Ehrlichia canis Genes and Vaccines

FIELD OF THE INVENTION

The invention pertains to the field of veterinary pathogens. More particularly, the present invention pertains to the sequence of specific genes of the bacterial canine pathogen *Ehrlichia canis* and the application of this technology to the development of a vaccine.

BACKGROUND OF THE INVENTION

The present invention relates to the sequence of genes from the *E. canis* bacterium, and the development of a vaccine against this organism.

Ehrlichia canis (*E. canis*) is a small gram-negative, obligately intracellular bacterium. This bacteria is the agent which causes canine monocytic ehrlichiosis (CME), a tick-borne disease which predominantly affects dogs. The most common carrier of *E. canis* is the brown dog tick *Rhipicephalus sanguineus.* The disease was described originally in Algeria in 1935. It was subsequently recognized in the United States in 1962, but is now known throughout much of the world. Canine monocytic ehrlichiosis caused much concern during the Vietnam War, when 160 military dogs died from the *E. canis* infection. There is no vaccination currently available against *E. canis*. It is a life threatening disease that continues to be an important health concern for veterinarians and pet owners alike.

Canine monocytic ehrlichiosis is an infectious blood disease. A reduction in cellular blood elements is the primary characteristic of the disease. *E. canis* lives and reproduces in the white blood cells (leukocytes). It eventually affects the entire lymphatic system, and devastates multiple organs. By targeting the white blood cells, these cells die

off rapidly. These dead blood cells migrate primarily to the spleen, which enlarges as a result. The bone marrow recognizes the loss of the white blood cells and works to form new, healthy cells. It sends out the cells prematurely, and these immature cells do not work properly. Often, these immature cells mimic those in leukemic patients, so the disease is misdiagnosed as leukemia. Canine monocytic ehrlichiosis may predispose dogs to various cancers.

There are three stages of canine monocytic ehrlichiosis. The first, acute stage mimics a mild viral infection. During the acute stage, most, if not all, of the damage is reversible and the animal is likely to recover. This is the stage where treatment is the most effective, stressing the need for early detection. Without treatment, however, the animal will progress into a subclinical (second) stage and/or to the chronic (final) stage. When the animal has reached the chronic stage, the bacterial organism has settled within the bone marrow. Many dogs in this stage suffer massive internal hemorrhage, or develop lethal complications such as sudden stroke, heart attack, renal failure, splenic rupture or liver failure.

E. canis can be cultured *in vitro* in a mammalian-derived cell line (DH82). Continued maintenance of these cells is difficult because the cell culture must be supplemented with primary monocytes (white blood cells found in bone marrow) every two weeks. The cultures are very slow growing, and the culture media is expensive.

Data concerning the genes in the *E. canis* genome has concentrated primarily on the 16S rRNA gene. Previous work has sequenced this gene, which is a ubiquitous component of the members of the ehrlichia family, as well as the majority of organisms worldwide. The high sequence homology between this gene throughout the living world makes it a poor candidate for vaccine development. It is necessary to find other genes within this genome if hope for a vaccine against this deadly disease can ever be realized.

Sequencing of the 16S rRNA gene indicates that *E. canis* is closely related (98.2% homology) to *E. chaffeensis*, the novel etiologic agent of human ehrlichiosis. Western blots of *E. canis* are similar when probed with antisera to *E. canis*, *E. chaffeensis* and *E.*

ewingi (another cause of human ehrlichiosis) indicating a close antigenic relationship between these three species (Chen *et al.*, 1994).

The indirect fluorescent antibody test (IFA) has been developed for detecting canine monocytic ehrlichiosis. IFA detects the presence of antibodies against the invading organism in a dog's blood. Unfortunately, this test is not always accurate. Sometimes, dogs will test negative in the acute phase because their immune system is delayed in forming antibodies. Another false negative may occur if there is a low titer in the chronic stage. An additional drawback of this test is the cross-reactivity found. The anti *E. canis* polyclonal antibody positively reacts with *E. chaffeensis*, undermining the specificity of the test. An alternative test, the Giesma smear, has been used to locate the actual organism in a dog's blood. Unfortunately, despite appropriate staining techniques and intensive film examination, the organisms frequently can not be located. The fallibility of these tests makes it essential to provide better diagnostic tools for this disease.

Due to difficulties in the detection of a tick bite, early diagnosis of infection, the suppression of host defenses and the nature of persistent infection of the disease, an effective vaccine against *E. canis* is urgently needed for dogs.

SUMMARY OF THE INVENTION

This invention discloses novel sequence data for *E. canis* genes. Specifically, a clone has been identified and sequenced. Four proteins termed ProA, ProB, ORF (an open reading frame with unknown function) and a cytochrome oxidase homolog, have been identified within this clone. In addition, a partial gene encoding a lipoprotein signal peptidase homolog has been discovered.

An embodiment of this invention includes the creation of a vaccine with this sequence and protein information. The proteins disclosed in this invention are extremely antigenic. Therefore, they have the potential to be extremely useful as a vaccine. The

types of vaccine made available by this novel technology include a DNA vaccine, a recombinant vaccine, and a T cell epitope vaccine.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the three clones identified in the library screen.

DESCRIPTION OF THE PREFERRED EMBODIMENT

E. canis causes a devastating canine disease. Currently, there is no vaccine available to prevent this disease. This invention provides the tools necessary to develop such a vaccine. More specifically, four genes have been identified from a genomic fragment of *E. canis*, named ProA, ProB, ORF and a cytochrome oxidase homolog. In addition, a partial gene coding for a lipoprotein signal peptidase homolog has been found. Any of these proteins can be utilized in an embodiment of this invention to develop a vaccine.

Screening an *E. canis* library

To identify genes in the *E. canis* genome, a genomic DNA expression library was constructed. An *E. canis* strain isolated from dogs with canine ehrlichiosis was grown in the dog cell line DH82 by a technique being known in the art, and incorporated by reference (Dawson *et al.*, 1991; Rikihisa, 1992). The cells were harvested and the chromosomal DNA extracted as described by a technique known in the art (Chang *et al.*, 1987; Chang *et al.*, 1989a; Chang *et al.*, 1989b; Chang *et al.*, 1993a; Chang *et al.*, 1993b). To construct the library, 200 μg of DNA was partially digested with *Sau*3A. DNA fragments from 3 to 8 kb were isolated and ligated to a plasmid, pHG165 (Stewart *et al.*, 1986). The plasmids were transformed into *E. coli* TB1 (Chang *et al.*, 1987).

The library was screened with polyclonal antibodies against *E. canis*. Polyclonal antibodies were generated from dogs that had been bitten by a tick harboring *E. canis*.

The polyclonal antibodies were preabsorbed with the lysate of an *E. coli* host strain. The library was plated on petri plates at a density of 1,000 colony forming units. Colonies were transferred to nitrocellulose and each filter was probed with 1 ml of the preabsorbed polyclonal antibodies. Positive colonies were identified with a second antibody consisting of an alkaline phosphatase-conjugated goat anti-rabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, MD), followed by color development with a substrate solution containing nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Positive clones were rescreened three times.

Three clones were isolated from this screening procedure (Figure 1). The longest genomic fragment (pCH4) encodes four complete genes and one partial gene. It completely encodes the proteins ProA, ProB, ORF and a cytochrome oxidase homolog, as well as containing the partial sequence of a lipoprotein signal peptidase homolog. ProA and ProB are located on a single operon. Restriction endonuclease digestion mapping and DNA sequencing were done by techniques known in the art, and incorporated by reference (Chang *et. al.*, 1987; Chang *et. al.*, 1989a; Chang *et. al.*, 1989b; Chang *et. al.*, 1993a; Chang *et. al.*, 1993b). Briefly, the DNA sequence was determined by automated DNA sequencing on the ABI PRISM Model 377 DNA system. The complete nucleotide sequences were determined on both strands by primer walking. The thermal cycling of the sequencing reactions utilized the Taq DyeDeoxy™ Terminator Cycle sequencing kit. Databases were searched for homologous proteins through the use of the BLAST network service of the National Center for Biotechnology Information (NCBI) (Althchul *et al.*, 1990; Gish *et al.*, 1993).

Sequence Information

The *E. canis* genes were sequenced. The cloned fragment contains 5,300 nucleotides, and codes for four proteins. There is also one partial gene at the carboxy terminus. SEQ. ID. NO. 1 is the entire nucleotide sequence. SEQ. ID. NO. 2 and 3 are the translation of nucleotides 12 through 533 from SEQ. ID. NO. 1 and code for a cytochrome oxidase homolog. Cytochrome oxidase is important in virulence, and therefore is a strong candidate for use in a vaccine. SEQ. ID. NO. 4 and 5 are the translation of nucleotides 939 through 2,252 from SEQ. ID. NO. 1 and code for ProA. SEQ. ID. NO. 6 and 7 are the

translation of nucleotides 2,258 through 3,664 from SEQ. ID. NO. 1 and code for ProB. Preliminary evidence indicates that ProA and ProB are proteases. SEQ. ID. NO. 8 and 9 are the translation of nucleotides 4,121 through 4,795 from SEQ. ID. NO. 1 and code for ORF, a protein with unknown function. SEQ. ID. NO. 10 and 11 are the translation of the complementary sequence of nucleotides 4,884 through 5,300 from SEQ. ID. NO. 1 and code for the partial sequence of a lipoprotein signal peptidase homolog. Lipoprotein signal peptidases are membrane proteins, and by nature may be less desirable for vaccine development. However, this protein is still worth pursuing in the creation of a vaccine.

Overexpression of ProA, ProB, ORF, cytochrome oxidase and the lipoprotein signal peptidase homolog

The *E. canis* antigens are overexpressed in a T7 promoter plasmid. The pRSET vector allows high level expression in *E. coli* in the presence of T7 RNA polymerase, which has a strong affinity for the T7 promoter. After subcloning the antigen genes into the pRSET vector, the subclones are transformed into an F' *E. coli* JM109 strain. For maximum protein expression, the transformants are cultured to O.D. 600=0.3, exposed to IPTG (1 mM) for one hour and then transfected with M13/T7 bacteriophages at a multiplicity of infection (MOI) of 5-10 plaque forming units (pfu) per cell. Time course studies indicate that maximum induction is reached two hours after induction.

The pellet is harvested by centrifugation and the cells are resuspended in 6M Guanidinium (pH 7.8). Cells are ruptured by French press and the total lysate is spun at 6000 rpm to separate cell debris by a technique known in the art, and hereby incorporated by reference (Chang *et al.*, 1993c). Immobilized metal ion affinity chromatography (IMIAC) is used to purify each of the proteins under denaturing conditions as described by the manufacturer (Invitrogen, San Diego, CA). The protein samples are separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized after staining with coomassie blue.

Vaccine Development

Prior to the present invention, no vaccine against *E. canis* had been developed. *E. canis* is endemic in dogs and closely related canidae in many parts of the world. Dogs in

North America are also increasingly at risk and the application of the present invention can potentially save the lives of thousands of dogs each year. An *E. canis* vaccine that can elicit cell-mediated immunity against this tick-borne disease of dogs is desperately needed.

DNA Vaccine

A DNA vaccine is constructed by subcloning the gene of interest into a eukaryotic plasmid vector. Candidate vectors include, but are not limited to, pcDNA3, pCI, VR1012, and VR1020. This construct is used as a vaccine.

Each of the newly identified genes, ProA, ProB, ORF, the cytochrome oxidase homolog, or the partial lipoprotein signal peptidase homolog can be used to create a DNA vaccine (reviewed in Robinson, 1997). In addition, any immunologically active portion of these proteins is a potential candidate for the vaccine. A plasmid containing one of these genes in an expression vector is constructed. The gene must be inserted in the correct orientation in order for the genes to be expressed under the control of eukaryotic promoters. Possible promoters include, but are not limited to, the cytomegalovirus (CMV) immediate early promoter, the human tissue plasminogen activator (t-PA) gene (characterized in Degen *et al.*, 1986), and the promoter/enhancer region of the human elongation factor alpha (EF-1 α) (characterized in Uetsuki *et al.*, 1989). Orientation is identified by restriction endonuclease digestion and DNA sequencing.

Expression of these gene products is confirmed by indirect immunofluorescent staining of transiently transfected COS cells. The same plasmid without these genes is used as a control. Plasmid DNA is transformed into *Escherichia coli* DH5α. DNA is purified by cesium chloride gradients and the concentration is determined by a standard protocol being known in the art, and incorporated by reference (Nyika *et al.*, 1998).

Once the vector is purified, the vector containing the DNA can be suspended in phosphate buffer saline solution and directly injected into dogs. Inoculation can be done via the muscle with a needle or intraveneously. Alternatively, a gene gun can be used to transport DNA-coated gold beads into cells by a technique known in the art, and hereby incorporated by reference (Fynan *et al.*, 1993). The rationale behind this type of vaccine

is that the inoculated host expresses the plasmid DNA in its cells, and produces a protein that raises an immune response. Each of the newly identified genes can be used to create a vaccine by this technique.

CpG molecules can be used as an adjuvant in the vaccine. This technique is known in the art, and is hereby incorporated by reference (Klinman *et al.*, 1997). Adjuvants are materials that help antigens or increase the immune response to an antigen. The motifs consist of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines. Oligonucleotides containing CpG motifs have been shown to activate the immune system, thereby boosting an antigen-specific immune response. This effect can be utilized in this invention by mixing the CpG oligonucleotides with the DNA vaccine, or physically linking the CpG motifs to the plasmid DNA.

Recombinant Vaccine

In order to develop a recombinant vaccine, each of the genes is individually subcloned into overexpression vectors, and then purified for vaccine development. ProA, ProB, ORF, the cytochrome oxidase homolog or the partial lipoprotein signal peptidase homolog is expressed in a plasmid with a strong promoter such as the tac, T5, or T7 promoter. Alternatively, immunologically active fragments of these proteins are used in the development of a vaccine. Each of these genes is subcloned into a plasmid and transformed into an *E. coli* strain as described above.

The recombinant protein is overexpressed using a vector with a strong promoter. Vectors for use in this technique include pREST (Invitrogen Inc., CA), pKK233-3 (Pharmacia, CA), and the pET system (Promega, WI), although any vector with a strong promoter can be used. After overexpression, the proteins are purified and mixed with adjuvant. Potential adjuvants include, but are not limited to, aluminum hydroxide, QuilA, or Montamide. The purified protein is used as immunogen to vaccinate dogs by a technique being known in the art, and incorporated by reference (Chang *et al.*, 1993c; Chang *et al.*, 1995). Briefly, the individual protein is expressed and purified from *E. coli.* Then, the dogs are injected intramuscularly or subcutaneously with the purified recombinant vaccine and adjuvant. This injection elicits an immune response.

T Cell Epitope Vaccine

Direct cell cytoxicity mediated by $CD8^+$ T lymphocytes (CTL) is the major mechanism of defense against intracellular pathogens. These effector lymphocytes eliminate infected cells by recognizing short peptides associated with MHC class I molecules on the cell surface. Exogenous antigens enter the endosomal pathway and are presented to $CD4^+$ T cells in association with class II molecules whereas endogenously synthesized antigens are presented to $CD8^+$ T cells in association with MHC class I molecules. *E. canis* is an intracellular pathogen that resides in monocytes and macrophages. The present invention develops novel ways of generating an *E. canis*-specific CTL response that would eliminate the organism from monocytes or macrophages of infected animals.

A strategy for increasing the protective response of a protein vaccine is to immunize with selective epitopes of the protein. The rationale behind this is that an epitope vaccine contains the most relevant immunogenic peptide components without the irrelevant portions. Therefore, a search is performed for the most highly antigenic portions of the newly identified proteins.

To identify T-cell epitopes from the newly discovered proteins, an initial electronic search for homologous sequences to known T-cell epitopes is performed. In addition, extensive T-cell epitope mapping is carried out. Each of the proteins, ProA, ProB, ORF, the cytochrome oxidase homolog, and the partial lipoprotein signal peptidase homolog, is tested for immunogenic peptide fragments. Mapping of T cell epitopes by a technique known in the art is hereby incorporated by reference (Launois *et al.*, 1994; Lee and Horwitz, 1999). Briefly, short, overlapping peptide sequences (9-20 amino acids) are synthesized over the entire length of the protein in question. These short peptide fragments are tested using healthy dogs which have been immunized with the protein of interest. Peripheral blood mononuclear cells from the dogs are tested for T cell stimulatory and IFN-γ inducing properties. Those fragments which elicit the strongest response are the best candidates for a T-cell epitope vaccine.

Once fragments are identified which will make the best epitopes, a recombinant adenylate cyclase of *Bordetella bronchiseptica* is constructed carrying an *E. canis* $CD8^+$ T cell epitope. The adenylate cyclase toxin (CyaA) of *Bordetella bronchiseptica* causes disease in dogs and elicits an immune response. In addition, CyaA is well suited for intracytoplasmic targeting. Its catalytic domain (AC), corresponding to the N-terminal 400 amino acid residues of the 1,706-residue-long protein, can be delivered to many eukaryotic cells, including cells of the immune system. Also, toxin internalization is independent of receptor-mediated endocytosis, suggesting that the catalytic domain can be delivered directly to the cytosol of target cells through the cytoplasmic membrane. The *Pseudomonas aeruginosa* exotoxin A (PE) is another toxin which could be used in this procedure to deliver peptides or proteins into cells, by a technique known in the art, and hereby incorporated by reference (Donnelly *et al.*, 1993).

Foreign peptides (16 residues) have been inserted into various sites of the AC domain of CyaA without altering its stability or catalytic and calmodulin-binding properties. Thus, protein engineering allows the design and delivery of antigens that specifically stimulate CTLs. The induction of specific $CD8^+$ T cells can play an important role in canine ehrlichiosis control due to the intracellular persistence of *E. canis* in monocytes.

The adenylate cyclase (AC) toxin (*cya)* gene of *B. bronchiseptica* has been cloned. A synthetic double-stranded oligonucleotide encoding a 9 to 20 amino acid class I T cell epitope of either ProA, ProB, ORF, the cytochrome oxidase homolog, or the partial lipoprotein signal peptidase homolog, is designed according to *B. bronchiseptica* codon usage. The complementary oligonucleotides are inserted in the hypervariable region of the cloned AC-coding sequence of the *cya*. This technique is known in the art in other systems, and is incorporated by reference (Sebo *et al.*, 1995; Guermonprez *et al.*, 1999).

Recombinant plasmids carrying the chimeric *cya* gene are sequenced to determine the copy number and orientation of the inserted epitope. A plasmid with a complete copy of the insert that specifies the T-cell epitope ($CD8^+$) in the correct orientation is chosen from the sequenced plasmids. The ability of the new chimeric protein to enter eukaryotic cells is necessary to ensure intracellular targeting of the epitopes (Fayolle *et al.*, 1996).

A vaccine can be created in one of two ways. Recombinant chimeric protein can be purified and used to inoculate dogs. Alternatively, an attenuated *B. bronchiseptica* strain that carries a T-cell epitope or *E. canis* gene by in-frame insertion into adenylate cyclase is created by allelic-exchange. Allelic-exchange is a technique known in the art, and is hereby incorporated by reference (Cotter and Miller, 1994).

Finally, protection against *E. canis* infection in dogs vaccinated with the adenylase cyclase- ProA, ProB, ORF, cytochrome oxidase homolog, or lipoprotein signal peptidase homolog chimeric protein is determined. Wild type and recombinant ACs and CyAs are diluted to working concentrations in PBS and the chimeric protein is injected into dogs either intramuscularly or subcutaneously. Alternatively, the T-cell epitope is inserted into the adenylate cyclase gene of an attenuated *B. bronchiseptica* strain in frame, and the dogs are given the live bacteria.

Recombinant antigens are promising candidates for human and animal vaccination against various pathogens. However, a serious drawback is the poor immunogenicity of recombinant antigens as compared to native antigens. A major challenge in the development of a new recombinant vaccine is, therefore, to have a new adjuvant system that increases the immunogenicity of antigens. Cytokines are powerful immunoregulatory molecules. Cytokines which could be used as adjuvants in this invention include, but are not limited to, IL-12 (interleukin-12), GM-CSF (granulocyte-macrophage colony stimulating factor), IL-1β (interleukin-1β) and γ-IFN (gamma interferon).

These cytokines can have negative side effects including pyrogenic and/or proinflammatory symptoms in the vaccinated host. Therefore, to avoid the side effects of a whole cytokine protein, an alternate approach is to use synthetic peptide fragments with the desired immunostimulatory properties. The nonapeptide sequence VQGEESNDK of IL-1β protein is endowed with powerful immuno-enhancing properties, and is discussed here to illustrate the use of a cytokine to increase immunogenicity.

This nonapeptide is inserted into the ProA, ProB, ORF, the cytochrome oxidase homolog, or the partial lipoprotein signal peptidase homolog protein and its immunogenicity is compared to that of the native protein. Reportedly, the insertion of this

sequence into a poorly immunogenic recombinant antigen increases the chance of a strong protective immune response after vaccination. This peptide could enhance the *in vivo* immune response against both T-dependent and T-independent antigens. The canine IL-1β sequence may mimic many immunomodulatory activities of the entire molecule of IL-1β while apparently lacking many of its undesirable proinflammatory properties. This strategy is employed to increase the immunogenicity of ProA, ProB, ORF, cytochrome oxidase, the partial lipoprotein signal peptidase homolog and other *E. canis* antigens.

Plasmid pYFC199 is derived from a pBR322 plasmid by the insertion of a fragment that includes the ProA, ProB, ORF, the cytochrome oxidase homolog, or the partial lipoprotein signal peptidase protein from *E. canis*. This plasmid contains a unique *Hind*III site where in-frame insertions encoding exogenous sequences can be inserted. Two complementary oligonucleotides, AGGCTTGTTCAGGGTGAAGAAGAATCCAACGACAAAAGCTT and AAGCTTTTGTCGTTGGATTCTTCACCCTGAACTTGCCA, that encode the canine IL-1β 163-171 peptide are annealed, cut with *Hind*III, and inserted into the pYFC199 *Hind*III site. The recombinant plasmid carrying the chimeric IL-1β gene is sequenced to determine the orientation of the inserted epitope.

The efficacy of the recombinant proteins as vaccines is tested in dogs. The purified protein is injected intraperitoneally into dogs. Specific pathogen free (SPF) dogs are divided into five groups: one group is given recombinant adenylate cyclase of *Bordetella bronchiseptica* carrying *E. canis* $CD8^+$ T cell epitopes derived from ProA, ProB, ORF, cytochrome oxidase homolog, or the partial lipoprotein signal peptidase homolog, one group is given recombinant adenylate cyclase of *Bordetella bronchiseptica* as a control, one group is given the ProA, ProB, ORF, cytochrome oxidase homolog, or the partial lipoprotein signal peptidase homolog protein plus a canine IL-1β 163-171 insert, one group is given a T cell epitope derived from ProA, ProB, ORF, cytochrome oxidase homolog, or the partial lipoprotein signal peptidase homolog alone, and the last group is given PBS as a negative control.

All animals are vaccinated (30-40 μg each) four times. The dogs are challenged ten days after the last vaccination with 10^7 *E. canis*. At day five postchallenge, approximately 1 ml blood from each dog is collected in an EDTA tube. Whether the vaccinated groups eliminate the organisms as compared to that of the control group is tested by culture and PCR. Two primers derived from the genes cloned can be used to amplify the gene product from the tissues or blood samples from these dogs. The internal primer can also be designed for use as an oligonucleotide probe to hybridize the PCR gene product.

This invention provides a badly needed vaccine against the *E. canis* bacterium. The vaccine can be used to protect dogs throughout the world from canine monocytic ehrlichiosis.

Accordingly, it is to be understood that the embodiments of the invention herein described are merely illustrative of the application of the principles of the invention. Reference herein to details of the illustrated embodiments are not intended to limit the scope of the claims, which themselves recite those features regarded as essential to the invention.

What is claimed is:

1. A recombinant DNA comprising said DNA selected from the group consisting of:

a) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

b) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

c) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

d) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

e) a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

f) any portion of said DNA above that encodes a protein that elicits an immune response against *E. canis*.

2. The recombinant DNA of claim 1 wherein said DNA encodes at least one immunogenic epitope.

3. A recombinant protein comprising said protein selected from the group consisting of:

a) a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

b) a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

c) a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

d) a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

e) a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

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f) any portion of any of the above proteins that elicits an immune response against *E. canis*.

4. The recombinant protein of claim 3 wherein said protein includes at least one immunogenic epitope.

5. A vaccine wherein said vaccine protects dogs against *E. canis* infection.

6. The vaccine of claim 5 comprising:

a) a vector capable of expressing a recombinant DNA inserted into said vector such that a recombinant protein is expressed when said vector is provided in an appropriate host; and

b) the recombinant DNA inserted into said vector wherein said DNA is selected from the group consisting of:

i. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

ii. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

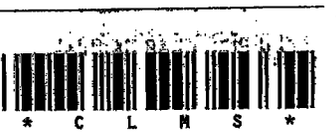
iii. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

v. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

vi. any portion of said DNA above that encodes a protein that elicits an immune response against *E. canis*.

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7. The vaccine of claim 6, wherein said DNA further comprises DNA that encodes CpG motifs.

8. The vaccine of claim 6 wherein said DNA further comprises a promoter selected from the group consisting of:

a) a cytomegalovirus (CMV) immediate early promoter;

b) a human tissue plasminogen activator gene (t-PA); and

c) a promoter/enhancer region of a human elongation factor alpha (EF-1 α).

9. The vaccine of claim 6, wherein said vector is selected from the group consisting of:

a) pcDNA3;

b) pC1;

c) VR1012; and

d) VR1020.

10. The vaccine of claim 6 wherein said vaccine is administered into said host by a method selected from the group consisting of:

a) intramuscular injection;

b) intraveneous injection; and

c) gene gun injection.

11. The vaccine of claim 10, wherein said host is a dog.

12. The vaccine of claim 5 comprising:

a) a recombinant protein that is selected from the group consisting of:

i. a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

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ii. a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

iii. a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv. a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

v. a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

vi. any portion of any of the above proteins that elicits an immune response against *E. canis*.

13. The vaccine of claim 12, wherein said vaccine further comprises adjuvants selected from the group consisting of:

a) aluminum hydroxide;

b) QuilA; and

c) Montamide.

14. The vaccine of claim 12 further comprising a cytokine operatively associated with said recombinant protein.

15. The vaccine of claim 14 wherein said cytokine is selected from the group consisting of:

a) interleukin-1β (IL-1β);

b) granulocyte-macrophage colony stimulating factor (GM-CSF);

c) gamma interferon (γ-IFN);

d) amino acids VQGEESNDK from the IL-Iβ protein; and

e) any portion of any of the cytokines above that elicits an improved immunogenic response against *E. canis*.

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16. The vaccine of claim 12 wherein said vaccine is administered into a host by a method selected from the group consisting of:

a) intramuscular injection; and

b) subcutaneous injection.

17. The vaccine of claim 16 wherein said host is a dog.

18. The vaccine of claim 5 comprising a recombinant protein that includes a T cell epitope wherein said T cell epitope comprises an amino acid peptide fragment of a protein selected from the group consisting of:

a) a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

b) a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

c) a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

d) a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

e) a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

f) any portion of any of the above proteins that elicits an immune response against *E. canis*.

19. The vaccine of claim 18 wherein said amino acid peptide fragment comprises nine to twenty amino acids.

20. The vaccine of claim 18 further comprising a recombinant DNA encoding a protein which is capable of being internalized into eukaryotic cells, including cells of the immune system.

21. The vaccine of claim 20 wherein said protein capable of being internalized into eukaryotic cells comprises a toxin selected from the group consisting of:

a) a recombinant adenylate cyclase of *Bordetella bronchiseptica*; and

b) a recombinant exotoxin A (PE) of *Pseudomonas aeruginosa.*

22. The vaccine of claim 18 wherein said vaccine is administered into a host by a method selected from the group consisting of:

a) intramuscular injection; and

b) subcutaneous injection.

23. The vaccine of claim 22 wherein said host is a dog.

24. A method of identifying a T cell epitope against *E. canis* comprising:

a) synthesizing overlapping peptide fragments over an entire length of a protein wherein said protein is selected from the group consisting of:

i. a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

ii. a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

iii. a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv. a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

v. a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

vi. any portion of any of the proteins above that elicits an immune response *against E. canis*;

b) testing said peptide fragment to determine if said peptide fragment elicits an immune response in a host animal; and

c) identifying said peptide fragment as said T cell epitope of *E. canis* if said fragment elicits an immune response.

25. The method of claim 24 wherein said peptide fragment comprises nine to twenty amino acids.

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26. A method of creating a vaccine against *E. canis* comprising:

a) selecting a vector capable of expressing a recombinant DNA inserted into said vector; and

b) inserting a recombinant DNA into said vector such that a recombinant protein is expressed when said vector is provided in an appropriate host wherein said DNA is selected from the group consisting of:

i. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

ii. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

iii. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

v. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

vi. any portion of said DNA above that encodes a protein that elicits an immune response against *E. canis*.

27. The method of claim 26, wherein said DNA further comprises DNA that encodes CpG motifs.

28. The method of claim 26 wherein said DNA further comprises a promoter selected from the group consisting of:

a) a cytomegalovirus (CMV) immediate early promoter;

b) a human tissue plasminogen activator gene (t-PA); and

c) a promoter/enhancer region of a human elongation factor alpha (EF-1 α).

29. The method of claim 26, wherein said vector is selected from the group consisting of:

a) pcDNA3;

b) pC1;

c) VR1012; and

d) VR1020.

30. The method of claim 26 wherein said vaccine is injected into said host in a manner selected from the group consisting of:

a) intramuscular injection;

b) intraveneous injection; and

c) gene gun injection.

31. The method of claim 30, wherein said host is a dog.

32. A method of creating a vaccine against *E. canis* comprising:

a) selecting a vector capable of expressing a recombinant protein inserted into said vector;

b) insertion of a recombinant DNA into said vector such that said recombinant protein is expressed when said vector is transformed into a bacterial strain wherein said DNA is selected from the group consisting of:

i. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

ii. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

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iii. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

v. a recombinant DNA that encodes a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

vi. any portion of said DNA above that encodes a protein that elicits an immune response against *E. canis*; and

c) harvesting said recombinant protein from said bacterial strain.

33. The method of claim 32, wherein said vaccine further comprises adjuvants selected from the group consisting of:

a) aluminum hydroxide;

b) QuilA; and

c) Montamide.

34. The method of claim 32, wherein said vaccine further comprises a promoter selected from the group consisting of:

a) tac;

b) T5; and

c) T7.

35. The method of claim 32, wherein said bacterial strain is *E. coli*.

36. The method of claim 32, wherein said vector is selected from the group consisting of:

a) pREST;

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b) pET; and

c) pKK233-3.

37. The method of claim 32 wherein said vaccine further comprises a cytokine operatively associated with said vaccine.

38. The method of claim 37 wherein said cytokine is selected from the group consisting of:

a) interleukin-1β (IL-1β);

b) granulocyte-macrophage colony stimulating factor (GM-CSF);

c) gamma interferon (γ-IFN);

d) amino acids VQGEESNDK from the IL-Iβ protein; and

e) any portion of any of the cytokines above that elicits an improved immunogenic response against *E. canis*.

39. The method of claim 32 wherein said vaccine is injected into said host in a manner selected from the group consisting of:

a) intramuscular injection; and

b) subcutaneous injection.

40. The method of claim 39 wherein said host is a dog.

41. A method of creating a T cell epitope vaccine comprising:

a) selecting a recombinant protein that includes a T cell epitope wherein said T cell epitope comprises an amino acid peptide fragment of a protein selected from the group consisting of:

i. a protein having an amino acid sequence as shown in SEQ. ID. NO. 3;

ii. a protein having an amino acid sequence as shown in SEQ. ID. NO. 5;

iii. a protein having an amino acid sequence as shown in SEQ. ID. NO. 7;

iv. a protein having an amino acid sequence as shown in SEQ. ID. NO. 9;

v. a protein having an amino acid sequence as shown in SEQ. ID. NO. 11; and

vi. any portion of any of the above proteins that elicits an immune response against *E. canis*;

b) identifying said T cell epitope from said protein;

c) incorporating said T cell epitope into a construct capable of expressing said epitope as a protein; and

d) harvesting said protein.

42. The method of claim 41 wherein said amino acid peptide fragment comprises nine to twenty amino acids.

43. The method of claim 41 wherein said construct capable of expressing said epitope further comprises a recombinant DNA encoding a protein which is capable of being internalized into eukaryotic cells, including cells of the immune system.

44. The method of claim 43 wherein said protein capable of being internalized into eukaryotic cells comprises a toxin selected from the group consisting of:

a) a recombinant adenylate cyclase of *Bordetella bronchiseptica*; and

b) a recombinant exotoxin A (PE) of *Pseudomonas aeruginosa*.

45. The method of claim 41 wherein said vaccine is injected into said host in a manner selected from the group consisting of:

a) intramuscular injection; and

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4 b) subcutaneous injection.

1 46. The method of claim 45 wherein said host is a dog.