

# **Recombinant subunit vaccine for *E. canis* - induced CME infections in canines**

## **Literature Review**

Canine monocytic ehrlichiosis (CME) can be caused by more than one *Ehrlichia* species. In the United States, *Ehrlichia* pathogens are most prevalent in the southeast, where three species of *Ehrlichia* are most commonly found: *E. canis*, *E. chaffeensis*, and *E. ewingii*. (O'Connor et. al., 2006) *Ehrlichia canis* is one of the *Ehrlichia* spp. that holds significance within the veterinary and human medical community. *E. canis* is an obligate, intracellular, gram-negative, dimorphic bacterium, transmitted through the brown dog tick, *Rhipicephalus sanguineus*. Various studies indicate that host susceptibility to *E. canis* infections is not just based on the virulence of the pathogen. Other factors such as tick-derived, local immunosuppressive factors, inoculum dose, epidermal entrance or repeated inoculations from multiple ticks must be considered. Experimentally, I.V. inoculated pathogens produce the same results as tick-inoculated pathogens. This confirms that the tick is not a necessary factor for *E. canis* infection, however, the tick may transmit other pathogens or biological factors with *E. canis* that can cause immunosuppression and/or contribute to the immunopathogenesis of *E. canis* infections. (Hess et. al., 2006) Once *E. canis* successfully invades the host, the canine can then become infected with CME, the most common tick borne infection in dogs. (Tsachey et. al., 2006) *E. canis* is primarily found within monocytes and macrophages of mammalian hosts as colonies of coccoid bodies, and is maintained in nature by persistent infection in wild and domestic canines. (Mavromatis et. al., 2006) *E. canis* now has worldwide distribution with the highest rates of morbidity and mortality in endemic areas. (Harrus et. al., 2003) The pathogen was first described in Algeria in 1935 and recognized as an organism of veterinary importance after the outbreak in 1963. The outbreak occurred in Singapore in British military dogs and in Vietnam in the United States military dogs. (Murray, 2003) One study documents the current distribution and the seroprevalence of *E. canis* in specific regions as follows: Israel 30%, Egypt 33%, Zimbabwe 42%, Spain 3% to 67% and Turkey 65%. (Tsachev, 2006)

*Ehrlichia* has recently emerged as a human pathogen, causing human monocytic ehrlichiosis (HME). HME infections are characterized by organ failure, coagulopathy and susceptibility to acquiring opportunistic infections. The agent that causes HME, *E. chaffeensis*, is genetically similar to *E. canis*. *E. chaffeensis* is zoonotic, meaning the pathogen can also cause infections in dogs. This reveals that zoonotic transmission and cross-infection between humans and dogs is a possibility. A deeper exploration of *E. canis* and successful vaccine development for canine infections can lead to a better understanding of human ehrlichiosis and to potential preventative control measures. (Hess et. al., 2006)

## Recombinant subunit vaccine for *E. canis* - induced CME infections in canines

*E. canis* infections are known to progress in three phases: the acute, the subclinical, and the chronic phase. (Morgan, 1997) Most acute stages occur in the warmer months, with infections characterized by reticuloendothelial perplasia, fever, splenomegaly, and thrombocytopenia. In acute infections, lesions tend to be nonspecific, but splenomegaly and discolored lungs are common signs. Dogs are able to recover from the acute stage of the infection with adequate treatment, and sometimes no treatment, and death in this stage is rare. While a strong antibody response may occur, often times the host still cannot clear the pathogen. If the host is not treated or inadequately treated, during the acute stage, then the host may progress into the subclinical stage, where they may become long-term asymptomatic carriers of the pathogen. Furthermore, persistent infections can progress into the chronic phase. In this phase, the host may have a poor response to treatment. Bone marrow failure and anemia can lead to opportunistic infections or death, due to massive hemorrhage. (Aiello, 1998)

Diagnosis of an *E. canis* infection often requires a platelet count as a screening test, a combination of observed, relevant clinical signs, a positive indirect serum fluorescent antibody titer and effective response to treatment. (Mavromatis et. al, 2006) The widely used and trusted *Handbook of Small Animal Practice* explains that definitive diagnosis is also made by western immunoblotting assays, and PCR assays. (Morgan, 1997) Serologic testing may be difficult in the acute stage because dogs may not become seropositive until 28 days after pathogen inoculation by the vector into the host, but in some cases, seropositivity can be seen in as early as 7 days of initial infection. (Greene, 2006) There are no current vaccines for *E. canis* infections; however, there are effective drug treatments available for positively diagnosed cases. Tetracycline is the drug of choice and is administered for a minimum of two weeks, for acute infections, and one to two months for chronic infections. Doxycycline is used in some cases where tetracycline fails, since doxycycline can better penetrate the intracellular space of infected cells. (Mavromatis et. al., 2006) Even with successful treatment and the absence of clinical signs, an *E. canis* antibody titer should be repeated to confirm seronegativity after six months from acquiring the virulent pathogen. Infections can persist even in the absence of clinical signs. (Aiello, 1998) Carrier states can also develop in canines from incomplete or ineffective host defenses. Subsequently, young dogs infected with *E. canis* do not seem to have significantly impaired immune responses or clinical signs during the first few months of experimental inoculation. (Hess et. al., 2006) Owners or veterinarians who do not treat infected canines exhibiting no clear evidence of infection inadvertently contribute to the persistence of *E. canis* in the host and within the environment. In the event of inadequate treatment, supportive therapy, such as platelet or whole-blood transfusion and fluid administration, can be used to help control hemorrhage, wasting and organ dysfunction. The best preventative measure against *E. canis* is to control the vector. Tick control is possible in the environment, as well as on the likely host. Regular tick-prevention treatments, including tick dips, are one way to control vector prevalence and possibility of infection. Preventative antimicrobial usage can also be used in endemic areas, such as kennels, to evade infection. (Aiello, 1998)

Persistent *E. canis* infections indicate that the pathogen must have biological mechanisms for evading host immune responses. *E. canis* is an aerobic organism that does not use glucose or fructose as a carbon or energy source, but instead uses amino acids as the main source. Since *E. canis* is a gram-negative bacterium, the pathogen lacks

peptidoglycan and lipopolysaccharide in the cell wall. However, *E. canis* has a protein-dominated cell wall structure to compensate for this lack of peptidoglycan. The structural rigidity of the outer wall of *E. canis* is likely dependent on covalent and noncovalent association between outer membrane proteins. In addition, this absence of peptidoglycan and lipopolysaccharide suggests that *E. canis* must have alternate structural and composition mechanisms for self-protection. *E. canis* may avoid host defenses by evading recognition by innate pattern recognition receptors, like Toll-like receptors 2 and 4. The alternation of the surface architecture and/or the expression of different protein variants are known crucial mechanisms of *E. canis*, used for persistence within the host. (Mavromatis et al., 2006)

*E. canis* antigenic diversity has been confirmed through immunoblot analysis of the IgG response to infections. Antigenic variation is crucial in influencing the severity of CME. (Greene, 2006) An operon of 22 paralogous major antigenic membrane proteins, commonly referred to as the p28/30 Omp, has been identified in *E. canis*. (Singu et al., 2006) Much of the altered surface architecture and expression of different protein variants observed with *E. canis* is a result of this large number of major antigenic proteins. *E. canis* is able to avoid host immune surveillance and establish persistent infections by differentiating the expression of these major antigenic proteins. (Mavromatis et al. 2006) A limited set of major *E. canis* immunoreactive glycoproteins has also been identified. Three common glycoproteins have been identified within this group of major immunoreactive proteins: gp36, gp140, and gp200. These glycoproteins appear to be of importance to evading host immune response through attachment to host cell (gp36 and gp140 are predicted to act as adhesions), and other potentially significant roles in ehrlichial pathobiology, including differential expression in the vector and the host. Twelve proteins containing tandem repeats have also been identified as important in pathogenicity and pathogen-host cell interactions. These proteins also account for the surface diversity and avoidance of immune recognition, due to the different allelic forms of tandem repeat proteins. Comparing the genome sequence of *E. canis* with other genomes revealed that *E. canis* shares 53 proteins of unknown function among other Rickettsiales and has 2 proteins that are unique to *E. canis*. (Mavromatis et al., 2006) Even with the complete *E. canis* genome sequence, there is still much to learn about ehrlichial genetics and the encoding for pathogenicity.

The p28/30 operon and the three glycoproteins, gp36, gp140 and gp200, referred to above are all highly antigenic and contribute to immune evasion. Another *E. canis* protein, VirB9, has been identified as a significant actor in facilitating intracellular survival. VirB9 is able to undergo moderate antigenic variation in the vector and in the host. However, virB9 is more highly conserved than proteins within the p28/30 operon. *E. canis* resides in the membrane-bound inclusion of monocytes and macrophages and is effective in avoiding fusion with lysosomes and degradation. VirB9 is suspected to play a crucial role in avoiding this fusion, and may also act to aid pathogen replication by encoding for type IV secretion machinery. (Felek et al., 2003)

Aside from antigenic variation and *E. canis* appropriation of type IV secretion machinery, canines often cannot clear *E. canis* infections because of general defective adaptive cell-mediated immunity (CMI). Deficiencies in T cells or MHC class II molecules are known to allow for more persistent infections. Experimentally administering anti-lymphocyte serum or cyclophosphamide to infected dogs caused the

infection to become more severe and lasting. Whether *E. canis* causes defective adaptive cell-mediated immunity, or if infections are just more likely to develop in canines with prior defects, is unknown. Regardless of how the host acquired deficiencies, any support for effective cellular immune response, can lead to possible control and eradication of the infection. Over-active humoral immune response is another characteristic of the host immune system that is indicative of persistent infection. (Hess et. al., 2006) Unlike humans, almost all canine lymphocytes present MHC class II receptors. MHC class II molecules interact with Th1 and Th2 T-cells and help regulate macrophage function. A study revealed that DH82 *E. canis*-infected macrophages had no MHC class II expression. This indicates that *E. canis* pathogenicity may also act through a mechanism that down-regulates MHC class II receptors. The predominant component of an effective immune response of dogs exposed to *E. canis* is the Th-1 type immune response, and the secretion of IFN-gamma, TNF-alpha and IL-2. Antibodies are also known to occasionally build effective host defense, despite dysfunctional cell-mediated immune responses. (Harrus et. al., 2003)

### **Description of Vaccine**

As mentioned above, there are currently no vaccines in production for canine monocytic ehrlichiosis. However, there are successful vaccines developed for another *Ehrlichia* spp., *E. ruminantium*. Since *E. canis* genome sequencing revealed almost complete synteny (88% of the proteome) with *E. ruminantium*, relevant information from *E. ruminantium* vaccines can be used in designing an effective vaccine against CME. (Mavromatis et. al., 2006) *Ehrlichia ruminantium* causes heartwater disease in ruminants. Heartwater, like *E. canis* infections, is infectious and tick-borne. Ruminants, particularly dairy cattle, in North America and around the world are at risk of acquiring *E. ruminantium* infections. Heartwater clinical signs include fever, the accumulation of fluid around the heart and throughout the body and edema. Death is likely for infected ruminants, if left untreated. Studies have revealed that ruminants are able to develop protective immunity against *E. ruminantium* after overcoming a preliminary infection, as is also the case with *E. canis* infection. The earliest procedure used to artificially acquire protective immunity consisted of infecting ruminants with the virulent organism in cryopreserved sheep blood, then treating with tetracycline once signs develop. An attenuated *E. ruminantium* vaccine was developed in 2005 that was shown to protect small ruminants against heartwater infection. The virulent organism was attenuated by propagation first in a canine macrophage cell line and then in a bovine endothelial cell line. Once inoculated with the attenuated vaccine, the test subjects did not present disease. Lethal doses of homologous virulent stock were administered and the test subjects appeared to be fully protected. By adapting the attenuated stock to a cell line from the natural host species, the vaccinated animal was able to obtain immunogenicity. Interestingly, the process of vaccine development and efficacy testing revealed that ticks are able to transmit attenuated organisms, and thus immunize naïve animals. (Zweygarth et. al., 2005)

Another study documents the production of an inactivated vaccine against heartwater. Inactivated *E. ruminantium* for the vaccine was produced using cultures of

ruminant endothelial cells and mass-producing the organism with the use of stir tanks. The vaccine was tested in field trials and yielded satisfactory results of the vaccine promoting protection against the homologous challenge. The researchers note that a recombinant vaccine would be ideal because of increased safety. However, development of a recombinant vaccine is difficult due to antigenic variation, as occurs with *E. canis*. Only one common outer membrane protein of *E. ruminantium* has been identified as a potential protein that could be used in recombinant vaccine development. (Marcelino et. al., 2006) There have been a few identified *E. canis* proteins (Omp p28/30, gp36, gp140, gp200 and virB9) that could confer incomplete or complete immunity, and are potential candidates for vaccines. The *E. canis* genome is also more conserved than most intracellular bacteria of the genus, which increases the probability of developing an effective recombinant vaccine against CME. (Mavromatis et. al., 2006) The conclusions drawn from vaccines against *E. ruminantium* can be used to further advances for development and production of a vaccine for the prevention and control of *E. canis*. (Marcelino et. al., 2006)

Based on the insight provided through *E. ruminantium* vaccine development and the supportive information that follows below, the decision was made to develop a recombinant subunit vaccine for *E. canis* infections. Infecting canines with virulent *E. canis* requires that follow-up treatment occur in order to overcome the induced infection. The major risk of obtaining immunity by this method is that the infected animal may not always be able to ward off the pathogen. Treatment could then become costly and prolonged. While this method is not vaccine-employed, the associated risk is the same as that from using attenuated vaccines. Despite the effectiveness of these vaccines, there is always the possibility that the attenuated pathogen will revert back to the virulent form and cause disease in the vaccinated animal. More specifically, because *E. canis* is an intracellular pathogen with complicated pathogenicity, the use of an attenuated vaccine would likely result in the vaccinated dog becoming sick or a carrier for the pathogen. Canines with defective MHC II machinery or CD8 T-cells are not successful in clearing *E. canis* infections, and *E. canis* either takes advantage or creates this defect. (Hess et. al., 2006) Regardless, administering an attenuated *E. canis* vaccine to a healthy dog can be as dangerous as administering an attenuated vaccine to any immunosuppressed animal. In addition, maintaining live or attenuated strains used for vaccine production is difficult, expensive and time-consuming. Inactive vaccines are safer than attenuated vaccines, however, they are less effective at mounting a successful immune response.

The primary disadvantage found in developing a recombinant subunit vaccine is high development costs associated with locating the genes, cloning them and expressing them efficiently in the vector. However, production costs are much lower with recombinant subunit vaccines than with the vaccine types mentioned above. Once vaccine development proves successful, the only challenges will be to grow and maintain the pathogen and the vector. Because the *E. canis* genome has been completely sequenced and possible protein vaccine candidates have already been identified, much of the timely and arduous tasks associated with recombinant subunit vaccine development have already been accomplished.

More specifically, the main challenge to creating an effective recombinant subunit vaccine for *E. canis* is antigenic variation. To combat this challenge, antigenic variation must be used as an advantage in vaccine development. This is possible through the

careful selection of antigens and of the subunit vector. As mentioned above, *E. canis*, unlike *E. ruminantium*, has a highly conserved genome and a few identified antigenic proteins known for their aid in host immune response evasion. The first of the five chosen antigens for the vaccine is Omp p28/30. Within the host, the proteins from the Omp p28/30 locus are post-translationally phosphorylated and glycosylated to produce multiple forms, and subsequently, antigenic variation. The p28/30 operon is shared in both *E. canis* and *E. chaffeensis* genomes. However, these similar *Ehrlichia* species express unique protein epitopes within this operon. The host cell-dependent protein expression in infected macrophages in both species is primarily from the Omp p28/30 - 19 and 20 genes. In experimentally infected tick cells, *E. canis* only expressed the p30 protein, while *E. chaffeensis* only expressed the p28 protein. P30 is likely an orthologue of p28 of *E. chaffeensis*, indicating an insertion mutation in the evolution from *E. chaffeensis* to *E. canis*. (Singu et. al., 2006) The decision to include the entire p28/30 operon in the vaccine was made based on previously discussed information: this genomic region contains most of the antigenic proteins of *E. canis*. In addition, because a distinguishing genomic difference in this region between *E. canis* and *E. chaffeensis* is known, this can be used as an advantage in testing for vaccine efficacy and confirming that memory was developed against *E. canis* and not against *E. chaffeensis*. The following section will discuss problems with distinguishing between these two *Ehrlichial* spp.

The next three chosen antigens are glycoproteins gp36, gp140, and gp200. These proteins are glycosylated within the host, forming different outer membrane patterns to evade host cell recognition. These common antigenic proteins were chosen as antigens, as well as the antigenic p28/30 operon, because with antigenic variation, no single antigen can be used to provide immunity against *E. canis*. These four antigens account for most of the problematic antigenic variation within *E. canis*, and are present in many identified strains. Recombinant subunit vaccines allow for purification of these antigens from the pathogen and elimination of any undesirable antigens. The ability to do this is what characterizes recombinant subunit vaccines as safe. To reiterate points discussed in the prior section, all four of these antigens act within *E. canis* to increase pathogenicity and the persistence of infection. One may argue that these chosen antigens may be damaging to the host. In response, because a safer and more controlled recombinant vaccine is being used, more freedom is allowed to include highly immunogenic agents that can produce stronger immune responses, and thus compose a more effective vaccine.

The final antigen chosen for the vaccine is virB9. Like other immunogenic proteins from the *E. canis* virB operon, virB9 contributes to pathogen-host cell interactions. (Mavromatis et. al., 2006) Dogs with CME were found to have antibodies to recombinant virB9. This confirms that virB9 is highly antigenic within the host, like the other four antigens. This also confirms that the infected hosts are still able to mount effective immune responses, and memory, to antigenic variants. Unlike the other four antigens, virB9 is nearly perfectly conserved between *E. canis* strains. Sequencing of virB9 strains from six different geographic locations revealed identical sequences between all strains. Vaccine development requires that at least one of the chosen antigenic epitopes be highly conserved. This will allow the vaccine to protect against all or most strains of *E. canis*. (Felek et. al, 2003) VirB9 is not just a valuable component for this vaccine because of sequence preservation, but also because the protein is

uniquely crucial to a major mechanism *E. canis* implements to evade host immune responses. The VirB operon and the virD4 gene encode for type IV secretion machinery, and are also where antigenic outer membrane protein genes are colocalized. These two genes are located in two separate loci in *E. canis*. This demonstrates that two independent conditions must be met to assemble the type IV machinery correctly. If type IV machinery is dysfunctional or assembled incorrectly, this may result in the failure to deliver effector molecules from *E. canis* into eukaryotic target cells. (Ohashi et. al., 2001) The type IV secretion system (TFSS) delivers virulence factors across the membranes of both the pathogen and the host cell and acts to ensure the survival of intracellular organisms, including *E. canis*. (Felek et. al., 2003) Therefore, by including this antigen, and the other four, a host immune response is intentionally selected that specifically acts to make intracellular survival difficult by decreasing the ability for *E. canis* to adhere and enter cells, preventing the correct assemblage of type IV machinery and creating a diverse response and recognition to variant antigens.

The subunit vector is essential in creating this diverse response and recognition to variant antigens. One of the stated advantages to recombinant subunit vaccines is that the mounted immune response is more likely to be based upon the pathogen's native conformation. Because the vaccine is attempting to make antigenic variation work favorably, this advantage actually presents a challenge to this. Matching one outer membrane protein pattern provided to the host through a vaccine with the pattern of any given *E. canis* strain that the host may encounter is highly unlikely. The vaccine must present the host immune system with various patterns in order to increase the likelihood of the vaccine-acquired immunity effectively combating many different antigenic *E. canis* strains. By increasing the range of variant antigens the host immune system can recognize, the speed of the adaptive immune response is boosted, particularly the CD8 T-cells, which are crucial to the elimination of *E. canis*. Therefore, *Escherichia coli* has been chosen as the subunit vector.

The genes encoding the five antigens will be isolated from the most highly conserved and prevalent strain of *E. canis* found in the canine host. These genes will be inserted into the genome of *E. coli* and allowed time for *E. coli* to provide the antigens from these genes. The antigens will be crudely purified and used as the major active serum component of the recombinant subunit vaccine. The number of generations per year is estimated as six times less in intracellular bacteria, such as *E. canis*, than in *E. coli*. Therefore, not much time is needed for *E. coli* to provide the antigens, as would be needed if an intracellular subunit vector was chosen instead. Ehrlichial proteins are able to be glycosylated by host cell machinery and *E. canis* likely has enzymes that mediate this protein glycosylation. *E. coli* can also perform glycosylation of recombinant forms of these proteins. Therefore, *E. coli* glycosylation machinery is able to act on *Ehrlichia* species and can be expected to create the same glycosylation patterns as *E. canis*. In addition, *Ehrlichia* spp. have a rate of 1% sequence divergence in 300 MY, while the estimated rate for *E. coli* is six times greater at 1% sequence divergence in 50 MY. This means that the need to encourage diversity through sequence divergence is fulfilled by *E. coli* and is in fact better equipped to present numerous antigenic patterns more efficiently than *E. canis*. (Mavromatis et. al., 2006) To preserve the native conformation of the original *E. canis* strain within the recombinant subunit vaccine is unbeneficial; the

presence of multiple conformations of the pathogen will better arm the vaccinated animal with the tools needed to battle antigenic variation.

A recombinant subunit vaccine was chosen instead of a recombinant vaccine that requires administering the whole *E. coli* organism to the vaccinated animal. Although *E. coli* has very low virulence and most animals constantly harbor the organism without ever becoming sick, using *E. coli* in the vaccine may be dangerous if the host immune response mounts an attack against the *E. canis* antigen-presenting *E. coli* organism. *E. coli* contains lipopolysaccharide (LPS), which the immune system recognizes and thus acts to produce cytokine THF-alpha, an inflammatory cytokine. THF-alpha circulating within the bloodstream can cause systemic inflammation, leading to shock, organ failure, and death. If the chance were taken to not purify the antigens, as a safety precaution, injectable epinephrine would have to be available if anaphylaxis should occur after vaccine administration.

In addition to the crudely purified serum containing the antigens, three effector adjuvants, Bay R1005, NAGO and CpG, will be added to the vaccine. Effector adjuvants enhance the immune response via biochemical pathways. Adjuvants work well with subunit vaccines to slow antigen release, which will aid in attaining a sustained immunity and will bind to receptors on dendritic cells and macrophages to produce inflammatory cytokines. Bay R1005 acts on B lymphocytes to increase their numbers, but it can signal the B lymphocytes to proliferate only after it binds to antigen, triggering the initial proliferation signal. BAY R1005 can also activate B-lymphocytes without active T-helper cells. NAGO enhances the interaction between antigen-presenting cells and T-cells, particularly the CD8 T-cell. (Los Alamos, 2005) CpG enhances the secretion of INF-gamma. By incorporating this adjuvant, INF-gamma is encouraged to act to up-regulate the MHC class II molecules that are deficient in *E. canis*-infected dogs. (Harrus et. al., 2003) Effective vaccines require that the vaccinated animal not become sick to the point where the immune system cannot adequately act to build memory. This adjuvant will add a small, yet significant, boost to the safety of this vaccine by assisting the natural defenses of the host to handle the potentially adverse effects of vaccine administration.

### **Description of Immunity Assessment**

The test subject population will include 21 dogs aged between 1 and 4 years, which initially test negative for anti-*Ehrlichia* antibody by ELISA. There will be three groups of seven dogs each; one will receive the actual recombinant subunit vaccine, one will be a negative control, receiving no vaccine and no live pathogen, and another will be a positive control, receiving an injection of the virulent, live pathogen. Dogs will be vaccinated on day 0, day 30 and day 60 and blood samples will be taken before immunization and one month after each vaccination dose, as dogs may not become seropositive until 28 days after vaccination/infection. (Hess et. al., 2006) The vaccine will be injected into the tibialis cranialis muscle of either hind leg. ((2)) Since recombinant subunit vaccines are not entirely efficient at inducing prolonged cytotoxic T-cell responses, administering only a few doses of the vaccine over a short period of time may prove to be less effective than presenting antigens over a sustained period of time. If the vaccine is deemed efficient through the development process, the dosage and timing of administration will have to be researched in an additional follow-up study.

An indirect immunofluorescence assay (IFA) with *E. canis* antigen as a substrate, is most often clinically used to screen for *E. canis* infections. Reports reveal that sera from canines infected with any *Ehrlichia* spp. can cross-react in immunoassays. Therefore, a positive seroreactivity result with an IFA indicates exposure to one of the most common Ehrlichial pathogens, and does not guarantee that *E. canis* is the infective agent. IFA results are reported in a quantitative fashion, unlike the commercially available ELISA test. The commercially available ELISA test detects *E. canis* antibody by using synthetic peptides from dominant epitomes. However, the synthetic peptide reagents meant to duplicate sequences in *E. canis* proteins are also expressed in strains of *E. chaffeensis*. The advantages of the commercially available ELISA are that it administers rapid results and is easy to use for in-clinic diagnosis. (O'Connor et. al., 2006)

Both of the two ELISA tests described above are inadequate to use, as they are currently available, to assess immunity in our vaccinated test subjects. While the major disadvantage to the IFA is the lack of specificity, the possibility of cross-reaction exists with virtually any ELISA test used to detect *Ehrlichia* spp. infections. This confusion can be avoided prior to vaccination by assessing the blood and confirming no seroreactivity of each of the 21 test subjects. If, after vaccine, there is seroreactivity, the assumption can be made that since purified *E. canis* antigens were used in the vaccine, the seroreactivity must be in reaction to *E. canis* and not another *Ehrlichia* spp. In addition, antigen p30 can be specifically tested for instead of just p28/30, which would distinguish *E. canis* from *E. chaffeensis*. The commercially available ELISA test uses synthetic peptides, which do not account for antigens that have diverged from the dominant epitome. ELISA tests that use cultured whole organisms as the substrate and that use recombinant major antigenic proteins have greater sensitivity of detecting *E. canis* - specific infections. (O'Connor et. al., 2006) The indirect IFA and sandwich ELISA used in this vaccine development, in combination, will test for specific antigens, use whole organisms and include recombinant major antigenic proteins.

The indirect ELISA test will be performed on the blood serum samples to test for GIG antibody and another sandwich ELISA will be performed to test for the vaccine antigens p28/30, gp36, gp140, gp200, and virB9. The vaccine antigens will be tested by coating 96 well ELISA plates with monoclonal antibodies specific to either the p28/30, gp36, gp140, gp200, or virB9 antigens. Five sandwich ELISAs will be conducted on the serum sample from each of the 21 test subjects. The microtiter plates will be coated with purified antibody to the antigen for 1 hour, the unbound antigen will be washed away and any open sites will be covered with milk protein to prevent any nonspecific binding to proteins. The vaccine antigen will be added to the plate to bind to antibody. After unbound antigen is removed from the plate, enzyme-labeled specific antibody will be added to a different epitope on the antigen (polyclonal rabbit serum from vaccinated rabbits) and the unbound antigen will be washed off again. Once the chromogenic substrate is added for the enzyme, the optical density can be calculated. The known antigen will be used as a positive control and no antigen, for the negative control. Based on OD values from the two controls, the test subject OD values will be statistically calculated for each of the five antigens separately and the titers grouped into three categories (thresholds yet to be determined): nonreactive titer, medium-titer and high-titer. (O'Connor et. al., 2006)

For the indirect ELISA test, the wells will be coated with the purified antigens (one at a time) and left to sit for 30 to 60 minutes. Like the sandwich ELISA, five tests for IgG specific to each of the antigens will be conducted on each of the 21 serum samples. The unbound antigen will be washed away and powdered milk will be added to cover any empty sites. After adding the serum sample, enough time will be allotted to allow the canine IgG to bind the antigen before washing. Anti-canine gamma chain that binds the Fc region of the canine IgG will be added. The unbound Ab-enzyme complexes will be removed and the chromogenic substrate added. For a negative control the antigen will be completely omitted and serum from a known infected dog will be used for a positive control. This ELISA test will determine whether or not there are IgG antibodies specific for p28/30, gp36, gp140, gp200 and virB9. One surveillance study in Bulgaria that used IFA for the detection of IgG marked serum samples at titers of 1:100 and higher as positive. Titers of 1:200, 1:400 and higher are indicative of a strong immune response to CME infection. (Tsachev, 2006) This same criterion will be employed for grouping titers. *E. canis* does not infect the mucosal system, therefore a mucosal response will not be stimulated through this vaccine and there will be no need to test for IgA. IgG has been chosen for testing because *E. canis* infects intracellular cells not located in the mucosa. IgG is an important antibody in protective immunity. IgG is responsible for opsonizing pathogens for engulfment by phagocytes, protecting against adhesions (such as gp36 and gp140), aid in neutralization and can activate the complement system. Although IgM is more efficient in the latter, a lot of IgG is required to be bound to the pathogen for activation. Therefore, if a substantial presence of IgG is detected, the assumption can be made that there is enough IgG to activate protective immunity, and thus, have successfully developed an effective vaccine.

An effective vaccine also requires that the host establish adequate memory to the pathogen. T-cells do not produce measurable antigen binding products, but they do go through two phases which can be measured: one in which they are activated and differentiated, and another, the effector phase, in which the function is expressed. In order to test for T-cell memory, flow cytometry with the fluorescence activated cell sorter will be used. An MHC peptide tetramer will be incorporated, which can be synthesized using recombinant MHC molecules containing one of our peptide epitopes (gp36, 140, 200, and virB9) with specific peptides bound to streptavidin using biotin. In order to make the tetramers, the bacterial enzyme BirA must be used. BirA recognizes specific amino acid sequences and the MHC : peptide complexes, which contain it, will be biotinylated to allow avidin binding. The complexes will be labeled with fluorochrome, so T-cells capable of binding can be detected. T-cells will be stained with antibodies specific for CD3 and CD8 and with a tetramer containing one of the epitopes used in the vaccine of the bacterium. Cells that are positive for both the CD8 staining and the specific tetramer staining will show up in the top right corner on the analysis graph.

In order to measure cytokine production an ELISPOT assay will be performed to measure the frequency of the T-cell response to our antigens. Specifically, the following cytokines will be tested for: INF-gamma, TNF-alpha, and TNF-beta. INF-gamma plays a major role in infection by intracellular pathogens through activating macrophages and recruiting them as effectors and recruiting antigen-presenting cells to the infection. This cytokine stimulates an increased expression of MHC class I and class II molecules, increasing the probability of infected cells being recognized as targets for cytotoxic

attack. IFN-gamma also decreases tryptophan in responsive cells, killing intracellular pathogens by “starving” them. TNF-alpha and TNF-beta work with IFN-gamma to activate macrophages and help to kill some target cells by interacting with TNFR-I. First cytokine-specific antibodies will bind to the surface of plastic wells and milk powder will be added to cover any remaining sites. Activated T-cells will be added and the cytokine secreted by some of the activated T-cells will be allowed to bind to the antibodies. This “captured” cytokine can be detected using a second cytokine specific antibody, which, coupled to an enzyme, causes a colored precipitate. T-cells that originally secreted cytokine will create a single spot of color for each cell. Plate incubation will be performed at 37 degrees Celsius for one hour and plates will be washed two times after incubation. The wells will be examined with phase contrast microscopy to determine the amount of colored spots produced as compared with the positive (purified cytokine) which would turn diffusely blue and the negative control (no cells), diffusely clear. ((3))

The dogs will be observed for 60 days (after all blood serum samples are taken) for symptoms of canine monocytic ehrlichiosis, including fever and weakness. Blood samples will be examined for signs of thrombocytopenia (low platelet count), anemia, and leukocytosis (elevated number of white blood cells). If clinical signs of CME occur in the test subjects, tetracycline and/or supportive therapy will be provided, if necessary. Any test subjects that undergo an adverse reaction to the vaccine, including pain in the area of injection, will also be treated. If any deaths occur, the bodies will be examined by necropsy for any signs of splenomegaly (indicative of CME) and the cause of death confirmed and documented. ((4))

This assessment of protective immunity follows Jenner’s original model consisting of three steps: an elicited immune response, challenging all dogs with the live bacteria *Ehrlichia canis*, and determining the severity of infection in vaccinated versus unvaccinated dogs. In the immunity assessment, an elicited immune response is confirmed from the correlated results of the tests described above. Because *E. canis* is highly antigenic, and five antigens were used, rather than just one or two, it is expected that through comparing test results for each of the test subjects, discrepancies will be found. Discovering a 100% perfectly elicited host immune response is not expected. Statistical analysis will be used to determine correlations between test results and to set up threshold values for negative, intermediate and positive elicited immune responses. (O’Connor et. al., 2006) While the positive control group is administered live *E. canis*, a follow-up study will also be conducted where all 21 vaccinated dogs are administered virulent *E. canis*. Based on clinical evaluation of signs and morbidity/mortality rates, the percentage of each of the three test subject groups that were able to clear *E. canis* infections, or ward off the pathogen before infection occurs, can be determined. It is expected that the negative control group will have the highest rates of morbidity/mortality and if the vaccine is effective, the group of dogs that were administered the recombinant subunit vaccine will have significantly lower rates than the negative control group.

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