discovered proteins, offers renewed promise for designing a broadly protective vaccine against geographically diverse strains [5,7].

Developing an effective vaccine for CME is complex due to the intricate biology of *E. canis* and its multifaceted interactions with both its canine host and the tick vector [26]. A major obstacle is the bacterium's significant antigenic variability, particularly within the TRP family. This variability complicates the identification of stable, broadly protective epitopes that can consistently elicit a robust immune response across different *E. canis* strains [5]. While certain proteins, including TRP19, show promise in inducing antibody production, their capacity to activate crucial Th1 cell-mediated immunity essential for combating intracellular bacteria warrants further investigation [5,9,10]. The wide antigenic variation observed in TRP36 among *E. canis* isolates, as well as the potential for genetic recombination due to co-infection, further undermines the utility of TRP36 as a universal vaccine antigen [16]. This suggests that a vaccine effective against one *E. canis* genotype may not confer sufficient protection against others, particularly in regions with high prevalence and diverse strains [5].

However, the primary challenge for TRP19 as a vaccine candidate is its ability to elicit a strong and sustained cell-mediated immune response, particularly a Th1 response [5]. Although TRP19 is a potent stimulator of humoral (Th2) immunity, a successful vaccine strategy against the intracellular bacterium *E. canis* requires a concurrent and robust Th1-mediated cellular response. This response is characterized by the production of cytokines such as IFN- γ , which activate macrophages and cytotoxic T lymphocytes essential for eradicating intracellular pathogens [26]. Experimental studies, particularly in mouse models, have demonstrated that rTRP19 can induce IFN- γ production and stimulate CD4+ T-cell differentiation in Th1 effector/memory cells. In addition, it induces antibody responses, causing a reduction in bacterial load [9,10,24]. These findings are encouraging, as they indicate that TRP19 possesses the intrinsic capacity to stimulate both arms of the adaptive immune system. However, despite these promising results, further research is imperative to fully elucidate the precise mechanisms by which TRP19 can optimally modulate Th1 cell differentiation and cytokine production in a natural canine host. This includes identifying specific T-cell epitopes within TRP19 and exploring various adjuvant formulations and delivery systems that can preferentially drive a protective Th1 response. Overcoming these challenges will be crucial for leveraging TRP19's inherent immunogenicity to develop a broadly effective and durable vaccine against the diverse *E. canis* genotypes prevalent in endemic regions.

Following *E. canis* infection, dogs develop a characteristic humoral immune response that is crucial for serodiagnosis and contributes to limiting, but not fully clearing, the infection [2,26]. An initial IgM surge in antibodies is typically detectable within 7 to 14 days post-infection [26], followed by a more sustained and robust IgG response, which becomes significant by 2–3 weeks and persists through the subclinical and chronic phases. This serves as the primary target for diagnostic tests such as indirect fluorescent antibody (IFA) assays [27,28]. Antibodies are directed against key immunoreactive proteins such as TRP19, which is highly conserved and consistently immunogenic [3,17]. While these antibodies may limit bacterial dissemination and target extracellular forms, their presence does not guarantee pathogen clearance, as the intracellular nature of *E. canis* necessitates a strong cell-mediated (Th1) response for clearance [26]. Furthermore, antibody-mediated mechanisms, such as the production of antiplatelet antibodies, can paradoxically contribute to the severe thrombocytopenia and immune-complex deposition observed in clinical disease [20,29].

Future research should expand on the current findings by optimizing adjuvant selection to specifically enhance Th1 cell-mediated immunity—such as through the use of saponin-based or TLR-agonist systems—to move beyond the humoral-focused aluminum hydroxide used here and better facilitate the intracellular killing of *E. canis*. To ensure broader translational relevance, subsequent trials must validate the vaccine's efficacy across diverse canine breeds and ages, moving from controlled intravenous challenges in beagles to field validation in endemic regions where dogs face natural tick-borne transmission. Furthermore, investigating the transmission blockade potential of the vaccine is critical, specifically determining if the complete bacterial elimination observed by day 14 effectively prevents ticks from acquiring the pathogen during a blood meal. Finally, the high conservation (98.8–100%) and immunodominance of rTRP19 highlight its dual utility as both a protective vaccine candidate and a robust diagnostic target, offering a pathway for developing sensitive ELISA-based tools for monitoring infection and vaccine-induced responses in global canine populations.

5. Conclusions

The rTRP19 vaccine prototypes reduced the clinical severity of experimental *E. canis* infection in dogs, as evidenced by a marked reduction in rickettsial load among all of the vaccinated subjects. The 100- μ g dose achieved complete bacterial clearance by day 7 after exposure to the disease, and both dose groups were free of detectable bacteria by day 14. This indicates the capacity of such vaccines to confer rapid, dose-dependent suppression of *E. canis* and promote pathogen clearance during the acute phase. Clinical fever was also mitigated in the high-dose group, further supporting the vaccine's effectiveness in reducing disease severity. Given the multifaceted challenges posed by *E. canis*—including complex pathogenesis, diverse clinical manifestations, and difficulties in vaccine development—these findings suggest that rTRP19 holds promise for alleviating the clinical signs and hematological abnormalities associated with canine monocytic ehrlichiosis. One way this is conducted is through the induction of robust antigen-specific antibody responses. Future studies should evaluate cellular immune correlates, durability of protection, and efficacy across diverse breeds and field conditions to refine this vaccine's protective potential. Although conserved antigens such as TRP19 provide potential for diagnostics and subunit vaccine design, overcoming antigenic variability and eliciting strong Th1 cell-mediated responses is crucial. Advancing delivery systems and deepening our understanding of host–pathogen interactions is essential to translate these findings into broadly protective vaccines that improve management strategies for CME.

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Data Availability Statement: The dataset is available on request from the authors. The raw data supporting the conclusions of this article will be made available by the authors on request.

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