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Compositions and methods of enhancing immune responses to Eimeria

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(54) **COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO EIMERIA**

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(58) **Field of Classification Search**

None

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(57) **ABSTRACT**

Vaccines comprising TRAP polypeptides and *Salmonella enteritidis* vectors comprising TRAP polypeptides are provided. The vaccines may also include a CD154 polypeptide capable of binding to CD40. Also provided are methods of enhancing an immune response against Apicomplexan parasites and methods of reducing morbidity associated with infection with Apicomplexan parasites.

18 Claims, 3 Drawing Sheets

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FIGURE 1

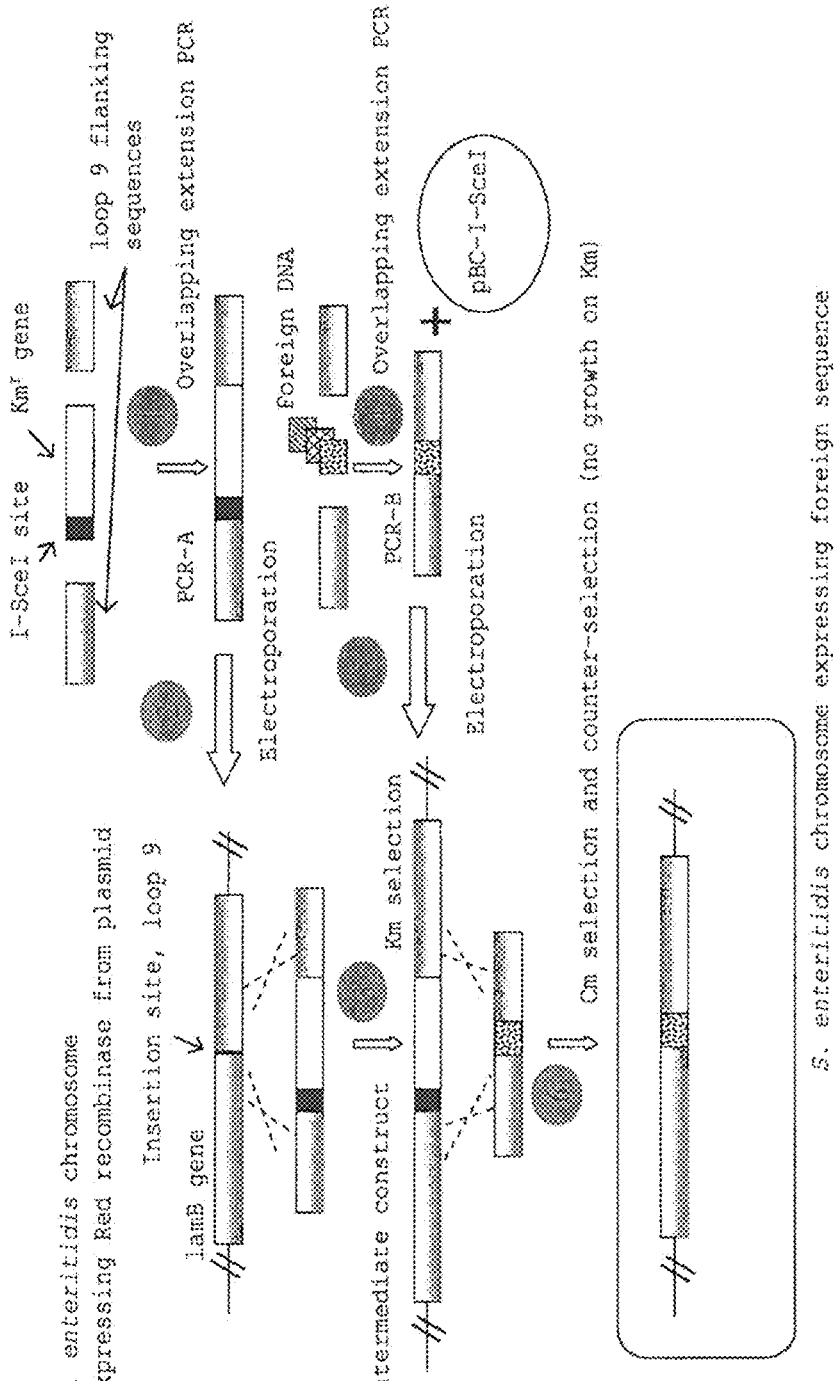
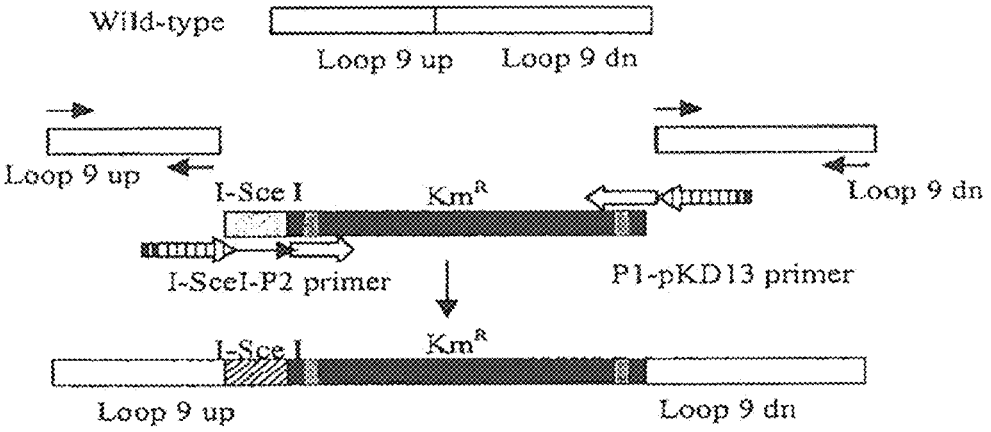
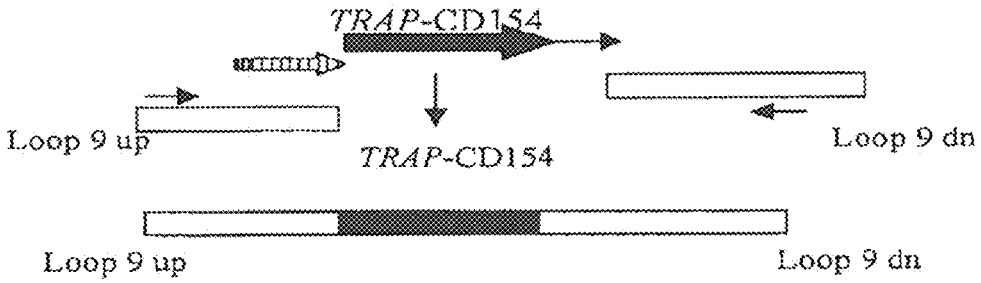

S. enteritidis chromosome
expressing Red recombinase from plasmid
Insertion site, loop 9
lamB gene
I-SceI site
Km^r gene
loop 9 flanking sequences
Overlapping extension PCR
PCR-A
Electroporation
foreign DNA
Overlapping extension PCR
PCR-B
pBC-I-SceI
Electroporation
Km selection
Intermediate construct
Cm selection and counter-selection (no growth on Km)
S. enteritidis chromosome expressing foreign sequence

FIGURE 2



PCR-A

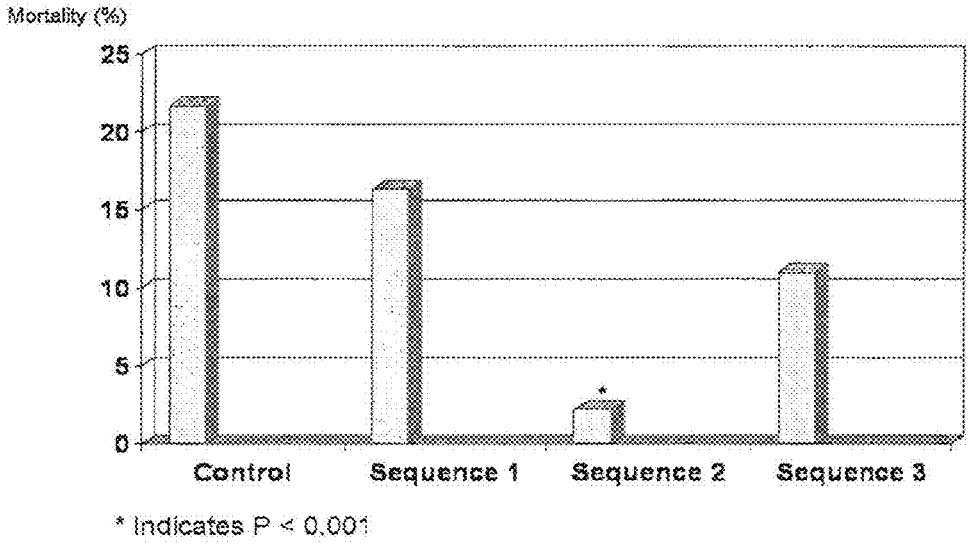
FIG. 2A



PCR-B

FIG. 2B

FIGURE 3


Mortality (%)
25
20
15
10
5
0
*
Control
Sequence 1
Sequence 2
Sequence 3
* Indicates P < 0.001

COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO EIMERIA

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a Continuation of U.S. patent application Ser. No. 12/740,608, filed Dec. 28, 2010 and issuing as U.S. Pat. No. 8,956,849 on Feb. 17, 2015, which application is a national stage filing under 35 U.S.C. 371 of International Application No. PCT/US2008/082254, filed Nov. 3, 2008, which claims the benefit of priority of U.S. Provisional Application Ser. No. 60/984,612, filed Nov. 1, 2007, all of which are incorporated herein by reference in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

None.

INTRODUCTION

Coccidiosis, an infectious disease of poultry, swine, and cattle caused by the Apicomplexan protozoal parasite *Eimeria*, presents problems worldwide. Coccidiosis is among the top ten infectious diseases of poultry in terms of its economic impact on the poultry industry. Other members of the Apicomplexan family also cause disease, including *Plasmodium, Cryptosporidium* and *Toxoplasma* which are the causative agents of malaria, cryptosporidiosis and toxoplasmosis, respectively. The vaccines currently available against *Eimeria* are based on controlled low dosage of essentially fully virulent but drug-sensitive *Eimeria* parasites. Vaccination with current *Eimeria*-based vaccines produces substantial vaccine-reaction morbidity and economic losses in vaccinated flocks. Thus an effective low-virulence vaccine against *Eimeria* is needed. An effective vaccine for *Eimeria* may also prove useful as a vaccine against other Apicomplexan parasites.

SUMMARY

A vaccine comprising a first polynucleotide sequence encoding a TRAP polypeptide or an immunogenic fragment thereof is disclosed. The TRAP polypeptide may comprise comprises SEQ ID NO:1, SEQ ID NO:2, or SEQ ID NO:3, or an immunogenic fragment thereof. The vaccines optionally further include a second polynucleotide sequence encoding a CD154 polypeptide capable of binding CD40. The CD154 polypeptides include fewer than 50 amino acids and comprise amino acids 140-149, or a homolog thereof.

Vaccines according to the present invention may be comprised within a vector, such as a virus, bacterium, or liposome. In one aspect, a vaccine comprising a *Salmonella enteritidis* comprising a first polynucleotide sequence encoding a TRAP polypeptide is provided.

In still another aspect, the invention includes methods of enhancing the immune response against an Apicomplexan parasite in a subject by administering a vaccine according to the present invention.

In a still further aspect, the invention includes methods of reducing morbidity associated with infection with an Apicomplexan parasite in a subject by administering a vaccine according to the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** depicts the scheme for making site-directed mutations in *Salmonella enteritidis*.

FIG. **2** depicts the design scheme of the overlapping extension PCR method used to generate the TRAP and TRAP-CD154 insertions into loop 9 of the lamB polynucleotide. FIG. **2**A shows the construction of the PCR-A construct using the overlapping PCR techniques described in the Examples. FIG. **2**B shows the construction of the PCR-B construct as described in the Examples.

FIG. **3** is a bar graph showing the percent mortality at five days post-infection with *Eimeria maxima* after inoculation with a *Salmonella* vector expressing the indicated *Eimeria* TRAP sequence.

DETAILED DESCRIPTION

Recombinant DNA technologies enable relatively easy manipulation of many bacterial and viral species. Some bacteria and viruses are mildly pathogenic or non-pathogenic, but are capable of generating a robust immune response. These bacteria and viruses make attractive vaccines for eliciting an immune response to antigens. Bacterial or viral vaccines may mimic a natural infection and produce robust and long lasting mucosal immunity. Vaccines are often relatively inexpensive to produce and administer. In addition, such vectors can often carry more than one antigen and may provide protection against multiple infectious agents.

In one aspect, a vaccine comprising a first polynucleotide sequence encoding a TRAP polypeptide or an immunogenic fragment thereof is provided. The TRAP polypeptide may comprise SEQ ID NO:11 or an immunogenic fragment of SEQ ID NO:11. A vaccine includes any composition comprising a polynucleotide encoding an antigenic polypeptide that is capable of eliciting an immune response to the polypeptide. In another aspect, the use of vectors, such as bacterial vectors, for vaccination and generation of immune responses against *Eimeria* or other Apicomplexan parasites such, as *Plasmodium* (the causative agent of malaria), *Toxoplasma*, and *Cryptosporidium* is disclosed. *Salmonella* strains make suitable vectors because bacterial genes may be mutated or attenuated to create bacteria with low to no pathogenesis to the infected or immunized subject, while maintaining immunogenicity.

A high molecular mass, asexual stage antigen from *Eimeria maxima* (EmTFP250) was demonstrated to be a target for maternal antibodies produced by breeding hens infected with this protozoan parasite. Analysis of the amino acid sequence of the antigen revealed a novel member of the TRAP (thrombospondin-related anonymous protein) family, containing 16 thrombospondin type-1 repeats and 31 epidermal growth factor-like calcium binding domains. EmTFP250 or TRAP also contains two low complex, hydrophilic regions rich in glutamic acid and glycine residues, and a transmembrane domain/cytosolic tail associated with parasite gliding motility that is highly conserved within apicomplexan microneme proteins. Several potential epitopes were selected and are identified in SEQ ID NO:1-3 and 11. Due to the conserved nature of this antigen, expression of these epitopes by a vector may induce protective immunity against multiple Apicomplexan parasites.

Salmonella may provide a useful vector because it can survive the gastrointestinal tract of the host and give rise to a mucosal immune response. Oral vaccines using a *Salmonella* vector produce a robust mucosal immune response and

are relatively easy to administer to both animals and humans. However, many of the current *Salmonella* vaccine strains are not as effective in generating a strong protective immune response as compared to their more virulent counterparts. Virulent strains provide a robust immune response but may also cause significant morbidity to the vaccinated subject. A *Salmonella* strain that could be used for effective mucosal, e.g., oral, vaccination would provide a vector that could be used to readily vaccinate a subject against one or more pathogenic agents, such as Apicomplexan parasites.

A *Salmonella enteritidis* strain useful as a vector, and various recombinant vectors made using this strain, are described. Specifically, a *Salmonella enteritidis* 13A (SE13A) capable of expressing an exogenous TRAP polypeptide is provided. In addition, a vaccine and methods of enhancing an immune response in a subject by administering the vaccine comprising a TRAP polynucleotide sequence encoding a TRAP polypeptide and a CD154 polynucleotide sequence encoding a polypeptide of CD154 or a homolog thereof that is capable of binding to CD40 are disclosed. The vaccines may be used to enhance an immune response against *Eimeria* or another Apicomplexan parasite, such as *Plasmodium, Toxoplasma* or *Cryptosporidium*, or may be used to reduce the morbidity associated with art infection caused by an Apicomplexan parasite.

A wild-type isolate of *Salmonella, Salmonella enteritidis* 13A (SE13A) (deposited with the American Type Culture Collection (ATCC) on Sep. 13, 2006, deposit number PTA-7871), was selected based upon its unusual ability to cause mucosal colonization and sub-mucosal translocation in chickens, permitting robust presentation of associated antigens or epitopes in commercial chickens. Importantly, this wild-type *Salmonella* isolate causes no clinically detectable disease or loss of performance in commercial chickens, indicating little disease-causing potential of the wild-type *Salmonella* in vertebrate animals.

The SE13A isolate may be further attenuated by inactivating at least one gene necessary for sustained replication of the bacteria outside of laboratory or manufacturing conditions. Attenuated or variant *Salmonella* strains that can be used as vectors are described below. SE13A was used to generate attenuated *Salmonella* strains to develop vaccines and generate enhanced immune responses, SE13A is invasive, non-pathogenic for poultry and causes no measurable morbidity. These features result in an enhanced immune response as compared to non-invasive bacterial vectors. Attenuation of SE13A by mutation of genes that limit the ability of the bacterium to spread may increase the safety of the vaccine. SE13A strains with mutations in aroA or htrA retain the ability to generate an immune response, but have limited replication in the host. Thus, the attenuation increases the safety of the vector without compromising the immunogenicity.

Mutations may be made in a variety of other *Salmonella* genes including, but not limited to, cya, crp, asd, cdt, phoP, phoQ, ompR, outer membrane proteins, dam, htrA or other stress related genes, aro, pur and gua. As shown in the Examples, mutations in aroA and htrA were found to attenuate SE13A. The aro genes are enzymes involved in the shikimate biosynthesis pathway or the aromatase pathway and aro mutants are auxotrophic for the aromatic amino acids tryptophan, tyrosine and phenylalanine. htrA is a stress response gene that encodes a periplasmic protease that degrades aberrant proteins. Mutants in htrA are also attenuated and display increased sensitivity to hydrogen peroxide.

The mutations in aroA and htrA described in the Examples are deletion mutations, but the mutations can be made in a variety of ways. Suitably, the mutations are non-reverting mutations that cannot be repaired in a single step. Suitable mutations include deletions, inversions, insertions and substitutions. A vector may include more than one mutation, for example a vector may contain mutations in both aroA and htrA. Methods of making such mutations are well known in the art.

Polynucleotides encoding TRAP polypeptide antigens and other antigens from any number of pathogenic organisms may be inserted into the vector (e.g., SE13A) and expressed by the bacteria. The expression of these polynucleotides by the vector will allow generation of antigenic polypeptides following immunization of the subject. The polynucleotides may be inserted into the chromosome of the bacteria or encoded on plasmids or other extrachromosomal DNA. Those of skill in the art will appreciate that numerous methodologies exist for obtaining expression of polynucleotides in vectors such as *Salmonella*. The polynucleotides may be operably connected to a promoter (e.g., a constitutive promoter, an inducible promoter, etc.) by methods known to those of skill in the art. Suitably, polynucleotides encoding TRAP antigens are inserted into a bacterial polynucleotide that is expressed. Suitably, the bacterial polynucleotide encodes a transmembrane protein, and the polynucleotide encoding the TRAP antigen is inserted into the bacterial polynucleotide sequence to allow expression of the TRAP antigen on the surface of the bacteria. For example, the polynucleotide encoding TRAP may be inserted in frame into the bacterial polynucleotide in a region encoding an external loop region of a transmembrane protein such that the bacterial polynucleotide sequence remains in frame. See Example 1.

Alternatively, the first polynucleotide encoding TRAP antigen may be inserted, into a polynucleotide encoding a secreted polypeptide. Those of skill in the art will appreciate that the polynucleotide encoding the TRAP antigen could be inserted in a wide variety of bacterial polynucleotides to provide expression and presentation of the TRAP antigen to the immune cells of a subject treated with the vaccine. In the Examples, a first polynucleotide encoding the TRAP polypeptide was inserted into loop 9 of the lamB gene of SE13A. The polynucleotide encoding the TRAP antigen may be included in a single copy or more than one copy. A bacterial vector containing multiple copies of the TRAP antigen inserted into loop 9 of lamB may also be generated. Alternatively, multiple copies of an epitope may be inserted into the bacterial vector at more than one location.

Suitably the first polynucleotide encodes a portion of the TRAP polypeptide or the entire TRAP polypeptide. The polynucleotide may be inserted into the vector. In the Examples, three polypeptides (SEQ ID NO: 1-3) were incorporated into SE13A. Suitably, the portion of the TRAP polypeptide inserted into the vector is an immunogenic fragment. An immunogenic fragment is a peptide or polypeptide capable of eliciting a cellular or humoral immune response. Suitably, an immunogenic fragment of TRAP may be 6 or more consecutive amino acids, 10 or more amino acids, 15 or more amino acids or 20 or more amino acids of the full-length protein sequence.

Other suitable epitopes for inclusion in a vaccine having TRAP comprised within a vector include, but are not limited to, polynucleotides encoding other *Eimeria*-related polypeptides. One of skill in the art will appreciate that a variety of sequences may be used in combination, with any other antigen and may also be used in conjunction with polypeptides encoding immune stimulatory peptides such as a polypeptide of CD154.

As described in more detail below, a vaccine including a vector may include a CD154 polypeptide that is capable of binding CD40 in the subject and stimulating the subject to respond to the vector and its associated antigen. Involvement of dendritic cells (DCs) is essential for the initiation of a powerful immune response as they possess the unique ability to activate naïve T cells, causing T cell expansion, and differentiation into effector cells. It is the role of the DC, which is an antigen presenting cell (APC) found in virtually all tissues of the body, to capture antigens, transport them to associated lymphoid tissue, and then present them to naïve T cells. Upon activation by DCs, T cells expand, differentiate into effector cells, leave the secondary immune organs, and enter peripheral tissues. Activated cytotoxic T cells (CTLs) are able to destroy virus-infected cells, tumor cells or even APCs infected with intracellular parasites (e.g., *Salmonella*) and have been shown to be critical in the protection against viral infection. CD40 is a member of the TNF-receptor family of molecules and is expressed on a variety of cell types, including professional antigen-presenting cells (APCs), such as DCs and B cells. Interaction of CD40 with its ligand CD154 is extremely important and stimulatory for both humoral and cellular immunity. Stimulation of DCs via CD40, expressed on the surface of DCs, can be simulated by anti-CD40 antibodies. In the body, however, this occurs by interaction with the natural ligand for CD40 (i.e. CD154) expressed on the surface of activated T-cells. Interestingly, the CD40-binding regions of CD154 have been identified. The CD40-binding region of CD154 may be expressed on the surface of a vector, such as a *Salmonella* vector, and results in an enhanced immune response against a co-presented peptide sequence.

As described above, polynucleotides encoding CD154 polypeptides may be inserted into the chromosome of the vector or maintained extrachromosomally. A CD154 polypeptide may be a portion of CD154 full-length protein or the entire CD154 protein. Suitably, the CD154 polypeptide is capable of binding CD40. One of skill in the art will appreciate that these polynucleotides can be inserted in frame in a variety of polynucleotides and expressed in different parts of the vector or may be secreted. The polynucleotide encoding a CD154 polypeptide capable of enhancing the immune response to TRAP may also encode the TRAP antigen. The polynucleotide encoding a CD154 polypeptide may be linked to the polynucleotide encoding the TRAP antigen, such that in the vector, the CD154 polypeptide and the TRAP antigen are present on the same polypeptide. In the Examples, a polynucleotide encoding a polypeptide of CD154 that is capable of binding to CD40 also encodes the TRAP antigen. See SEQ ID NOS: 1, 2, 3 and 11 in the attached sequence listing. In the Examples, the polynucleotides (SEQ ID NO: 13-15) encoding the TRAP antigen and the polynucleotide encoding the CD154 polypeptide are both inserted in loop 9 of the lamB gene. Those of skill in the art will appreciate that bacterial polynucleotides encoding other transmembrane proteins and other loops of the lamB gene may also be used.

As discussed above, a CD154 polynucleotide encoding a CD154 polypeptide that is capable of enhancing the immune response to the antigen may be included in the vaccine. Suitably, the CD154 polypeptide is fewer than 50 amino acids long, more suitably fewer than 40, fewer than 30 or fewer than 20 amino acids in length. The polypeptide may be between 10 and 15 amino acids, between 10 and 20 amino acids or between 10 and 25 amino acids in length. The CD154 sequence and CD40 binding region are not highly conserved among the various species. The CD154 sequences of chicken and human are provided in SEQ ID NO:10 and SEQ ID NO:4, respectively.

The CD40 binding regions of CD154 have been determined for a number of species, including human, chicken, duck, mouse and cattle and are shown in SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:7, SEQ ID NO:8, and SEQ ID NO:9, respectively. Although there is variability in the sequences in the CD40 binding region between species, the human CD154 polypeptide was able to enhance the immune response in chickens. Therefore, one may practice the invention using species specific CD154 polypeptides or a heterologous CD154 polypeptide.

In the Examples, several SE13A recombinant bacteria were generated. In each of the SE13A strains containing both the TRAP and CD154 polynucleotides, the TRAP polypeptide and the CD154 polypeptide were encoded on the same polynucleotide and were in frame with each other and with the *Salmonella* lamB polynucleotide in which they were inserted. In alternative embodiments, the CD154 polypeptide and the TRAP polypeptide may be encoded by distinct polynucleotides. SE13A aroA htrA TRAP contains a deletion in aroA and htrA and encodes both the TRAP epitope (SEQ ID NO: 1-3) and optionally the CD154 polypeptide (SEQ ID NO:4) inserted into loop 9 of lamB.

Compositions comprising an attenuated *Salmonella* strain and a pharmaceutically acceptable carrier are also provided. A pharmaceutically acceptable carrier is any carrier suitable for in vivo administration. Examples of pharmaceutically acceptable carriers suitable for use in the composition include, but are not limited to, water, buffered solutions, glucose solutions or bacterial culture fluids. Additional components of the compositions may suitably include, for example, excipients such as stabilizers, preservatives, diluents, emulsifiers and lubricants. Examples of pharmaceutically acceptable carriers or diluents include stabilizers such as carbohydrates (e.g., sorbitol, mannitol, starch, sucrose, glucose, dextran), proteins such as albumin or casein, protein-containing agents such as bovine serum or skimmed milk and buffers (e.g., phosphate buffer). Especially when such stabilizers are added to the compositions, the composition is suitable for freeze-drying or spray-drying.

Methods of enhancing immune responses in a subject by administering a vaccine containing a TRAP polypeptide and a CD154 polypeptide capable of binding to CD40 and activating CD40 are also provided. The vaccine comprising the polynucleotide encoding a CD154 polypeptide capable of binding to CD40 is administered to a subject in an amount effective to enhance the immune response of the subject to the vaccine. Suitably, the vaccine contains a polynucleotide encoding a polypeptide including amino acids 140-149 of the human CD154 polypeptide (SEQ ID NO:4) or a homolog thereof. Therefore, a homologue of amino acid 140-149 derived from one species may be used to stimulate an immune response in a distinct species.

Several suitable polypeptides are identified herein. Suitably, the polynucleotide encodes a CD154 polypeptide from the same species as the subject. Suitably, a polynucleotide encoding the polypeptide of SEQ ID NO:5 is used in human subjects, a polynucleotide encoding the polypeptide of SEQ ID NO:6 is used in chickens, a polynucleotide encoding the polypeptide of SEQ ID NO:7 is used in ducks, a polynucleotide encoding the polypeptide of SEQ ID NO:8 is used in mice, and a polynucleotide encoding the polypeptide of SEQ ID NO:9 is used in cows. In the Examples, the human CD154 polypeptide (SEQ ID NO:5) was used in a chicken

vaccine and was demonstrated to enhance the immune response to a foreign antigen. Thus other heterologous combinations of CD154 polypeptides and subjects may be useful in the methods of the invention. The CD154 polypeptide may be used to enhance the immune response in the subject to any foreign antigen or antigenic polypeptide present in the vaccine in addition to the TRAP polypeptide. One of skill in the art will appreciate that the CD154 polypeptide could be used to enhance the immune response to more than one antigenic polypeptide present in a vaccine.

The polypeptide from CD154 stimulates an immune response at least in part by binding to its receptor, CD40. The Examples used a polypeptide homologous to the CD154 polypeptide which is expressed on immune cells of the subject and which is capable of binding to the CD40 receptor on macrophages and other antigen presenting cells. Binding of this ligand-receptor complex stimulates macrophage (and macrophage lineage cells such as dendritic cells) to enhance phagocytosis and antigen presentation while increasing cytokine secretions known to activate other local immune cells (such as B-lymphocytes). As such, molecules associated with the CD154 peptide are preferentially targeted for immune response and expanded antibody production.

Potential vectors for use in the methods include, but are not limited to, *Salmonella* (*Salmonella enteritidis*), *Shigella, Escherichia* (*E. coli*), *Yersinia, Bordetella, Lactococcus, Lactobacillus, Bacillus, Streptococcus, Vibrio* (*Vibrio cholerae*), *Listeria*, adenovirus, poxvirus, herpesvirus, alphavirus, and adeno-associated virus.

In addition, methods of enhancing an immune response against an Apicomplexan parasite and methods of reducing morbidity associated with subsequent infection with an Apicomplexan parasite are disclosed. Briefly, the methods comprise administering to a subject a vaccine comprising a first polynucleotide sequence encoding a TRAP polypeptide in an effective amount. The TRAP polypeptides may include SEQ ID NO: 1-3 and 11. The insertion of the TRAP polypeptides into the vector may be accomplished in a variety of ways known to those of skill in the art, including but not limited to the scarless site-directed mutation system described in BMC Biotechnol. 2007 Sep. 17; 7(1): 59, Scarless and Site-directed Mutagenesis in *Salmonella enteritidis* chromosome, which is incorporated herein by reference in its entirety. The vector may also be engineered to express the TRAP polypeptides in conjunction with other polypeptides capable of enhancing the immune response as discussed above, such as in SEQ ID NO:4 and SEQ ID NO: 10. In particular, a polypeptide of CD154 capable of binding CD40 may be expressed by the vector to enhance the immune response of the subject to the TRAP polypeptide. Optionally, the vector is a bacterium, such as *Salmonella enteritidis*.

The useful dosage of the vaccine to be administered will vary depending on the age, weight and species of the subject, the mode and route of administration and the type of pathogen against which an immune response is sought. The composition may be administered in any dose sufficient to evoke an immune response. For bacterial vaccines, it is envisioned that doses ranging from 10^3 to 10^{10} bacteria, from 10^4 to 10^9 bacteria, or from 10^5 to 10^7 bacteria are suitable. The composition may be administered only once or may be administered two or more times to increase the immune response. For example, the composition may be administered two or more times separated by one week, two weeks, or by three or more weeks. The bacteria are suitably viable prior to administration, but in some embodiments the bacteria may be killed prior to administration. In some embodiments, the bacteria may be able to replicate in the subject, while in other embodiments the bacteria may not be capable of replicating in the subject.

For administration to animals or humans, the compositions may be administered by a variety of means including, but not limited to, intranasally, mucosally, by spraying, intradermally, parenterally, subcutaneously, orally, by aerosol or intramuscularly. Eye-drop administration or addition to drinking water or food are additionally suitable. For chickens, the compositions may be administered in ovo.

Some embodiments of the invention provide methods of enhancing immune responses in a subject. Suitable subjects may include, but are not limited to, vertebrates, suitably mammals, suitably a human, and birds, suitably poultry such as chickens. Other animal models of infection may also be used. Enhancing an immune response includes, but is not limited to, inducing a therapeutic or prophylactic effect that is mediated by the immune system of the subject. Specifically, enhancing an immune response may include, but is not limited to, enhanced production of antibodies, enhanced class switching of antibody heavy chains, maturation of antigen presenting cells, stimulation of helper T cells, stimulation of cytolytic T cells or induction of T and B cell memory.

It is envisioned that several epitopes or antigens from the same or different pathogens may be administered in combination in a single vaccine to generate an enhanced immune response against multiple antigens. Recombinant vaccines may encode antigens from multiple pathogenic microorganisms, viruses or tumor associated antigens. Administration of vaccine capable of expressing multiple antigens has the advantage of inducing immunity against two or more diseases at the same time. For example, live attenuated bacteria, such as *Salmonella enteritidis* 13A, provide a suitable vector for eliciting an immune response against multiple antigens.

Bacterial vaccines may be constructed using exogenous polynucleotides encoding antigens which may be inserted into the bacterial genome at any non-essential site or alternatively may be carried on a plasmid using methods well known in the art. One suitable site for insertion of polynucleotides is within external portions of transmembrane proteins or coupled to sequences that target the exogenous polynucleotide for secretory pathways. One example of a suitable transmembrane protein for insertion of polynucleotides is the lamB gene. In the Examples, TRAP and CD154 polynucleotides were inserted into loop 9 of the lamB sequence.

Exogenous polynucleotides include, but are not limited to, polynucleotides encoding antigens selected from pathogenic microorganisms or viruses and include polynucleotides that are expressed in such a way that an effective immune response is generated. Such polynucleotides may be derived from pathogenic viruses such as influenza (e.g., M2e, hemagglutinin, or neuraminidase), herpesviruses (e.g., the genes encoding the structural proteins of herpesviruses), retroviruses (e.g., the gp160 envelope protein), adenoviruses, paramyxoviruses, coronaviruses and the like. Exogenous polynucleotides can also be obtained from pathogenic bacteria, e.g., genes encoding bacterial proteins such as toxins, and outer membrane proteins. Further, exogenous polynucleotides from parasites, such as other Apicomplexan parasites are attractive candidates for use of a vector vaccine.

Polynucleotides encoding polypeptides involved in triggering the immune system may also be included in a vector, such as a live attenuated *Salmonella* vaccine. The polynucleotides may encode immune system molecules known for

their stimulatory effects, such as an interleukin, Tumor Necrosis Factor, an interferon, or another polynucleotide involved in immune-regulation. The vaccine may also include polynucleotides encoding peptides known to stimulate an immune response, such as the CD154 polypeptide described herein.

The following examples are meant only to be illustrative and are not meant as limitations on the scope of the invention or of the appended claims.

EXAMPLES

Example 1. Construction of TRAP and TRAP/CD154 Inserts

Strains and Culture Conditions

All plasmids were first maintained in TOP 10 *E. coli* cells (Invitrogen, Carlsbad, Calif., USA) unless described otherwise. *Salmonella enteritidis* 13A was used for introduction of mutations. *Salmonella enteritidis* strain 13A was a field isolate available from USDA/APHIS/NVSL and deposited with the ATCC as deposit number PTA-7871. Bacteria carrying plasmid pKD46 were grown at 30° C. Other bacteria were grown at 37° C. Plasmid curing was conducted at 37° C.

Luria-Bertaui (LB) media was used for routine growth of cells, and SOC media (Invitrogen, Carlsbad, Calif., USA) was used for phenotypic expression after electroporation. When appropriate, the following antibiotics were added to the media: ampicillin (Amp) at 100 μg/ml kanamyein (Km) at 50 μg/ml, and chloramphenicol (Cm) at 25 μg/ml.

Plasmids

Plasmids pKD46, pKD13, and pBC-I-SceI were described previously (Datsenko and Wanner, PNAS 2000, 97:6640-6645 and Kang et al. J Bacteriol 2004, 186:4921-4930, both, of which are incorporated herein by reference in their entireties). Plasmid pKD46 encodes Red recombinase enzymes which mediate homologous recombination of incoming linear DNA with chromosomal DNA. This plasmid also contains the Ampicillin resistance gene and is temperature-sensitive so that it requires 30° C. for maintenance in the cell. Plasmid pKD13 served as a template for amplification of the Km resistance (Km^r) gene used in overlapping PCR. Plasmid pBC-I-SceI, which is maintained in the cell at 37° C., produces the I-SceI enzyme, which cleaves the following 18 base pair, rare recognition sequence: 5'-TAGGGATAACAGGGTAAT-3' (SEQ ID NO: 16). Plasmid pBC-I-SceI also contains the chloramphenicol resistance (Cm^r) gene.

PCR

All primers used for PCR are listed in Table 1. Typically, PCR was performed using approximately 0.1 μg of purified genomic, plasmid or PCR-generated DNA (Qiagen, Valencia, Calif., USA), 1× cloned Pfu polymerase buffer, 5U Pfu polymerase (Stratagene La Jolla, Calif., USA), 1 mM dNTPs (GE Healthcare Bio-Sciences Corp., Piscataway, N.J.), and 1.2 μM of each primer in a total volume of 50 μL. The DNA engine thermal cycler (Bio-Rad, Hercules, Calif., USA) was used with the following amplification conditions: 94° C. for 2 minutes; 30 cycles of 94° C. sec for 30 sec, 58° C. for 60 sec, 72° C. for 90 sec per 1 kb; and 72° C. for 10 minutes for final extension. Each PCR product was gel purified (Qiagen, Valencia, Calif., USA) and either eluted in 25 μL EB buffer for preparation of templates used in overlapping extension PCR or in 50 μL EB buffer, ethanol precipitated and suspended in 5 μL of ddH_2O for electroporation into *S. enteritidis*.

TABLE 1

Primer sequences

Primer	Amplified region	Primer sequence
Iam-up-f	loop 9 up	5'TGTACAAGTGGACGCCAATC 3' (SEQ ID NO: 12)
Iam-up-r		5'*GTTATCGCCGTCTTTGATATAGCC* 3' (SEQ ID NO: 18)
Iam-dn-f	loop 9 dn	5'*ATTTCCCGTTATGCCGCAGC* 3' (SEQ ID NO: 19)
Iam-du-r		5'GTTAAACAGAGGGCGACGAG 3' (SEQ ID NO: 20)
Km-f	I-SceI/Kmc gene	5'*GCTATATCAAAGACGGCGATAAC***TAACTATAACGGTCCTAAGGT AGCGA**ATTTCCGGGGATCCGTCGA 3' (SEQ ID NO: 21)
Km-r		5'*GCTGCGGCATAACGGGAAATT*GTAGGCTGGAGCTGCTTCG 3' (SEQ ID NO: 22)
Kan4f	inside Kmc gene:	5'CAAAAGCGCTCTGAAGTTCC 3' (SEQ ID NO: 23)
Kan4r	sequencing	5'GCGTGAGGGGATCTTGAAGT 3' (SEQ ID NO: 24)
SEQ1 hCD154 up reverse	SEQ1 hCD154/loop 9 up	5'GGAGGACGCAACCGCCGCGGTCGGAAAACCACCACCGGAGGA *GGAGTTATCGCCGTCTTTGATATAGCC*3' (SEQ ID NO: 25)
SEQ1hCD 154 down forward	SEQ1hCD154/loop 9 down	5'CCGCGGCGGTTGCGTCCTCCTCCTGGGCAGAAAAAGGTTATTAT ACCATGTCTTCCTCCTCC*ATTTCCCGTTATGCCGCAGC*3' (SEQ ID NO: 26)
SEQ2 hCD154 up reverse	SEQ2-hCD154/ loop 9 up	5'TTTTCTTCTTCTTCTTCCGGTTCCGGACGTTCATGACCTTCTTCGG CTTTCGGCTGAACCGCCGGGGTTTCCGGCGCCGCGGAGGAGGAG *TTATCGCCGTCTTTGATATAGCC*3' (SEQ ID NO: 27)

TABLE 1-continued

Primer sequences

Primer	Amplified region	Primer sequence
SEQ2 hCD154 up reverse	SEQ2-hCD154/ loop 9 down	5'ACCGGAAGAAGAAGAAGAAAAAAAAGAAGAAGGTGGTGGTTT TCCGACCGCGGCGGTTGCGTCCTCCTCCTGGGCAGAAAAAGGTTA TTATACCATGTCTTCCTCCTCCA*TTTCCCGTTATGCCGCAGC*3' (SEQ ID NO: 28)
SEQ3 Hcd154 up reverse	SEQ3 hCD154/loop 9 up	5'GCAACACCACCACCAACCGCCGCGATCAGCAGAACACCACCAA CACCACCCGCAACCGCCGCGGTCGGAAAACCACCACCGGAGGAG *GAGTTATCGCCGTCTTTGATATAGCC*3' (SEQ ID NO: 29)
SEQ3 Hcd154 up reverse	SEQ3-hCD154/ loop 9 down	5'GGCGGTTGGTGGTGGTGTTGCGGCGTTTACCTCCGGTGGTGGTG GTGCGGGTGCGCAGGAATCCTCCTCCTGGGCAGAAAAAGGTTAT TATACCATGTCTTCCTCCTCC*ATTTCCCGTTATGCCGCAGC*3' (SEQ ID NO: 30)
Iam 3f	outer regions of loop	5'GCCATCTCGCTTGGTGATAA 3' (SEQ ID NO: 31)
Iam 3r	9: sequencing	5'CGCTGGTATTTTGCGGTACA 3' (SEQ ID NO: 32)

In Table 1, italicized nucleotides are complementary to either side of the lamB gene Loop 9 insertion site, which corresponds to nucleotide 1257 using *S. typhimurium* as an annotated reference genome. Bold font nucleotides represent the I-SceI recognition site in the Km-f primer.

Electroporation

Transformation of pKD46 into *S. enteritidis* was the first step carried out so that Red recombinase enzymes could be used for mediating recombination of subsequent mutations. Plasmid pKD46 was harvested from *E. coli* BW25113 (Datsenko and Wanner, PNAS 2000, 97:6640-6645) using a plasmid preparation kit (Qiagen Valencia, Calif., USA). Then 0.5 μL of pKD46 DNA was used for transformation into *S. enteritidis* 13A which had been prepared for electroporation. (Datsenko and Wanner, PNAS 2000, 97:6640-6645). Briefly, cells were inoculated into 10-15 mL of 2×YT broth and grown at 37° C. overnight. Then 100 μL of overnight culture was re-inoculated into 10 mL fresh 2×YT broth at 37° C. for 3-4 hours. Cells to be transformed with pKD46 plasmid were heated at 50° C. for 25 minutes to help inactivate host restriction. Cells were washed five times in ddH_2O water and resuspended in 60 μL of 10% glycerol. Cells were then pulsed at 2400-2450 kV for 1-6 ms, incubated in SOC for 2-3 hours at 30° C. and plated on LB media with appropriate antibiotics. *S. enteritidis* transformants with pKD46 were maintained at 30° C. When these transformants were prepared for additional electroporation reactions, all steps were the same except that 15% arabinose was added to induce Red recombinase enzymes one hour prior to washing, and cells did not undergo the 50° C. heat step.

Loop 9 up-I-SceI/Km^r-Loop 9 Down Construct

Introduction of I-SceI enzyme recognition site along with the Km^r gene into loop 9 of the lamB gene was done by combining the Red recombinase system (Datsenko and Wanner, PNAS 2000, 97:6640-6645, which is incorporated herein by reference in its entirety) and overlapping PCR (Horton et al., BioTechniques 1990, 8:528-535, which is incorporated herein by reference in its entirety). The insertion site corresponds to nucleotide 1257 of the lamB gene using *Salmonella typhimurium* LT2 (*S. typhimurium*) as an annotated reference genome. First, the upstream and downstream regions immediately flanking the loop 9 insertion site (loop 9 up and loop 9 down, respectively) were amplified separately. Primers used were lam-up-f and lam-up-r for loop 9 up and lam-dn-f and lam-dn-r for loop 9 down. Then the Km^r gene from pKDB plasmid was amplified using primers Km-f and Km-r. Here, the I-SceI enzyme site was synthetically added to the 5' end of Km-f primer then preceded by a region complimentary to the loop-up-r primer. Likewise, a region complimentary to the loop-dn-f primer was added to the 5' end of Km-r primer. The complimentary regions allow all 3 PCR products to anneal when used as templates in one PCR reaction. FIG. **2***a* represents this design scheme. PCR fragments consisting of loop 9 up-I-SceI/Km^r-loop 9 down sequence (PCR-A) were electroporated into *S. enteritidis* cells, which harbored pKD46 and were induced by arabinose, and then plated on LB with Km plates. To verify the correct sequence orientation of the mutation, we performed colony PCR with primer pairs Kan4F/lam3f and Kan4R/lam3r, where Kan4F and Kan4R are Km^r gene-specific primers and lam3f and lam3r are primers located outside the lamB loop 9 region. These PCR fragments were gel purified (Qiagen, Valencia, Calif., USA) and used for DNA sequencing.

Loop 9 up-TRAP-CD154-Loop 9 Down Construct

The final overlapping PCR fragment, PCR-B, contained the added TRAP antigen in combination with CD154 sequences flanked by loop 9 up and down regions (FIG. **2***b*). Combination sequences consisted of TRAP polynucleotide encoding SEQ ID NO: 1-3 and CD154 along with spacers such as Serine (Ser) residues.

To shorten the amount of steps for construction of the next fragment, the TRAP-CD154 sequence was synthetically added to the 5' end of the lam-dn-f primer and preceded by the complimentary region to the loop-up-r primer. The previously used PCR product for loop 9 up could be used together with the newly constructed PCR product in which the TRAP-CD154s were incorporated at the 5' end of loop 9 down to perform the final PCR reaction. However, for other insert sequences, an extra PCR step was needed, due to the longer lengths of insert sequences, to amplify loop 9 up with added nucleotides specific to insertion sequences connected to loop-up-r primer. The coding sequence for Gly (GGT) and Serine (TCC) as well as all other amino acids were chosen based on compiled data of the most frequently used codons in *E. coli* and *Salmonella typhimurium* proteins. See Table 1 for further details of primer design.

I-SceI Site/Km^r Insertion Mutation

The first mutation step involved designing a PCR fragment, PCR-A, which would serve as the carrier of the I-SceI site/Km^r cassette to be inserted into the lamB site. PCR-A consisted of the I-SceI enzyme recognition site adjacent to the Km^r gene with approximately 200-300 bp of flanking DNA on each end homologous to the upstream and downstream regions of lamB loop 9 insertion site (loop 9 up and loop 9 down, respectively). The fragment was introduced into *S. enteritidis* cells expressing Red recombinase enzymes and Km^r colonies were selected. After screening a few colonies by colony PCR, positive clones were sequenced for the desired inserted I-SceI site/Km^r sequence, and the identified mutant was selected and designated as SE164.

Genomic Replacement of I-SceI/Km^r with TRAP-CD154s

The second mutation step required constructing a PCR fragment, referred to as PCR-B and shown in FIG. **2**B, consisting of the final insertion sequence, the TRAP-CD154s, flanked by lamB homologous fragments. PCR-B amplicons have no selection marker and must be counter-selected after replacement for the previous I-SceI site/Km^r mutation in SE164. Plasmid pBC-I-SceI encodes the Cm^r gene and the I-SceI enzyme, which will cut the genome at the I-SceI site of SE164. Therefore, pBC-I-SceI was electroporated into SB164 along with PCR-B. After recombination of PCR-B to replace PCR-A, positive clones were chosen based on the ability to grow on Cm but not on Km. After DNA sequencing of mutants to confirm successful recombination of PCR-B, the strains were designated Sequence 1, Sequence 2 and Sequence 3. Ten random clones for each of the TRAP-CD154 insertions were used for PCR with lam 3f and lam 3r then digested using unique restriction enzymes sites for each insertion sequence and 100% of clones tested by digestion were positive for the desired mutation sequence. Sequencing results demonstrated that the insertion of TRAP-CD154 was exactly into the loop 9 region without the addition of extraneous nucleotides in each case. The inserts of the TRAP-CD154 vaccines are as follows: TRAP-CD154 (SEQ ID NO:33); TRAP-US-CD154 (SEQ ID NO:34); TRAP-DS-CD154 (SEQ ID NO:35).

Example 2. Attenuation of TRAP-CD154 Mutants/Inserts

Attenuation of SE13A was achieved by deletion mutation of the aroA gene and/or the htrA gene. Mutation of the aroA gene, a key gene in the chorismic acid pathway of bacteria, results in a severe metabolic deficiency which affects seven separate biochemical pathways. Mutation of the htrA gene reduces the cell's ability to withstand exposure to low and high temperatures, low pH, and oxidative and DNA damaging agents and reduces the bacteria's virulence.

To achieve deletion mutations in SE13A, the target gene sequence in the bacterial genome of *S. enteritidis* was replaced with the Km resistant gene sequence. This was completed using overlapping extension PCR and electroporation of the PCR products as described above. The Km resistance gene was targeted into the genomic region containing the genes of interest (aroA or htrA) by flanking the Km resistance gene with 200-300 base pairs of sequences homologous to the genes of interest. Once Km resistant mutants were obtained, the aroA and htrA deletion mutations were confirmed by DNA sequencing. Analogous aroA- and htrA-*Salmonella* strains were deposited with the American Type Culture Collection on Sep. 13, 2006 (Deposit No. PTA-7872 and Deposit No. PTA-7873, respectively). The attenuated strains were previously tested in vivo with regards to clearance time. Both of the attenuated strains had quicker clearance times than did the wildtype 13A strain, but both were able to colonize the liver, spleen, and cecal tonsils of chickens after oral infection. Attenuated strains comprising the TRAP-CD154s and lacking both aroA and htrA were isolated.

Example 3. Protection of Chicks from Mortality after *Eimeria* Infection

Day-of-hatch chicks (n=280) were orally vaccinated with about 1×10^8 cfu of the *Salmonella* isolates comprising the three distinct polynucleotides encoding the TRAP polypeptides of SEQ ID NO: 1-3 or saline control. At 21 days of age, the chicks were orally challenged with 10^4 sporulated oocysts of *Eimeria maxima*. The chicks were monitored daily post challenge. As depicted in FIG. **3**, mortality of chicks at day 5 post, challenge was reduced as compared to non-vaccinated animals irrespective of the vaccine strain given. The mortality was as follows: TRAP (SEQ ID NO:1) 7/43 (16.3%); TRAP US(SEQ ID NO:2) 1/46 (2.2.%); TRAP DS (SEQ ID NO:3) 6/43 (11%); Control (unvaccinated) 10/46 (21.7%). Surprisingly, the chicks vaccinated with a *Salmonella* comprising TRAP polypeptide of SEQ ID NO:2 demonstrated marked and significantly reduced mortality as compared to control non-vaccinated chicks ($P<0.001$). Necropsy was performed and indicated that all mortality was related to the *Eimeria maxima* infection.

In a repeat experiment, mortality in the vaccinated bird (6/48) was significantly lower than the controls (17/50) and performance was better in the vaccinated chicks, but the difference was not significant.

In addition, serum was collected from immunized birds and an ELISA for TRAP performed. A robust TRAP specific antibody response was generated in the birds vaccinated with TRAP-US (SEQ ID NO:2).

Example 4. Morbidity Associated with Vaccination is Limited

To evaluate the efficacy of TRAP US-CD154 (SEQ ID NO:34) as a potential vaccine candidate, a similar study was completed to investigate morbidity associated with vaccination. Broiler chickens were orally vaccinated with 1×10^8 cm/bird of the *Salmonella* vaccine with TRAP US and CD154 insert (SEQ ID NO:34) or sham vaccinated with saline. Coccidia challenge was performed with sporulated oocytes of *Eimeria maxima* (10^5 sporulated oocysts/bird) at three weeks post-vaccination. Body weight gain and lesions were evaluated 7 days post-challenge. Immunized birds showed a significant ($p<0.01$) improvement in performance. Immunized birds had about a 31% weight gain as compared to unvaccinated controls. Thus, vaccination with a *Salmonella*-based vaccine comprising a TRAP polypeptide and a CD154 polypeptide capable of binding CD40 may protect birds from morbidity and mortality associated with *Eimeria* infection.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 35

<210> SEQ ID NO 1
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Eimeria maxima

<400> SEQUENCE: 1

Gly Gly Gly Phe Pro Thr Ala Ala Val Ala
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<210> SEQ ID NO 2
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Eimeria maxima

<400> SEQUENCE: 2

Ala Ala Pro Glu Thr Pro Ala Val Gln Pro Lys Pro Glu Glu Gly His
1 5 10 15

Glu Arg Pro Glu Pro Glu Glu Glu Glu Glu Lys Lys Glu Glu Gly Gly
20 25 30

Gly Phe Pro Thr Ala Ala Val Ala
35 40

<210> SEQ ID NO 3
<211> LENGTH: 40
<212> TYPE: PRT
<213> ORGANISM: Eimeria maxima

<400> SEQUENCE: 3

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1 5 10 15

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20 25 30

Gly Gly Gly Ala Gly Ala Gln Glu
35 40

<210> SEQ ID NO 4
<211> LENGTH: 261
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

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1 5 10 15

Leu Pro Ile Ser Met Lys Ile Phe Met Tyr Leu Leu Thr Val Phe Leu
20 25 30

Ile Thr Gln Met Ile Gly Ser Ala Leu Phe Ala Val Tyr Leu His Arg
35 40 45

Arg Leu Asp Lys Ile Glu Asp Glu Arg Asn Leu His Glu Asp Phe Val
50 55 60

Phe Met Lys Thr Ile Gln Arg Cys Asn Thr Gly Glu Arg Ser Leu Ser
65 70 75 80

Leu Leu Asn Cys Glu Glu Ile Lys Ser Gln Phe Glu Gly Phe Val Lys
85 90 95

Asp Ile Met Leu Asn Lys Glu Glu Thr Lys Lys Glu Asn Ser Phe Glu
100 105 110

Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala Ala His Val Ile Ser
115 120 125

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Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln Trp Ala Glu Lys Gly
    130                 135                 140

Tyr Tyr Thr Met Ser Asn Asn Leu Val Thr Leu Glu Asn Gly Lys Gln
145                 150                 155                 160

Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr Ala Gln Val Thr
                165                 170                 175

Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala Pro Phe Ile Ala Ser
            180                 185                 190

Leu Cys Leu Lys Ser Pro Gly Arg Phe Glu Arg Ile Leu Leu Arg Ala
        195                 200                 205

Ala Asn Thr His Ser Ser Ala Lys Pro Cys Gly Gln Gln Ser Ile His
    210                 215                 220

Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala Ser Val Phe Val Asn
225                 230                 235                 240

Val Thr Asp Pro Ser Gln Val Ser His Gly Thr Gly Phe Thr Ser Phe
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Gly Leu Leu Lys Leu
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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 5

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<211> LENGTH: 11
<212> TYPE: PRT
<213> ORGANISM: Gallus gallus

<400> SEQUENCE: 6

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1               5                   10


<210> SEQ ID NO 7
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Anas sp.

<400> SEQUENCE: 7

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<210> SEQ ID NO 8
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<212> TYPE: PRT
<213> ORGANISM: Mus sp.

<400> SEQUENCE: 8

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<400> SEQUENCE: 10

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1               5                   10                  15

Ser Pro Ser Thr Met Lys Met Phe Met Cys Phe Leu Ser Val Phe Met
            20                  25                  30

Val Val Gln Thr Ile Gly Thr Val Leu Phe Cys Leu Tyr Leu His Met
        35                  40                  45

Lys Met Asp Lys Met Glu Glu Val Leu Ser Leu Asn Glu Asp Tyr Ile
    50                  55                  60

Phe Leu Arg Lys Val Gln Lys Cys Gln Thr Gly Glu Asp Gln Lys Ser
65                  70                  75                  80

Thr Leu Leu Asp Cys Glu Lys Val Leu Lys Gly Phe Gln Asp Leu Gln
                85                  90                  95

Cys Lys Asp Arg Thr Ala Ser Glu Glu Leu Pro Lys Phe Glu Met His
            100                 105                 110

Arg Gly His Glu His Pro His Leu Lys Ser Arg Asn Glu Thr Ser Val
        115                 120                 125

Ala Glu Glu Lys Arg Gln Pro Ile Ala Thr His Leu Ala Gly Val Lys
    130                 135                 140

Ser Asn Thr Thr Val Arg Val Leu Lys Trp Met Thr Thr Ser Tyr Ala
145                 150                 155                 160

Pro Thr Ser Ser Leu Ile Ser Tyr His Glu Gly Lys Leu Lys Val Glu
                165                 170                 175

Lys Ala Gly Leu Tyr Tyr Ile Tyr Ser Gln Val Ser Phe Cys Thr Lys
            180                 185                 190

Ala Ala Ala Ser Ala Pro Phe Thr Leu Tyr Ile Tyr Leu Tyr Leu Pro
        195                 200                 205

Met Glu Glu Asp Arg Leu Leu Met Lys Gly Leu Asp Thr His Ser Thr
    210                 215                 220

Ser Thr Ala Leu Cys Glu Leu Gln Ser Ile Arg Glu Gly Gly Val Phe
225                 230                 235                 240

Glu Leu Arg Gln Gly Asp Met Val Phe Val Asn Val Thr Asp Ser Thr
                245                 250                 255

Ala Val Asn Val Asn Pro Gly Asn Thr Tyr Phe Gly Met Phe Lys Leu
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<210> SEQ ID NO 11
<211> LENGTH: 70
<212> TYPE: PRT
<213> ORGANISM: Eimeria maxima

<400> SEQUENCE: 11

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1               5                   10                  15

Glu Arg Pro Glu Pro Glu Glu Glu Glu Glu Lys Lys Glu Glu Gly Gly
            20                  25                  30

Gly Phe Pro Thr Ala Ala Val Ala Gly Gly Val Gly Gly Val Leu Leu
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<400> SEQUENCE: 12

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<210> SEQ ID NO 13
<211> LENGTH: 120
<212> TYPE: DNA
<213> ORGANISM: Eimeria maxima

<400> SEQUENCE: 13

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<210> SEQ ID NO 14
<211> LENGTH: 120
<212> TYPE: DNA
<213> ORGANISM: Eimeria maxima

<400> SEQUENCE: 14

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gcggttggtg gtggtgttgc ggcgtttacc tccggtggtg gtggtgcggg tgcgcaggaa 120

<210> SEQ ID NO 15
<211> LENGTH: 210
<212> TYPE: DNA
<213> ORGANISM: Eimeria maxima

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ccggaagaag aagaagaaaa aaaagaagaa ggtggtggtt ttccgaccgc ggcggttgcg 120

ggtggtgttg gtggtgttct gctgatcgcg gcggttggtg gtggtgttgc ggcgtttacc 180

tccggtggtg gtggtgcggg tgcgcaggaa 210

<210> SEQ ID NO 16
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 16

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<210> SEQ ID NO 17
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 17

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<210> SEQ ID NO 18
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: loop 9 up

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<220> FEATURE:
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<210> SEQ ID NO 20
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<210> SEQ ID NO 21
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<212> TYPE: DNA
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<210> SEQ ID NO 22
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: I-SceI/Kmr gene

<400> SEQUENCE: 22

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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: inside Kmr gene: sequencing

<400> SEQUENCE: 23

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<210> SEQ ID NO 24
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<213> ORGANISM: Artificial Sequence
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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gatatagcc 69

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<220> FEATURE:
<223> OTHER INFORMATION: SEQ1hCD154/ loop 9 down

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ccatttcccg ttatgccgca gc 82

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<220> FEATURE:
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 28

accggaagaa gaagaagaaa aaaaagaaga aggtggtggt tttccgaccg cggcggttgc 60

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tgccgcagc 129

<210> SEQ ID NO 29
<211> LENGTH: 113
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SEQ3 hCD154/ loop 9 up

<400> SEQUENCE: 29

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<210> SEQ ID NO 30
<211> LENGTH: 129
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: SEQ3-hCD154/ loop 9 down

<400> SEQUENCE: 30

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tgccgcagc 129

<210> SEQ ID NO 31
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: outer regions of loop 9: sequencing

<400> SEQUENCE: 31

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<210> SEQ ID NO 32
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: outer regions of loop 9: sequencing

<400> SEQUENCE: 32

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<211> LENGTH: 87
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: TRAP-CD154

<400> SEQUENCE: 33

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ggttattata ccatgtcttc ctcctcc 87

<210> SEQ ID NO 34
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 34

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cgtccggaac cggaagaaga agaagaaaaa aaagaagaag gtggtggttt tccgaccgcg 120

gcggttgcgt cctcctcctg ggcagaaaaa ggttattata ccatgtcttc ctcctcc 177

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gcgcaggaat cctcctcctg ggcagaaaaa ggttattata ccatgtcttc ctcctcc 177

We claim:

1. A vaccine comprising a vector comprising a first polynucleotide sequence encoding a thrombospondin-related anonymous protein (TRAP) polypeptide consisting of SEQ ID NO: 1, SEQ ID NO: 2 or SEQ ID NO: 3.

2. The vaccine of claim **1**, wherein the vector is selected from the group consisting of a virus, a bacterium, and a liposome.

3. The vaccine of claim **2**, wherein the vector is a bacterium.

4. The vaccine of claim **3**, the bacterium comprising the TRAP polypeptide on its surface.

5. The vaccine of claim **3**, wherein the bacterium is selected from the group consisting of *Salmonella* species, *Bacillus* species, *Escherichia* species, and *Lactobacillus* species.

6. The vaccine of claim **3**, wherein the first polynucleotide is inserted into a polynucleotide sequence encoding an external portion of a transmembrane protein.

7. The vaccine of claim **1**, wherein the vaccine comprises more than one copy of the first polynucleotide sequence.

8. A method of inducing an immune response against an Apicomplexan parasite in a subject comprising administering to the subject the vaccine of claim **1** in an amount effective to induce the immune response of the subject to the Apicomplexan parasite.

9. The method of claim **8**, wherein the vector is selected from the group consisting of a virus and a bacterium.

10. The method of claim **8**, wherein the vector expresses the TRAP polypeptide on its surface.

11. The method of claim **8**, wherein the vector is selected from the group consisting of *Salmonella* species, *Bacillus* species, *Escherichia* species, and *Lactobacillus* species.

12. The method of claim **8**, wherein the first polynucleotide is inserted into a polynucleotide sequence encoding an external portion of a transmembrane protein.

13. The method of claim **8**, wherein the vaccine comprises more than one copy of the first polynucleotide sequence.

14. The method of claim **8**, wherein the vaccine is administered by a method selected from the group consisting of oral, intranasal, parenteral, and in ovo.

15. The method of claim **8**, wherein the immune response comprises an enhanced antibody response or an enhanced T cell response.

16. The method of claim **8**, wherein the subject is member of a poultry species or is a mammal.

17. The method of claim **8**, wherein the vector comprising the vaccine is killed prior to administration to the subject or is not capable of replicating in the subject.

18. The vaccine of claim **1**, wherein the TRAP polypeptide consists of SEQ ID NO: 2.

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