



HU000030655T2



(19) **HU**

(11) Lajstromszám: **E 030 655**

(13) **T2**

**MAGYARORSZÁG**  
Szellemi Tulajdon Nemzeti Hivatala

## **EURÓPAI SZABADALOM** **SZÖVEGÉNEK FORDÍTÁSA**

(21) Magyar ügyszám: **E 08 843740**

(51) Int. Cl.: **A61K 390/12**

(2006.01)

(22) A bejelentés napja: **2008. 11. 03.**

**A61K 38/04**

(2006.01)

(96) Az európai bejelentés bejelentési száma:  
**EP 20080843740**

(86) A nemzetközi (PCT) bejelentési szám:

**PCT/US 08/082254**

(97) Az európai bejelentés közzétételi adatai:  
**EP 2214701 A2 2009. 05. 07.**

(87) A nemzetközi közzétételi szám:

**WO 09059298**

(97) Az európai szabadalom megadásának meghirdetési adatai:  
**EP 2214701 B1 2016. 08. 03.**

(30) Elsőbbségi adatai:

**984612 P**

**2007. 11. 01.**

**US**

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Készítmények és eljárások eimeria elleni immunválasz fokozására

Az európai szabadalom ellen, megadásának az Európai Szabadalmi Közlönyben való meghirdetésétől számított kilenc hónapon belül, felszólalást lehet benyújtani az Európai Szabadalmi Hivatalnál. (Európai Szabadalmi Egyezmény 99. cikk(1))

A fordítást a szabadalmas az 1995. évi XXXIII. törvény 84/H. §-a szerint nyújtotta be. A fordítás tartalmi helyességét a Szellemi Tulajdon Nemzeti Hivatala nem vizsgálta.



(12)

(11)

EP 2 214 701 B1

## EUROPEAN PATENT SPECIFICATION

(45) Date of publication and mention of the grant of the patent:  
**03.08.2016 Bulletin 2016/31**

(21) Application number: **08843740.5**

(22) Date of filing: **03.11.2008**

(51) Int Cl.:  
**A61K 39/012 (2006.01)**      **A61K 38/04 (2006.01)**

(86) International application number:  
**PCT/US2008/082254**

(87) International publication number:  
**WO 2009/059298 (07.05.2009 Gazette 2009/19)**

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

ZUSAMMENSETZUNGEN UND VERFAHREN ZUR VERSTÄRKUNG VON IMMUNREAKTIONEN GEGENÜBER EIMERIA

COMPOSITIONS ET PROCÉDÉS POUR AMPLIFIER DES RÉPONSES IMMUNITAIRES À L'EIMERIA

(84) Designated Contracting States:  
**AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MT NL NO PL PT RO SE SI SK TR**

(30) Priority: **01.11.2007 US 984612 P**

(43) Date of publication of application:  
**11.08.2010 Bulletin 2010/32**

(60) Divisional application:  
**16175274.6**

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(56) References cited:  
**WO-A2-03/004683**      **WO-A2-03/004684**

(87) References cited:  

- WALLACH M ET AL: "MATERNAL IMMUNIZATION WITH GAMETOYTE ANTIGENS AS A MEANS OF PROVIDING PROTECTIVE IMMUNITY AGAINST EIMERIA MAXIMA IN CHICKENS", INFECTION AND IMMUNITY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, US, vol. 60, no. 5, 1 May 1992 (1992-05-01), pages 2036-2039, XP009036953, ISSN: 0019-9567**

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- SCHNEIDER J ET AL: "A prime-boost immunisation regimen using DNA followed by recombinant modified vaccinia virus Ankara induces strong cellular immune responses against the Plasmodium falciparum TRAP antigen in chimpanzees", VACCINE, ELSEVIER LTD, GB, vol. 19, no. 32, 14 September 2001 (2001-09-14), pages 4595-4602, XP004303150, ISSN: 0264-410X, DOI: DOI:10.1016/S0264-410X(01)00260-2
- WITCOMBE D M ET AL: "Molecular characterisation of EmTFP250: A novel member of the TRAP protein family in *Eimeria maxima*", INTERNATIONAL JOURNAL OF PARASITOLOGY, PERGAMON PRESS, GB, vol. 33, no. 7, 1 July 2003 (2003-07-01), pages 691-702, XP002313641, ISSN: 0020-7519, DOI: DOI:10.1016/S0020-7519(03)00086-9
- COX MANDY M ET AL: "Scarless and site-directed mutagenesis in *Salmonella enteritidis* chromosome", BMC BIOTECHNOLOGY, BIOMED CENTRAL LTD. LONDON, GB, vol. 7, no. 1, 17 September 2007 (2007-09-17), page 59, XP021035645, ISSN: 1472-6750
- LI WEI: "Synergistic antibody induction by antigen-CD40 ligand fusion protein as improved immunogen.", IMMUNOLOGY JUN 2005 LNKD-PUBMED:15885127, vol. 115, no. 2, June 2005 (2005-06), pages 215-222, XP002632788, ISSN: 0019-2805
- HARCOURT J L ET AL: "CD40 ligand (CD154) improves the durability of respiratory syncytial virus DNA vaccination in BALB/c mice", VACCINE, ELSEVIER LTD, GB, vol. 21, no. 21-22, 20 June 2003 (2003-06-20), pages 2964-2979, XP004429697, ISSN: 0264-410X, DOI: DOI:10.1016/S0264-410X(03)00119-1
- MENDOZA R B ET AL: "Cutting Edge: Immunostimulatory effects of a plasmid expressing CD40 ligand (CD154) on Gene Immunization", JOURNAL OF IMMUNOLOGY, AMERICAN ASSOCIATION OF IMMUNOLOGISTS, US, vol. 159, no. 12, 15 December 1997 (1997-12-15), pages 5777-5781, XP002286228, ISSN: 0022-1767
- VEGA MARIO I ET AL: "A *Salmonella typhi* OmpC fusion protein expressing the CD154 Trp14O-Ser149 amino acid strand binds CD40 and activates a lymphoma B-cell line", IMMUNOLOGY, BLACKWELL PUBLISHING, OXFORD, GB, vol. 110, no. 2, 1 October 2003 (2003-10-01), pages 206-216, XP002558957, ISSN: 0019-2805, DOI: DOI:10.1046/J.1365-2567.2003.01717.X [retrieved on 2003-09-18]
- DU A ET AL: "Efficacy of a DNA vaccine delivered in attenuated *Salmonella typhimurium* against *Eimeria tenella* infection in chickens", INTERNATIONAL JOURNAL OF PARASITOLOGY, PERGAMON PRESS, GB, vol. 35, no. 7, 1 June 2005 (2005-06-01), pages 777-785, XP004926570, ISSN: 0020-7519, DOI: DOI:10.1016/J.IJPARA.2005.03.005
- KONJUFCA VJOLLCA ET AL: "A recombinant attenuated *Salmonella enterica* serovar Typhimurium vaccine encoding *Eimeria acervulina* antigen offers protection against *E. acervulina* challenge.", INFECTION AND IMMUNITY DEC 2006 LNKD- PUBMED:16982843, vol. 74, no. 12, December 2006 (2006-12), pages 6785-6796, XP002632790, ISSN: 0019-9567
- POGONKA THOMAS ET AL: "A single dose of recombinant *Salmonella typhimurium* induces specific humoral immune responses against heterologous *Eimeria tenella* antigens in chicken", INTERNATIONAL JOURNAL OF PARASITOLOGY, PERGAMON PRESS, GB, vol. 33, no. 1, 1 January 2003 (2003-01-01), pages 81-88, XP002372984, ISSN: 0020-7519, DOI: DOI:10.1016/S0020-7519(02)00251-5
- HAYES L J ET AL: "Chlamydia trachomatis major outer membrane protein epitopes expressed as fusions with LamB in an attenuated aro A strain of *Salmonella typhimurium*; their application as potential immunogens.", JOURNAL OF GENERAL MICROBIOLOGY JUL 1991 LNKD-PUBMED:1720166, vol. 137, no. 7, July 1991 (1991-07), pages 1557-1564, XP002632791, ISSN: 0022-1287
- WANG J ET AL: "Immunogenicity of viral B-cell epitopes inserted into two surface loops of the *Escherichia coli* K12 LamB protein and expressed in an attenuated aroA strain of *Salmonella typhimurium*", VACCINE, ELSEVIER LTD, GB, vol. 17, no. 1, 1 January 1999 (1999-01-01), pages 1-12, XP004139026, ISSN: 0264-410X, DOI: DOI:10.1016/S0264-410X(98)00153-4
- O'CALLAGHAN D ET AL: "Immunogenicity of foreign peptide epitopes expressed in bacterial envelope proteins", RESEARCH IN MICROBIOLOGY, ELSEVIER, AMSTERDAM, NL, vol. 141, no. 7-8, 1 September 1990 (1990-09-01), pages 963-969, XP023925305, ISSN: 0923-2508, DOI: DOI:10.1016/0923-2508(90)90136-E [retrieved on 1990-09-01]
- VERMEULEN A N: "PROGRESS IN RECOMBINANT VACCINE DEVELOPMENT AGAINST COCCIDIOSIS A REVIEW AND PROSPECTS INTO THE NEXT MILLENNIUM", INTERNATIONAL JOURNAL OF PARASITOLOGY, PERGAMON PRESS, GB, vol. 28, no. 7, 1 July 1998 (1998-07-01), pages 1121-1130, XP001024960, ISSN: 0020-7519, DOI: DOI:10.1016/S0020-7519(98)00080-0

- JENKINS M C: "Progress on developing a recombinant coccidiosis vaccine", INTERNATIONAL JOURNAL OF PARASITOLOGY, PERGAMON PRESS, GB, vol. 28, no. 7, 1 July 1998 (1998-07-01), pages 1111-1119, XP002311785, ISSN: 0020-7519
- WITCOMBE ET AL.: 'Eimeria maxima TRAP family protein EmTFP250: subcellular localisation and induction of immune responses by immunization with a recombinant C-terminal derivative.' INT JOUR PARASITOLOGY vol. 34, no. 7, 2004, pages 861 - 872, XP008133629
- SEO ET AL.: 'Mucosal humoral immunity to experimental *Salmonella enteritidis* infection in the chicken crop.' AVIAN DISEASES vol. 46, no. 4, 2002, pages 1015 - 1020, XP008133683
- DANFORTH H D ET AL: "Genetically engineered antigen confers partial protection against avian coccidial parasites", POULTRY SCIENCE, CHAMPAIGN, IL, US, vol. 68, 1 January 1989 (1989-01-01), pages 1643-1652, XP002098106, ISSN: 0032-5791

**Description****INTRODUCTION**

5 [0001] 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.

**15 SUMMARY**

15 [0002] Vaccines according to the invention, and uses thereof according to the invention, are defined in the claims. 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 CD 154 polypeptides include fewer than 50 amino acids and comprise amino acids 140-149, or a homolog thereof.

20 [0003] 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.

25 [0004] In still another aspect, the invention includes the use of vaccines according to the invention in methods of enhancing the immune response according to claim 14.

30 [0005] Also disclosed herein are 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****[0006]**

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

**40 DETAILED DESCRIPTION**

45 [0007] 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.

50 [0008] 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.

55 [0009] 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 International journal for

Parasitology 34 (2004) 861-872. 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.

**[0010]** *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.

**[0011]** 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 an infection caused by an Apicomplexan parasite.

**[0012]** A wild-type isolate of *Salmonella*, *Salmonella enteritidis* 13A (SE13A) (deposited with the American Type Culture Collection (ATCC) on September 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.

**[0013]** 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.

**[0014]** Mutations may be made in a variety of other *Salmonella* genes including, but not limited to, *cya*, *crp*, *asd*, *cat*, *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.

**[0015]** 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.

**[0016]** 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.

[0017] 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.

[0018] 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.

[0019] 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.

[0020] As described in more detail below, a vaccine including a vector may include a CD 154 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 naive 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 CD 154 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.

[0021] As described above, polynucleotides encoding CD 154 polypeptides may be inserted into the chromosome of the vector or maintained extrachromosomally. A CD 154 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 CD 154 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 CD 154 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.

[0022] 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 CD 154 sequence and CD40 binding region are not highly conserved among the various species. The CD 154 sequences of chicken and human are provided in SEQ ID NO:10 and SEQ ID NO:4, respectively.

[0023] 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 CD 154 polypeptide as defined in the claims.

[0024] 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 CD 154 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 CD 154 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*.

[0025] 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.

[0026] 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 CD 154 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.

[0027] 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 CD 154 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 described herein. The CD 154 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.

[0028] 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 CD 154 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 CD 154 peptide are preferentially targeted for immune response and expanded antibody production.

[0029] 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.

[0030] 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 Sept, 17: 7(1): 59, Scarless and Site-directed Mutagenesis in *Salmonella enteritidis* chromosome. 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*.

[0031] 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.

**[0032]** 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.

**[0033]** Some embodiments of this disclosure 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.

15 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.

**[0034]** 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 20 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.

**[0035]** Bacterial vaccines may be constructed using exogenous polynucleotides encoding antigens which may be 25 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.

30 **[0036]** 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 35 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.

**[0037]** Polynucleotides encoding polypeptides involved in triggering the immune system may also be included in a 40 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 CD 154 polypeptide described herein.

**[0038]** 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.

## 45 EXAMPLES

### Example 1. Construction of TRAP and TRAP/CD154 inserts.

#### 50 Strains and Culture Conditions

**[0039]** All plasmids were first maintained in TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA, 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.

**[0040]** Luria-Bertani (LB) media was used for routine growth of cells, and SOC media (Invitrogen, Carlsbad, CA, USA) was used for phenotypic expression after electroporation. When appropriate, the following antibiotics were added to the media: ampicillin (Amp) at 100µg/ml, kanamycin (Km) at 50µg/ml, and chloramphenicol (Cm) at 25µg/ml.

## Plasmids

**[0041]** 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). 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 ( $\text{Km}^r$ ) 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 ( $\text{Cm}^r$ ) gene.

## PCR

**[0042]** All primers used for PCR are listed in Table 1. Typically, PCR was performed using approximately 0.1 $\mu\text{g}$  of purified genomic, plasmid or PCR-generated DNA (Qiagen, Valencia, CA, USA), 1x cloned *Pfu* polymerase buffer, 5U *Pfu* polymerase (Stratagene La Jolla, CA, USA), 1mM dNTPs (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and 1.2 $\mu\text{M}$  of each primer in a total volume of 50  $\mu\text{L}$ . The DNA engine thermal cycler (Bio-Rad, Hercules, CA, 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, CA, USA) and either eluted in 25 $\mu\text{L}$  EB buffer for preparation of templates used in overlapping extension PCR or in 50 $\mu\text{L}$  EB buffer, ethanol precipitated and suspended in 5 $\mu\text{L}$  of ddH<sub>2</sub>O for electroporation into *S. enteritidis*.

**Table 1. Primer sequences**

Primer	Amplified region	Primer sequence
lam-up-f lam-up-r	loop 9 up	5'TGTACAAGTGGACGCCAAC 3' (SEQ ID NO:17) 5'GTTATGCCGTCTTGATATAGCC 3' (SEQ ID NO:18)
lam-dn-f lam-dn-r	loop 9 dn	5'ATTTCCCGTTATGCCGCAGC 3' (SEQ ID NO:19) 5'GTTAACAGAGGGCGACGAG 3' (SEQ ID NO:20)
Km-f Km-r	I-SceI/ $\text{Km}^r$ gene	5'GCTATATCAAAGACGGCGATAACTAACTATAACGGTCCTAAGGT AGCGAATTCCGGGGATCCGTGA 3' (SEQ ID NO:21) 5'GCTGCCATAACGGGAAATTGTTAGGCTGGAGCTGCTTCG 3' (SEQ ID NO:22)
Kan4f Kan4r	inside $\text{Km}^r$ gene: sequencing	5'CAAAAGCGCTCTGAAGTTCC 3' (SEQ ID NO:23) 5'GCGTGAGGGGATCTGAAGT 3' (SEQ ID NO:24)
SEQ1 hCD154 up reverse	SEQ1 hCD154/ loop 9 up	5'GGAGGACGCAACGCCCGCGTCGGAAAACCACCCGGAGGA GGAGTTATGCCGTCTTGATATAGCC3' (SEQ ID NO:25)
SEQ1hCD 154 down forward	SEQ1hCD154/ loop 9 down	5'CCGCGGCGGTTGCGCTCCTCCTGGCAGAAAAAGGTTATTAT ACCATGTCTCCTCCATTCCCGTTATGCCGCAGC3' (SEQ ID NO:26)
SEQ2 hCD154 up reverse	SEQ2-hCD154/ loop 9 up	5'TTTCTTCTTCTTCCGGTTCCGGACGTTCATGACCTCTCGG CTTCGGCTGAACCGCCGGGTTCCGGCGCGAGGAGGAG TTATGCCGTCTTGATATAGCC3' (SEQ ID NO:27)
SEQ2 hCD154 up reverse	SEQ2-hCD154/ loop 9 down	5'ACCGGAAGAAGAAGAAGAAAAAAAAGAAGAAGGTGGTGGTT TCCGACCGCGCGGGTTGCGCTCCTCCTGGCAGAAAAAGGTTA TTATACCATGTCTCCTCCATTCCCGTTATGCCGCAGC3' (SEQ ID NO:28)
SEQ3 Hcd154 up reverse	SEQ3 hCD154/ loop 9 up	5'GCAACACCAACCAACCGCCCGATCAGCAGAACACCACCAA CACCAACCGCAACCGCCCGGGTCGGAAAACCACCCACCGGAGGAG GAGTTATGCCGTCTTGATATAGCC3' (SEQ ID NO:29)

(continued)

Primer	Amplified region	Primer sequence
SEQ3 hCD154 up reverse	SEQ3-hCD154/loop 9 down	5'GGCGGGTTGGTGGTGGTGTGCGGCGTTACCTCCGGTGGTGGTGTGCGGGTGCAGGAATCCTCCTGGCAGAAAAAGGTTAT TATACCATGTCTTCCTCCTCCATTCCCGTTATGCCGCAGC3' (SEQ ID NO:30)
Iam 3f	outer regions of loop 9: sequencing	5'GCCATCTCGCTTGGTGATAA 3' (SEQ ID NO:31)
Iam 3r		5'CGCTGGTATTTGCGGTACA 3' (SEQ ID NO:32)

**[0043]** 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-Scel recognition site in the Km-f primer.

## Electroporation

**[0044]** 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, CA, USA). Then 0.5 $\mu$ L 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 10-15mL of 2X YT broth and grown at 37°C overnight. Then 100 $\mu$ L of overnight culture was re-inoculated into 10mL fresh 2X 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<sub>2</sub>O water and resuspended in 60 $\mu$ L of 10% glycerol. Cells were then pulsed at 2400-2450kV for 1-6ms, 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-Seel/ Km<sup>r</sup>- Loop 9 down Construct

**[0045]** 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) and overlapping PCR (Horton et al., BioTechniques 1990, 8:528-535). 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 pKD13 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. Figure 2a 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, CA, USA) and used for DNA sequencing.

### Loop 9 up- TRAP -CD154 - Loop 9 down Construct

**[0046]** The final overlapping PCR fragment, PCR-B, contained the added TRAP antigen in combination with CD 154 sequences flanked by loop 9 up and down regions (Figure 2b). Combination sequences consisted of TRAP polynucleotide encoding SEQ ID NO:1-3 and CD 154 along with spacers such as Serine (Ser) residues.

**[0047]** 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<sup>r</sup> insertion mutation

**[0048]** The first mutation step involved designing a PCR fragment, PCR-A, which would serve as the carrier of the I-SceI site/ Km<sup>r</sup> cassette to be inserted into the *lamB* site. PCR-A consisted of the I-SceI enzyme recognition site adjacent to the Km<sup>r</sup> gene with approximately 200-300bp 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<sup>r</sup> colonies were selected. After screening a few colonies by colony PCR, positive clones were sequenced for the desired inserted I-SceI site/ Km<sup>r</sup> sequence, and the identified mutant was selected and designated as SE164.

#### Genomic Replacement of I-SceI/ Km<sup>r</sup> with TRAP-CD154s

**[0049]** The second mutation step required constructing a PCR fragment, referred to as PCR-B and shown in Figure 2B, 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<sup>r</sup> mutation in SE164. Plasmid pBC-I-SceI encodes the Cm<sup>r</sup> 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 SE164 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-CD 154 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.

**[0050]** 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. **[0051]** 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 September 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-CD 154s and lacking both *aroA* and *htrA* were isolated.

#### Example 3. Protection of chicks from mortality after *Eimeria* infection

**[0052]** Day-of-hatch chicks (n=280) were orally vaccinated with about 1 x 10<sup>8</sup> 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<sup>4</sup> sporulated oocysts of *Eimeria maxima*. The chicks were monitored daily post challenge. As depicted in Figure 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.

[0053] 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.

[0054] 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

[0055] 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  $1 \times 10^8$  cfu/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 oocysts 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

##### [0056]

<110> BOARD OF TRUSTEES OF THE UNIVERSITY OF ARKANSAS

<120> COMPOSITIONS AND METHODS OF ENHANCING IMMUNE RESPONSES TO EIMERIA

<130> 013961-9038 WO00

<140> New International Application

<141> 2008-11-03

<150> US 60/984,612

<151> 2007-11-01

<160> 35

<170> PatentIn version 3.5

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EP 2 214 701 B1

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45

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20 25 30

10 Ile Thr Gln Met Ile Gly Ser Ala Leu Phe Ala Val Tyr Leu His Arg  
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15 Arg Leu Asp Lys Ile Glu Asp Glu Arg Asn Leu His Glu Asp Phe Val  
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20 Leu Leu Asn Cys Glu Glu Ile Lys Ser Gln Phe Glu Gly Phe Val Lys  
85 90 95

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

30 Met Gln Lys Gly Asp Gln Asn Pro Gln Ile Ala Ala His Val Ile Ser  
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35 Glu Ala Ser Ser Lys Thr Thr Ser Val Leu Gln Trp Ala Glu Lys Gly  
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40 Leu Thr Val Lys Arg Gln Gly Leu Tyr Tyr Ile Tyr Ala Gln Val Thr  
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45 Phe Cys Ser Asn Arg Glu Ala Ser Ser Gln Ala Pro Phe Ile Ala Ser  
180 185 190

50

55

EP 2 214 701 B1

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10 Leu Gly Gly Val Phe Glu Leu Gln Pro Gly Ala Ser Val Phe Val Asn  
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**EP 2 214 701 B1**

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1 5 10 15

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

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

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

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

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

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

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

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

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

50

55

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

5 Lys Ala Gly Leu Tyr Tyr Ile Tyr Ser Gln Val Ser Phe Cys Thr Lys  
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10 Ala Ala Ala Ser Ala Pro Phe Thr Leu Tyr Ile Tyr Leu Tyr Leu Pro  
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15 Met Glu Glu Asp Arg Leu Leu Met Lys Gly Leu Asp Thr His Ser Thr  
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45 Gly Phe Pro Thr Ala Ala Val Ala Gly Gly Val Gly Gly Val Leu Leu  
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## Claims

1. A vaccine comprising a first polynucleotide sequence encoding a TRAP polypeptide or an immunogenic fragment thereof, wherein the first polynucleotide encodes a TRAP polypeptide which is SEQ ID NO:2 or encodes an immunogenic fragment of SEQ ID NO:2.
2. The vaccine of claim 1, further comprising a second polynucleotide sequence encoding a CD154 polypeptide capable of binding CD40, the CD154 polypeptide having fewer than 50 amino acids and comprising:
  - (i) amino acids 140-149 of SEQ ID NO:4,
  - (ii) SEQ ID NO:5,
  - (iii) SEQ ID NO:6,
  - (iv) SEQ ID NO:7,
  - (v) SEQ ID NO:8, or
  - (vi) SEQ ID NO:9.
3. The vaccine of claim 2, wherein the vaccine comprises more than one copy of the first polynucleotide sequence, more than one copy of the second polynucleotide sequence or more than one copy of the first polynucleotide sequence and more than one copy of the second polynucleotide sequence.
- 45                   4. The vaccine of any of claims 2-3, wherein the first polynucleotide sequence is linked in frame to the second polynucleotide sequence.
- 50                   5. The vaccine of any of claims 1-4, wherein the first polynucleotide is comprised within a vector.
6. The vaccine of any of claims 1-5, wherein the first polynucleotide is comprised within a bacterium, the bacterium comprising the TRAP polypeptide on its surface.
- 55                   7. The vaccine of any of claims 1-6, wherein the first polynucleotide is comprised within a bacterium and the bacterium is selected from the group consisting of *Salmonella* species, *Bacillus* species, *Escherichia* species, and *Lactobacillus* species.
8. The vaccine of claim 7, wherein the bacterium is *Salmonella enteritidis* selected from strains deposited as ATCC

PTA-7871, ATCC PTA-7872 and ATCC PTA-7873.

9. The vaccine of any one of the preceding claims, wherein the first polynucleotide sequence is inserted into a polynucleotide sequence encoding an external portion of a transmembrane protein.

5

10. Use of a vaccine of any one of claims 1-9 in the preparation of a medicament for enhancing the immune response against an Eimerian parasite in a subject, the enhancing the immune response comprising administering to the subject the vaccine in an amount effective to enhance the immune response of the subject to the Eimerian parasite.

10

11. The use of claim 10, wherein the enhanced immune response comprises an enhanced antibody response or an enhanced T cell response.

12. The use of any of claims 10-11, wherein the vaccine is not capable of replicating.

15

13. The use of any one of claims 10-12, wherein the Eimerian parasite is *Eimeria maxima*.

14. A vaccine of any of claims 1-9 for use in a method of enhancing the immune response against an Eimerian parasite in a poultry, the method comprising administering to the poultry the vaccine in an amount effective to enhance the immune response of the poultry to the Eimerian parasite.

20

### Patentansprüche

1. Impfstoff, der eine erste Polynucleotidsequenz umfasst, die für ein TRAP-Polypeptid oder ein immunogenes Fragment davon kodiert, wobei das erste Polynucleotid für ein TRAP-Polypeptid kodiert, das Seq.-ID Nr. 2 ist, oder für ein immunogenes Fragment von Seq.-ID Nr. 2 kodiert.

25

2. Impfstoff nach Anspruch 1, der weiters eine zweite Polynucleotidsequenz umfasst, die für ein CD154-Polypeptid kodiert, das in der Lage ist, CD40 zu binden, wobei das CD154-Polypeptid weniger als 50 Aminosäuren aufweist und Folgendes umfasst:

30

(i) Aminosäuren 140-149 der Seq.-ID Nr. 4,  
 (ii) Seq.-ID Nr. 5,  
 (iii) Seq.-ID Nr. 6,  
 (iv) Seq.-ID Nr. 7,  
 (v) Seq.-ID Nr. 8 oder  
 (vi) Seq.-ID Nr. 9.

35

3. Impfstoff nach Anspruch 2, wobei der Impfstoff mehr als eine Kopie der ersten Polynucleotidsequenz, mehr als eine Kopie der zweiten Polynucleotidsequenz oder mehr als eine Kopie der ersten Polynucleotidsequenz und mehr als eine Kopie der zweiten Polynucleotidsequenz umfasst.

40

4. Impfstoff nach einem der Ansprüche 2-3, wobei die erste Polynucleotidsequenz im Raster mit der zweiten Polynucleotidsequenz verbunden ist.

45

5. Impfstoff nach einem der Ansprüche 1-4, wobei das erste Polynucleotid in einem Vektor enthalten ist.

6. Impfstoff nach einem der Ansprüche 1-5, wobei das erste Polynucleotid in einem Bakterium enthalten ist, wobei das Bakterium das TRAP-Polypeptid auf seiner Oberfläche aufweist.

50

7. Impfstoff nach einem der Ansprüche 1-6, wobei das erste Polynucleotid in einem Bakterium enthalten ist und das Bakterium aus der aus *Salmonella*-Spezies, *Bacillus*-Spezies, *Escherichia*-Spezies und *Lactobacillus*-Spezies bestehenden Gruppe ausgewählt ist.

55

8. Impfstoff nach Anspruch 7, wobei das Bakterium *Salmonella enteriditis* ist, das aus den als ATCC PTA-7871, ATCC PTA-7872 und ATCC PTA-7873 hinterlegten Stämmen ausgewählt ist.

9. Impfstoff nach einem der vorangegangenen Ansprüche, wobei die erste Polynucleotidsequenz in eine Polynucleo-

tidsequenz insertiert ist, die für einen externen Teil eines Transmembranproteins kodiert.

5 10. Verwendung eines Impfstoffs nach einem der Ansprüche 1-9 zur Herstellung eines Medikaments zur Stärkung der Immunantwort gegen einen Eimeria-Parasiten in einem Individuum, wobei die Stärkung der Immunantwort das Verabreichen des Impfstoffs an das Individuum in einer Menge umfasst, die wirksam ist, um die Immunantwort des Individuums gegen den Eimeria-Parasiten zu verstärken.

10 11. Verwendung nach Anspruch 10, wobei die verstärkte Immunantwort eine verstärkte Antikörperantwort oder eine verstärkte T-Zell-Antwort umfasst.

15 12. Verwendung nach einem der Ansprüche 10-11, wobei der Impfstoff nicht replikationsfähig ist.

20 13. Verwendung nach einem der Ansprüche 10-12, wobei der Eimeria-Parasit Eimeria maxima ist.

14. Impfstoff nach einem der Ansprüche 1-9 zur Verwendung in einem Verfahren zur Stärkung der Immunantwort gegen einen Eimeria-Parasiten in einem Federvieh, wobei das Verfahren das Verabreichen des Impfstoffs an das Federvieh in einer Menge umfasst, die wirksam ist, um die Immunantwort des Federviehs auf den Eimeria-Parasiten zu verstärken.

#### Revendications

1. Vaccin comprenant une première séquence polynucléotidique codant pour un polypeptide TRAP ou un de ses fragments immunogéniques, dans lequel le premier polynucléotide code pour un polypeptide TRAP qui est SEQ ID NO:2 ou code pour un fragment immunogénique de SEQ ID NO:2.

2. Vaccin selon la revendication 1, comprenant en outre une seconde séquence polynucléotidique codant pour un polypeptide CD154 capable de se lier à CD40, le polypeptide CD154 ayant moins de 50 acides aminés et comprenant :

30 (i) les acides aminés 140 à 149 de SEQ ID NO:4,  
 (ii) SEQ ID NO:5,  
 (iii) SEQ ID NO:6,  
 (iv) SEQ ID NO:7,  
 35 (v) SEQ ID NO:8, ou  
 (vi) SEQ ID NO:9.

3. Vaccin selon la revendication 2, lequel vaccin comprend plus d'une copie de la première séquence polynucléotidique, plus d'une copie de la seconde séquence polynucléotidique ou plus d'une copie de la première séquence polynucléotidique et plus d'une copie de la seconde séquence polynucléotidique.

4. Vaccin selon n'importe lesquelles des revendications 2-3, dans lequel la première séquence polynucléotidique est reliée dans le cadre à la seconde séquence polynucléotidique.

45 5. Vaccin selon n'importe lesquelles des revendications 1-4, dans lequel le premier polynucléotide est compris dans un vecteur.

6. Vaccin selon n'importe lesquelles des revendications 1-5, dans lequel le premier polynucléotide est compris dans une bactérie, la bactérie comprenant le polypeptide TRAP à sa surface.

50 7. Vaccin selon n'importe lesquelles des revendications 1-6, dans lequel le premier polynucléotide est compris dans une bactérie et la bactérie est sélectionnée dans le groupe constitué par les espèces *Salmonella*, les espèces *Bacillus*, les espèces *Escherichia*, et les espèces *Lactobacillus*.

55 8. Vaccin selon la revendication 7, dans lequel la bactérie est *Salmonella enteritidis* sélectionnée parmi les souches déposées sous ATCC PTA-7871, ATCC PTA-7872 et ATCC PTA-7873.

9. Vaccin selon l'une quelconque des revendications précédentes, dans lequel la première séquence polynucléotidique

est insérée dans une séquence polynucléotidique codant pour une partie externe d'une protéine transmembranaire.

5        10. Utilisation d'un vaccin selon l'une quelconque des revendications 1-9 dans la préparation d'un médicament destiné à améliorer la réponse immunitaire contre un parasite *Eimeria* chez un sujet, l'amélioration de la réponse immunitaire comprenant l'administration au sujet du vaccin en une quantité efficace pour améliorer la réponse immunitaire du sujet au parasite *Eimeria*.

10        11. Utilisation selon la revendication 10, dans laquelle la réponse immunitaire améliorée comprend une réponse des anticorps améliorée ou une réponse des lymphocytes T améliorée.

15        12. Utilisation selon n'importe lesquelles des revendications 10-11, dans laquelle le vaccin n'est pas capable de réplication.

15        13. Utilisation selon l'une quelconque des revendications 10-12, dans laquelle le parasite *Eimeria* est *Eimeria maxima*.

20        14. Vaccin selon l'une quelconque des revendications 1-9 destiné à être utilisé dans une méthode permettant d'améliorer la réponse immunitaire contre un parasite *Eimeria* chez la volaille, la méthode comprenant l'administration à la volaille du vaccin en une quantité efficace pour améliorer la réponse immunitaire de la volaille au parasite *Eimeria*.

25

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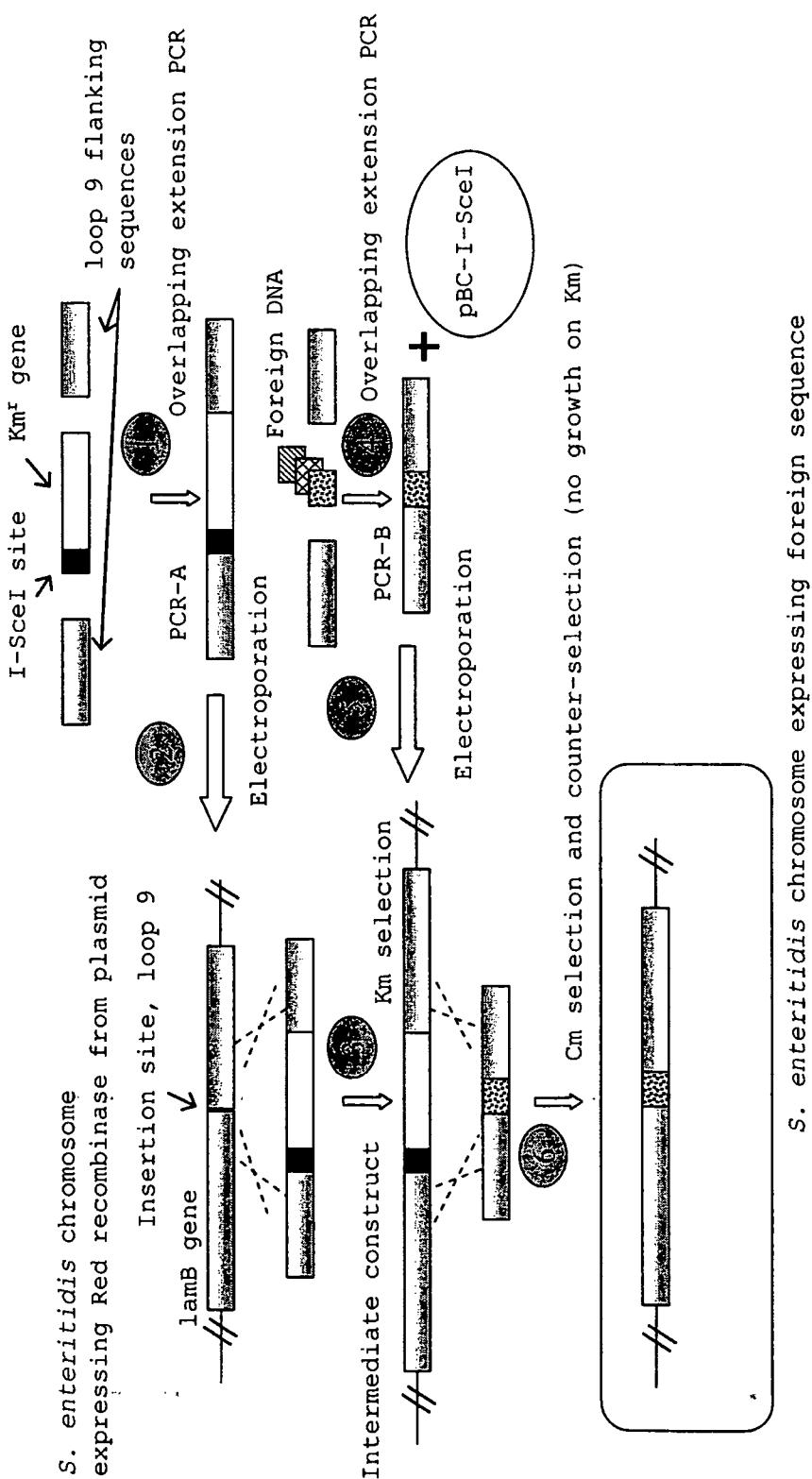
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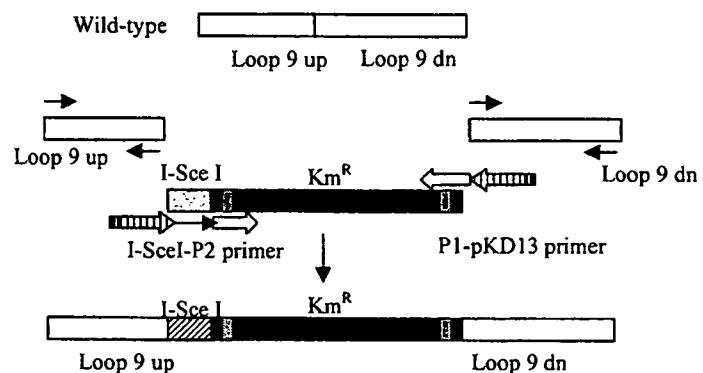
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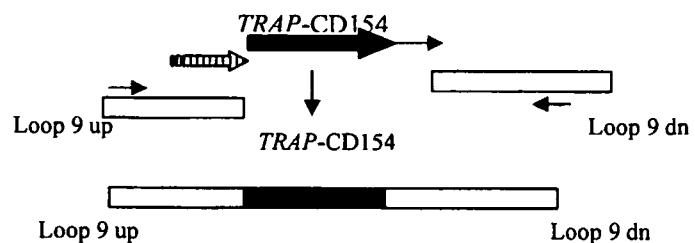
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**FIG. 1**



PCR-A

FIG. 2A



PCR-B

FIG. 2B

Mortality at 5 days post-challenge of broiler chickens challenged at 21 days of age with  $10^4$ /bird of sporulated oocyst of *E. maxima*.

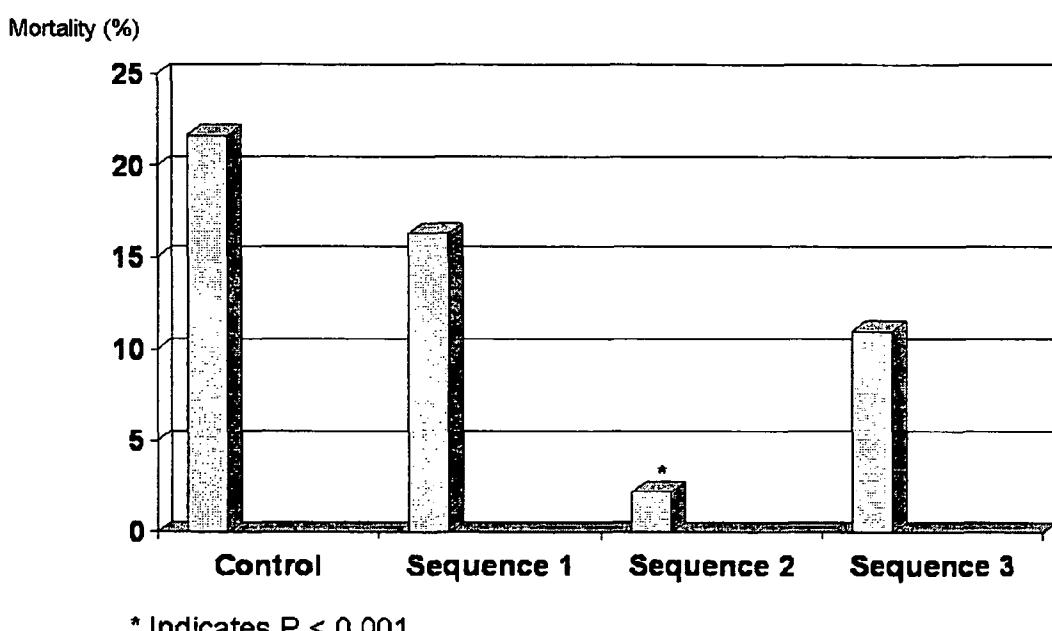


Figure 3: In this experiment, day-of-hatch chicks were either not vaccinated (control) or vaccinated with the Salmonella vector expressing Sequence 1, 2, or 3 from the *Eimeria maxima* TRAP protein as described in the appendix. All chicks were challenged with exactly the same dose of *Eimeria maxima* at day 21. Necropsy confirmed that all mortality was related to *Eimeria maxima* infection. Mortality was markedly and significantly reduced in the group vaccinated with the vector expressing sequence 2.

**REFERENCES CITED IN THE DESCRIPTION**

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**Patent documents cited in the description**

- US 60984612 B [0056]

**Non-patent literature cited in the description**

- *BMC Biotechnol.*, 17 September 2007, vol. 7 (1), 59 [0030]
- **DATSENKO ; WANNER.** *PNAS*, 2000, vol. 97, 6640-6645 [0041] [0044] [0045]
- **KANG et al.** *J Bacteriol*, 2004, vol. 186, 4921-4930 [0041]
- **HORTON et al.** *BioTechniques*, 1990, vol. 8, 528-535 [0045]

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EP2214701

**KÉSZÍTMÉNYEK ÉS ELJÁRÁSOK EIMERIA ELLENI IMMUNVÁLASZ  
FOKOZÁSÁRA**

Szabadalmi igénypontok



SZTNH-100048666

1. Vakcina, amely tartalmaz egy első polinukleotid-szekvenciát, amely kódol egy TRAP polipeptidet vagy annak egy immunogén fragmensét, ahol az első polinukleotid kódolja a SEQ ID NO:2 (azaz 2-es azonosítási számú szekvencia) szerinti TRAP polipeptidet, vagy kódolja a SEQ ID NO:2 egy immunogén fragmensét.
2. Az 1. igénypont szerinti vakcina, amely tartalmaz továbbá egy második polinukleotid-szekvenciát, amely kódol egy olyan CD154 polipeptidet, amely képes CD40-hez kötődni, a CD154 polipeptid 50-nél kevesebb aminosavat tartalmaz, és tartalmazza:
  - (i) a SEQ ID NO:4 140-149. aminosavait,
  - (ii) a SEQ ID NO:5-öt,
  - (iii) a SEQ ID NO:6-öt,
  - (iv) a SEQ ID NO:7-öt,
  - (v) a SEQ ID NO:8-öt, vagy
  - (vi) a SEQ ID NO:9-öt.
3. A 2. igénypont szerinti vakcina, ahol a vakcina egynél több kópiában tartalmazza az első polinukleotid-szekvenciát, egynél több kópiában tartalmazza a második polinukleotid-szekvenciát, vagy egynél több kópiában tartalmazza az első polinukleotid-szekvenciát és egynél több kópiában tartalmazza a második polinukleotid-szekvenciát.
4. A 2-3. igénypontok bármelyike szerinti vakcina, ahol az első polinukleotid-szekvencia in frame kapcsolódik a második polinukleotid-szekvenciához.

6. Az 1-4. igénypontok bármelyike szerinti vakcina, ahol az első polinukleotidot egy vektor tartalmazza.
6. Az 1-5. igénypontok bármelyike szerinti vakcina, ahol az első polinukleotidot egy baktérium tartalmazza, a baktérium tartalmazza a TRAP polipeptidet a felszínén.
7. Az 1-6. igénypontok bármelyike szerinti vakcina, ahol az első polinukleotidot baktérium tartalmazza, és a baktériumot a *Salmonella*-fajok, *Bacillus*-fajok, *Escherichia*-fajok és *Lactobacillus*-fajok által alkotott csoporthból választjuk.
8. A 7. igénypont szerinti vakcina, ahol a baktérium az ATCC PTA-7871, ATCC PTA-7872 és ATCC PTA-7873 számon letétbe helyezett törzsek közül választott *Salmonella enteritidis*.
9. Az előző igénypontok bármelyike szerinti vakcina, ahol az első polinukleotid-szekvencia egy olyan polinukleotid-szekvenciába van beinszertálva, amely egy transzmembrán fehérje egy externális részletét kódolja.
10. Az 1-9. igénypontok bármelyike szerinti vakcina alkalmazása olyan gyógyszer előállításában, amely alkalmazható egy alanyban *Eimeria* parazita elleni immunválasz fokozására, ahol az immunválasz fokozása tartalmazza a vakcina olyan mennyiségének beadását az alanynak, amely hatásosan fokozza az alanynak az *Eimeria* parazita elleni immunválaszát.
11. A 10. igénypont szerinti alkalmazás, ahol a fokozott immunválasz tartalmaz egy fokozott antitestes választ vagy egy fokozott T-sejtes választ.
12. A 10-11. igénypontok bármelyike szerinti vakcina alkalmazása, ahol a vakcina nem képes replikálódásra.
13. A 10-12. igénypontok bármelyike szerinti alkalmazás, ahol az *Eimeria* parazita *Eimeria maxima*.

14. Az 1-9. igénypontok bármelyike szerinti vakcina baromfiban *Eimeria* parazita elleni immunválasz fokozására szolgáló eljárásban történő alkalmazásra, az eljárás tartalmazza a vakcina olyan mennyiségének beadását a baromfinak, amely hatásosan fokozza a baromfinak az *Eimeria* parazita elleni immunválaszát.