(54) Title: DIFFERENTIATING THERAPEUTIC COMPOSITION

(57) Abstract: The present invention provides a method of tagging a subject to which at least a first antigen has been administered or will be administered, wherein the antigen is derived from an agent capable of infecting the subject and is able to either generate or assist in generating a therapeutic effect in the subject, the method comprising the step of: administering to the subject a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen
"Differentiating Therapeutic Composition"

Field of the Invention

[0001] The present invention is directed to a differentiating therapeutic composition and methods of its use in the detection of immunized and non-immunized subject. More particularly the invention provides a vaccine for immunising a subject that provides a marker to indicate that immunisation has occurred. The invention also relates to a method for immunising a subject with a vaccine such that the immunisation can be detected.

Background Art

[0002] Control programmes that use vaccination for viral outbreaks and infections must have an effective system to monitor for continued presence of viral infection within the population. However, vaccination complicates large scale surveillance for the spread of the infection by serological means, as both vaccinated and exposed subjects produce antibody specific to the virus. The antigenic similarity between the infecting virulent field strain of the virus and the viral vaccine (particularly if killed virus is used as a vaccine) hampers the discrimination between infected and vaccinated subjects as vaccination results in the occurrence and persistence of antibodies that are indistinguishable between infected and vaccinated subjects.

[0003] There are many viral diseases (e.g. Foot and Mouth Disease, Avian Influenza (Al), Newcastle disease, West Nile virus and feline immunodeficiency virus (FIV)), where monitoring of disease outbreaks and spread is hampered by the inability to distinguish infected from vaccinated subjects. Currently, the control of avian influenza spread is being managed increasingly by use of H5 avian influenza vaccination under government controlled programs in countries such as China, Indonesia, Vietnam and Hong Kong.

[0004] There is increasing world interest in DIVA (differentiating infected and vaccinated animals) vaccination strategies. For example, the joint WHO/FAO/OIE meetings on avian influenza strain H5N1 HPAI have recommended all vaccination is practiced using a DIVA, so spread of infection can be monitored. However, as
discussed below, current DIVA methods are difficult to upscale and often have problems with the differentiation of vaccination from infection with other circulating viral strains.

[0005] Current methods of monitoring include physical tagging of vaccinated animals, the use of sentinel animals, virological testing and the use of recombinant heterologous vaccines. However, these current methods have a number of limitations.

[0006] The physical tagging of vaccinated animals involves the individual identification of vaccinated subjects by physical means such as ear tags, leg bands or wing tags. However, these methods are difficult to apply on a large scale due to logistical and economic reasons. The use of unvaccinated sentinel animals is also logistically and economically difficult in many affected countries that have small scale village stocks of at-risk animals, such as poultry flocks or individual cattle. Furthermore, there is also a risk that if sentinels become infected with the virus there is increased risk of spread to humans.

[0007] Virological testing of individuals via screening and detection of live virus or RT-PCR surveillance testing is a very expensive and infrastructure heavy process, which is unsuitable for many countries, particularly poorer countries, where diseases such as avian influenza and foot and mouth disease are well established. The methods also suffer from scale-up problems. Furthermore, Virology for detection of virus and RT-PCR testing only provides information relating to the current infection of an individual subject, and does not allow analysis of the infection and/or vaccination history of that subject.

[0008] Recently, a number of recombinant heterologous vaccines termed "differentiating infected from vaccinated animals" or DIVA vaccines have been developed. After vaccination with such recombinant vaccines, vaccinated birds produce a different N antibody response than for naturally infected birds. Differentiating antibody tests are then used to determine if the subject has been infected with the wild-type virus or the recombinant virus (e.g. H5N2 vaccines may be used in areas with H5N1 infection and then birds tested for antibody to N1 as an indicator of exposure, or use of the heterologous vaccine H7N3 to vaccinate
against H7N1 disease which allows vaccinated birds to be identified by presence of N3 antibody.

[0009] However, other low pathogenic avian influenza viruses such as H9N2, H6N1, etc are circulating in water-birds and poultry in many countries currently facing wide-spread H5N1 infection. Vaccination of birds against H5N1 with, for example, a H5N2 vaccine, will not prevent subsequent infection of that bird with a low pathogenic avian influenza virus such as H6N1. As a result, DIVA antibody tests based on the N subtype antibody will give false positives if tested for N1. Furthermore, some countries are using both homologous H5N1 vaccines and heterologous H5N2 vaccines in their vaccination programs that complicate use of the DIVA serology testing. Additionally, there are 16 H types and 9 N types that may combine into avian flu subtypes, thus many new tests and new vaccine subtypes would be required for the method to be extended to each new virus subtype.

[0010] The present invention addresses a need in the art for new viral vaccines which allows the differentiation of vaccinated from infected subjects capable of at least ameliorating one or more of the problems attendant with the prior art.

General

[0011] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. The invention includes all such variations and modifications. The invention also includes all of the steps, features, formulations and compounds referred to or indicated in the specification, individually or collectively and any and all combinations or any two or more of the steps or features.

[0012] Each document, reference, patent application or patent cited in this text is expressly incorporated herein in their entirety by reference, which means that it should be read and considered by the reader as part of this text. That the document, reference, patent application or patent cited in this text is not repeated in this text is merely for reasons of conciseness.
Any manufacturer's instructions, descriptions, product specifications, and product sheets for any products mentioned herein or in any document incorporated by reference herein, are hereby incorporated herein by reference, and may be employed in the practice of the invention.

The present invention is not to be limited in scope by any of the specific embodiments described herein. These embodiments are intended for the purpose of exemplification only. Functionally equivalent products, formulations and methods are clearly within the scope of the invention as described herein.

The invention described herein may include one or more range of values (e.g., size, concentration etc). A range of values will be understood to include all values within the range, including the values defining the range, and values adjacent to the range which lead to the same or substantially the same outcome as the values immediately adjacent to that value which defines the boundary to the range.

Throughout this specification, unless the context requires otherwise, the word "comprise" or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers. It is also noted that in this disclosure and particularly in the claims and/or paragraphs, terms such as "comprises", "comprised", "comprising" and the like can have the meaning attributed to it in U.S. Patent law; e.g., they can mean "includes", "included", "including", and the like; and that terms such as "consisting essentially of and "consists essentially of have the meaning ascribed to them in U.S. Patent law, e.g., they allow for elements not explicitly recited, but exclude elements that are found in the prior art or that affect a basic or novel characteristic of the invention.

Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.
Summary of the Invention

[0018] In one form, the present invention provides a method of tagging a subject to which at least a first antigen has been administered or will be administered, wherein the antigen is derived from an agent capable of infecting the subject and is able to either generate or assist in generating a therapeutic effect in the subject, the method comprising the step of: administering to the subject a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen.

[0019] In a more preferred form, the present invention provides a method of tagging a subject that has received a first antigen derived from an infectious agent capable of infecting the subject, which antigen is capable of generating in the subject or at least assisting in the generation of a therapeutic response, the method comprising the step of: administering to the subject a tagging antigen that is detectable in the subject and which is capable of distinguishing treated from untreated subjects, which antigen is expressed exogenously from the first antigen.

[0020] In another form the invention provides a method of tagging a subject that has been administered a first antigen derived from a virus capable of infecting the subject, the method comprising the step of:

(a) administering to the subject a tagging antigen that is not derived from an organism capable of naturally infecting the subject; and

(b) wherein the tagging antigen evokes a detectable response in the subject.

[0021] In selecting the first antigen and the tagging antigen neither should interfere with the other in terms of eliciting a detectable signal such as a positive antibody response.

[0022] The present invention also provides a method of immunising a subject against an infectious agent, said method comprising the steps of:

(a) administering a first antigen to the subject, wherein the first antigen is able to either generate or assist in generating a therapeutic effect in the subject, and
(b) administering to the subject a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen identified in step (a).

[0023] More preferably the method of immunising a subject against a virus, comprises the steps of:

(a) administering to the subject at least a first antigen derived from a virus capable of infecting the subject that delivers or generates a therapeutic benefit;

(b) simultaneously administering to the subject a tagging antigen that is not derived from an organism capable of naturally infecting the subject; and

(c) wherein the tagging antigen evokes a detectable immune response in the subject.

[0024] The present invention also provides a method of immunising a subject against a virus, the method comprising the step of: administering to the subject a single dosage form comprising (a) a first antigen derived from a virus capable of infecting the subject and (b) a tagging antigen that is not derived from an organism capable of naturally infecting the subject, wherein the tagging antigen evokes a detectable immune response in the subject.

[0025] The present invention also provides a pharmaceutically acceptable formulation:

(a) a first antigen which is able to either generate or assist in generating a therapeutic effect in the subject, and

(b) a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen identified in step (a).

[0026] More preferably, the pharmaceutically acceptable formulation comprises:

(a) a first antigen derived from a virus capable of infecting the subject;

(b) a tagging antigen that is not derived from an organism capable of naturally infecting the subject, wherein the tagging antigen evokes a detectable immune response in the subject.
The present invention also provides a method of tagging a vaccine against a virus capable of infecting a subject, the method comprising the step of: combining a first antigen which is able to either generate or assist in generating a therapeutic effect in the subject, and a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen.

More preferably the method of tagging a vaccine comprises the step of:
(a) contacting the vaccine with a tagging antigen that is not derived from an organism capable of naturally infecting the subject; and
(b) wherein the tagging antigen evokes a detectable immune response in the subject.

The present invention also provides a method of identifying a subject who has been vaccinated with a formulation as herein described, the method comprising the step of: assaying a sample from the subject to detect the tag.

More preferably the identification method comprises the steps of:
(a) assaying a sample from a subject for antibodies to the tagging antigen; and
(b) wherein the presence of antibodies to the tagging antigen indicates that they have been immunized with at least the tagging antigen.

The present invention also provides a kit for immunising a subject comprising:
(a) a first antigen derived from a virus capable of infecting the subject; and
(b) a tagging antigen that is not derived from an organism capable of naturally infecting the subject.

The present invention also provides a kit comprising: a means for detecting antibodies generated against a tagging antigen that is not derived from an organism capable of infecting the subject.
Other aspects and advantages of the invention will become apparent to those skilled in the art from a review of the ensuing description, which proceeds with reference to the following illustrative drawings.

5 Brief Description of the Drawings

[0034] Figure 1 illustrates that Equivac™ T vaccinated chickens are positive for tetanus toxoid (TT) antibodies.

[0035] Figure 2 illustrates that TT + Alum vaccinated chickens display positive TT antibodies following vaccinations.

[0036] Figure 3 illustrates that TT + Alum vaccinated ducks display positive TT antibodies following booster vaccination.

[0037] Figure 4 illustrates the level of TT antibodies present in the sera of broiler chickens (35 days old) collected from abattoirs in comparison to positive controls from vaccinated chickens.

[0038] Figure 5 illustrates the level of TT antibodies present in the sera of broiler breeders (1.5 to 2 years old) collected from abattoirs in comparison to positive controls from vaccinated chickens.

[0039] Figure 6 illustrates the level of TT antibodies present in the sera of layers (1.5 to 2 years old) collected from abattoirs in comparison to positive controls from vaccinated chickens.

[0040] Figure 7 illustrates the level of TT antibodies present in the sera of Northwest Australian Sentinel chickens (older than 1 year) in comparison to positive controls from vaccinated chickens.

[0041] Figure 8 illustrates the level of TT in Hong Kong/Mainland China chickens (90 days old).

[0042] Figure 9 illustrates Kununurra duck TT antibody levels.

[0043] Figure 10 illustrates Jandakot duck TT antibody levels.
Figure 1 illustrates chicken TT antibody levels following co-delivery of avian influenza and TT vaccines.

Figure 12 illustrates HA titres in co-vaccinated chickens.

Figure 13 illustrates TT and AI titres in co-vaccinated ducks.

Figure 14 illustrates TT and AI titres in co-vaccinated ducks.

Figure 15 illustrates TT levels in vaccinated chickens.

Figure 16 illustrates HI titres in chickens following a combined vaccination of TT and AI vaccines.

**Detailed Description of the Invention**

According to the present invention the inventors have revealed that the problems with prior art can be addressed at least in part by tagging an animal with a marker or reporter that is detectable in a subject, wherein that tag is able to generate its signal independently of the expression of the first antigen. By generating a signal independently of the expression of the first antigen, the therapeutic effect of the first antigen used to treat subjects can easily be replaced without a need to substantially alter the detection system that is used in the methodologies of the invention. Moreover, by separating expression of the first antigen from the development of a detectable signal by the tag, a greater level of control can be exerted over the role of the tag and the effect of the first antigen.

**Method of tagging a subject**

According to the invention there is provided a method of tagging a subject to which at least a first antigen has been administered or will be administered, wherein the antigen is derived from an agent capable of infecting the subject and is able to either generate or assist in generating a therapeutic effect in the subject, the method comprising the step of: administering to the subject a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen.
In a more preferred form, the present invention provides a method of tagging a subject that has received a first antigen derived from an infectious agent capable of infecting the subject, which antigen is capable of generating in the subject or at least assisting in the generation of a therapeutic response, the method comprising the step of: administering to the subject a tagging antigen that is detectable in the subject and which is capable of distinguishing treated from untreated subjects, which antigen is expressed exogenously from the first antigen.

In another form, the invention provides a method of tagging a subject that has been administered with a first antigen derived from a virus capable of infecting the subject, the method comprising the step of:

(a) administering to the subject a tagging antigen that is not derived from an organism capable of naturally infecting the subject; and

(b) wherein the tagging antigen evokes a detectable response in the subject.

The virus against which the first antigen is therapeutically effective can be any virus that can infect a subject and cause disease. Preferably, the virus is a virus that can infect animal species, including but not restricted to humans and/or other higher mammals, fish and birds. The virus may be an infective virus selected from the following: a member of the Picornaviridae virus family such as an aphthovirus (e.g. the virus responsible for foot and mouth disease), a member of the Orthomyxoviridae virus family such as avian influenza, a member of the Retroviridae virus family such as FIV, a member of the Paramyxoviridae virus family such as the virus responsible for Newcastle disease, a member of the Rhabdoviridae family such as the lyssavirus causing rabies, a member of the Parvoviridae family such as the virus associated with gastroenteritis, and a member of the Papovaviridae family such as papillomaviruses responsible for tumours and warts. In one particular form of the present invention the virus is avian influenza.

In selecting the tagging antigen care should be exercised in ensuring that said antigen does not interfere with first antigen at least in terms of eliciting a
detectable signal such as a positive antibody response. The tagging antigen may however promote the first antigen response.

[0056] As used herein the phrase "antigen that is not derived from an organism capable of naturally infecting the subject" refers generally to an antigen derived from an organism that is not widely observed and found to infect members of a cohort of which the subject is a member. In accordance with this understanding, the detectability of the antigen must not be masked by a natural infected state in the subject. More particularly, the signal generated by the tagging antigen must exceed any noise generated by an innate infection from the organism in members of the cohort to which the subject belongs. Thus, the tagging antigen must be detectable in an immunized animal. Preferably, the noise from the inadvertent presence of the tagging antigen will be low in the population of animals treated. Further the tagging antigen should not induce an adverse immunological response that will harm the subject.

[0057] In the selection of the first antigen and the tagging antigen, care needs to be taken to ensure that the two antigens do not interfere with each other in terms of either's capacity to elicit positive antibody responses.

[0058] The subject may be varied but is preferably selected from the group comprising: mammals, birds and fish. More preferably, the subject is a mammal such as farm animals including sheep, goats, pigs, cows, horses, llamas, household pets such as dogs and cats, and primates; humans; birds, such as chickens, geese and ducks and fish.

[0059] The method of the present invention may be employed, for example, when a subject has been vaccinated with a first antigen from an infective virus and is protected against further infection from that virus. The subject may later be "tagged" by vaccination with the tagging antigen to indicate that, if antibodies are detected to the first antigen in that subject, these antibodies result from vaccination not infection.

[0060] While one of ordinary skill will appreciate that the tagging antigen may be endogenously expressed with the first antigen, in a preferred form of the
invention the tagging antigen is exogenously expressed relative to the first antigen.

[0061] As used herein the phrase "which antigen is expressed exogenously from the first antigen" refers generally to expression of a first antigen in a manner which does not involve or depend upon the means for producing the tagging antigen. Preferably, the tagging antigen is expressed independently to the first antigen. More preferably, a second expression vehicle is employed to express the tagging antigen.

[0062] The present invention also provides a method of tagging a subject that has been administered with a first antigen derived from an infectious agent capable of infecting the subject, which antigen is capable of generating in the subject a therapeutic response, the method comprising the step of: administering to the subject a tagging antigen that is detectable in the subject and which is capable of distinguishing treated from untreated subjects, which antigen is expressed exogenously from the first antigen.

[0063] According to the method of the invention the first antigen will generally provide the subject with a therapeutic benefit. Preferably, the first antigen is used to generate a protective or a neutralising response as might be achieved from a vaccine, however, any type of therapeutic benefit can be achieved by the first antigen. In accordance with this effect of the first antigen, one of ordinary skill will appreciate that the first antigen may comprise multiple antigenic parts capable of generating multiple immunological responses. The first antigen may also be a single antigen. However, it will be appreciated that subjects will generally be administered with a plurality of antigens such as a mixture of two or more antigens. In this regard, some vaccines are designed to contain multiple antigens to evoke the most potent antibody response in the subject.

[0064] In a highly preferred form of the invention the first antigen may be derived from a source selected from the group comprising: whole inactivated virus or live attenuated virus or a viral agent that has been recombinantly manufactured to delete its virulence, while still preserving its native immunological characteristics. Preferably, the first antigen is derived from a naturally occurring
virus. However, it may also be derived from a genetically modified or recombinant
form of a naturally occurring virus.

[0065] Preferably, the first antigen is capable of evoking protective antibodies in
the subject and thus preventing the subject from infection with the virus. When
the virus is avian influenza virus, hemagglutinin in combination with
neuraminidase and/or an envelope protein from West Nile virus may comprise the
first antigen. Alternatively, the first antigen may be selected from the group
comprising: internal conserved viral antigens, e.g. matrix or nucleoprotein. Another
example of a first antigen, when the virus is avian influenza, is a combination of
H5 and N2.

Tagging system

[0066] As noted herein a requirement of the tagging system is that the tag is
capable of distinguishing immunized from non-immunized animals. Tags that may
be employed in the method of the invention will be known to those of ordinary
skill. They include any method of marking or reporting the presence of an
immunized animal from a non-immunized animal. Desirably, the tag will be an
innocuous marker with no discernible toxicity that is strongly detectable and can
enable the rapid isolation of a population of immunized subjects. For example,
the marker may be a genetic marker or an expressed protein marker or an
immunological marker.

[0067] To the extent that the tagging system relies upon genetic markers, the
profile of such markers can be obtained by techniques such as Restriction
Fragment Length Polymorphisms (RFLPs), Randomly Amplified Polymorphic
DNAs (RAPDs), Arbitrarily Primed Polymerase Chain Reaction (AP-PCR), DNA
Amplification Fingerprinting (DAF), Sequence Characterized Amplified Regions
(SCARs), Amplified Fragment Length Polymorphisms (AFLPs), Simple Sequence
Repeats (SSRs) which are also referred to as Microsatellites, and Single
Nucleotide Polymorphisms (SNPs). For example, see Berry, Don, et al.,
"Assessing Probability of Ancestry Using Simple Sequence Repeat Profiles:
Applications to Maize Hybrids and Inbreds", Genetics, 2002, 161:813 824, and
Berry, Don, et al., "Assessing Probability of Ancestry Using Simple Sequence
Repeat Profiles: Applications to Maize Inbred Lines and Soybean Varieties”, Genetics, 2003, 165: 331-342, which are incorporated by reference herein.

[0068] To the extent that the tagging system depends on a protein marker system the tag may be a protein that is not ordinarily expressed in the subject population, but for which there is a biological test. For example, the protein may be detected by a marked monoclonal antibody. Alternatively, the tag may be a sequence for green, yellow or blue fluorescent protein genes (GFP, YFP, and BFP, respectively).

[0069] In a highly preferred form of the invention the tagging system is based on an immunological marker. Preferably the immunological marker is a tagged antigen derived from an organism that is incapable of infecting the subject. For example, the tagging antigen for a foot and mouth disease vaccine could comprise an antigen from an organism that doesn't naturally infect cattle. Alternatively, the antigen could be an artificial antigen.

[0070] Preferably, the tagging antigen is derived from the causative organism of tetanus for example tetanus toxoid. In this regard, avian species are not susceptible to the tetanus toxoid (the toxic dose for birds is approximately 350,000 times the toxic dose for horses). Furthermore, avian species are not infected or affected by Clostridium tetani, but they do produce an antibody response to the antigen. Alternatively, the tagging antigen could be selected from another organism not infecting or affecting the species, such as the diphtheria toxoid from Corynebacterium diphthehae.

[0071] Preferably, the tagging antigen is an existing antigen that is already in mass production and has already been registered for administration to humans and/or animals. Even more preferably, the tagging antigen is amenable to large scale production at relatively low cost.

[0072] Preferably, the tagging antigen is incapable of infecting any member of a given genus or species of subject and thus may be used in a wide range of vaccines or vaccines that are capable of immunising a diverse range of subjects.
Administration of antigens

[0073] The present invention also provides a method of immunising a subject against an infectious agent, said method comprising the steps of:

(a) administering a first antigen to the subject, wherein the first antigen is able to either generate or assist in generating a therapeutic effect in the subject, and

(b) administering to the subject a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen identified in step (a).

[0074] When delivered to an animal, the first antigen and the marker or reporter can be co-administered or administered simultaneously or sequentially in any order.

[0075] Preferably, the first antigen and tagging antigen are co-administered or administered simultaneously. Thus, the present invention also provides a method of immunising a subject against a virus, the method comprising the steps of:

(a) administering to the subject a first antigen derived from a virus capable of infecting the subject; and

(b) co-administering or simultaneously administering to the subject a tagging antigen that is not derived from an organism capable of naturally infecting the subject, wherein the tagging antigen evokes a detectable immune response in the subject.

[0076] For the purposes of the present invention simultaneous administration means administration at substantially the same time or within 24 hours.

[0077] When the antigens are administered simultaneously it is preferable that the antigens be administered in a single dosage form.

[0078] The present invention also provides a method of immunising a subject against an infectious agent, said method comprising the steps of:
(a) administering a first antigen to the subject, wherein the first antigen is able to either generate or assist in generating a therapeutic effect in the subject; and

(b) administering to the subject a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen identified in step (a).

[0079] More preferably, the method of immunising a subject against a virus, comprises the steps of:

(a) administering to the subject at least a first antigen derived from a virus capable of infecting the subject that delivers or generates a therapeutic benefit;

(b) simultaneously administering to the subject a tagging antigen that is not derived from an organism capable of naturally infecting the subject, wherein the tagging antigen evokes a detectable immune response in the subject.

[0080] The present invention also provides a method of immunising a subject against a virus, the method comprising the step of: administering to the subject a single dosage form comprising (a) a first antigen derived from a virus capable of infecting the subject and (b) a tagging antigen that is not derived from an organism capable of naturally infecting the subject, wherein the tagging antigen evokes a detectable immune response in the subject.

[0081] The single dosage form may comprise an admixture or other combination of a formulation comprising the first antigen and a formulation comprising the tagging antigen. Alternatively, the single dosage form may comprise a formulation containing the first antigen and the tagging antigen.

[0082] According to the invention recombinant constructs comprising the first antigen or the tagging antigen may be delivered to a subject in, for example, a live attenuated virus or a non-pathogenic virus related to the infective virus. Such live attenuated virus or non-pathogenic virus multiplies in the vaccinated subject and can provide continuous antigenic stimulation over a period of time by expression of both the first and tagging antigen. The virus expressing the recombinant
construct of the first antigen and tagging antigen may also be further treated to provide an inactivated whole virus vaccine.

[0083] The first and/or tagging antigen may also be in the form of a DNA vaccine. Thus, the first antigen and/or the tagging antigen may be delivered as naked DNA for vaccination. Preferably, the DNA vaccine comprises a recombinant construct comprising the first or the tagging antigen. The DNA vaccine construct may further comprise plasmid DNA.

[0084] Alternatively, the first antigen and tagging antigen may be administered simultaneously as two or more separate dosage forms. In this form of the invention the subject receives multiple dosage forms simultaneously.

[0085] The selection of the first antigen and tagging antigen and the manner of their administration is important in that the antigens should not interfere with the actions of each other upon administration. Preferably, the first antigen and tagging antigen work co-operatively or synergistically in that the action of at least one of the antigens is enhanced by the presence of the other. Most preferably, the tagging antigen boosts the response of the vaccinated subject to the first antigen, whilst still evoking a detectable immune response against itself.

[0086] Preferably, the antigens are delivered in a single dose but the antigens may be delivered in more doses over a period of time. Multiple doses may be administered as is required to maintain a state of immunity to the infective virus. Generally, where multiple administrations are necessary a vaccination regimen may comprise an initial dose of the vaccine followed by from one to four booster inoculations given at intervals of two to four weeks. Following an initial vaccination, subjects may receive a boost in about 4 weeks.

30 Formulations/route of administration/dosages

[0087] The present invention also provides a pharmaceutically acceptable formulation:

(a) a first antigen which is able to either generate or assist in generating a therapeutic effect in the subject, and
(b) a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen identified in step (a).

More preferably, the pharmaceutically acceptable formulation comprises:

(a) a first antigen derived from a virus capable of infecting the subject;
(b) a tagging antigen that is not derived from an organism capable of naturally infecting the subject, wherein the tagging antigen evokes a detectable immune response in the subject.

Preferably, the formulation is a vaccine that is able to confer protective immunity on the subject. Even more preferably, the formulation has therapeutic qualities in that it can be administered to a subject that has already been infected with the virus and causes an improvement in the health of the subject.

The first and/or tagging antigen may be conjugated or linked to another peptide or to a polysaccharide. For example, immunogenic proteins well-known in the art may be employed. Useful immunogenic proteins include keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA), ovalbumin, human serum albumin (HAS), human gamma globulin, chicken immunoglobulin G and bovine gamma globulin. Useful immunogenic polysaccharides include group A Streptococcal polysaccharide, C-polysaccharide from group B Streptococci, or the capsular polysaccharides of Streptococcus pneumoniae or group B Streptococci.

The formulations of the present invention may be administered by any suitable route, with the specific route being largely dictated by the manner in which the antigens have been formulated for delivery. Thus, the antigens may be administered parenterally, usually subcutaneously (SC) intramuscularly (IM), intravenously (IV), intraperitoneally (IP) or intradermally (ID) in an appropriate vehicle. Other modes of administration, however, such as oral delivery, intranasal delivery or delivery by suppository, are also acceptable. Alternatively, the formulations of the present application may be delivered in ovo directly into embryonated eggs for vaccines against avian diseases.
Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers (such as those based on Ringer's dextrose), and the like. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%.

Intranasal formulations may include vehicles that neither cause irritation to the nasal mucosa nor significantly disturb ciliary function. Diluents such as water, aqueous saline or other known substances can be employed with the subject invention. The nasal formulations may also contain preservatives such as, but not limited to, chlorobutanol and benzalkonium chloride. A surfactant may be present to enhance absorption of the subject proteins by the nasal mucosa.

Additionally, the vaccine may be delivered orally. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Capsules, tablets and pills for oral administration to a patient may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.
Oral solid dosage forms as described herein are described generally in Martin, Remington's Pharmaceutical Sciences, 18th Ed. (1990 Mack Publishing Co. Easton PA 18042) at Chapter 89, which is herein incorporated by reference. Solid dosage forms include tablets, capsules, pills, troches or lozenges, cachets or pellets. Also, liposomal or proteinoid encapsulation may be used to formulate the present compositions (as, for example, proteinoid microspheres reported in U.S. Patent No. 4,925,673). Liposomal encapsulation may be used and the liposomes may be derivatised with various polymers (E.g., U.S. Patent No. 5,013,556). A description of possible solid dosage forms for the therapeutic is given by Marshall, in Modern Pharmaceutics, Chapter 10, Banker and Rhodes ed., (1979), herein incorporated by reference. In general, the formulation will include inert ingredients which allow for protection against the stomach environment, and release of the biologically active material in the intestine.

Oral liquid preparations may be in the form of, for example, aqueous or oily suspension, solutions, gels, emulsions, syrups or elixirs, or a product for reconstitution with water or other suitable vehicle before use. Such preparations may contain conventional additives such as suspending agents, emulsifying agents, non-aqueous vehicles (which may include edible oils), or preservatives. Alternatively, the vaccine may be in the form of a component of a food carrier material, such as a pudding or yogurt. In particular a live vaccine would require packaging in a form that would allow delivery into the human alimentary tract as whole virions that could be taken up in at least the first part of the alimentary tract, i.e., the oral cavity, or at other sites, such as the intestine. Said formulation may include gelatin, cellulose, or a variety of other excipients as ingredients, or the formulation may be a gel or a food carrier such as a pudding or similar formulation that would include the virus as a component. As another embodiment, flavourings, emulsifiers, or other additives may be included in the formulation of the product, the delivery vehicle or other components of the packaged material. The vaccine of the present invention can be packaged as a solution, as single doses, as a paste or gel, or in a food or nutritional substance in a plastic container, pillow-pack, tear-pack, straw tube packaging or other suitable packaging for the liquid, gel or food carrier formulation.
[0098] The dosage or effective amount is sufficient to prevent, ameliorate or reduce the infection of the subject with the infective virus and/or generate an antibody response to the tagging antigen. The effective amount is readily determined by one skilled in the art. The active ingredient may typically range from about 0.01 % to about 95% (w/w) of the composition, or even higher or lower if appropriate! Preferably, the active amount of the first antigen and the tagging antigen will be less than about 10% (w/w) of the composition, more preferably less than about 1% (w/w) of the composition, most preferably less than about 0.05% (w/w) of the composition.

[0099] The amount of antigen in each dose is selected as an amount that induces an immuno-protective response to the first antigen and an immune response to the tagging antigen without significant adverse side effects in a typical vaccination subject. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 0.1-1000 µg of protein, preferably 0.2-200 µg. An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of antibody titres and other responses in subjects.

[00100] The quantity to be administered depends upon factors such as the age, weight and physical condition of the subject considered for vaccination. The quantity also depends upon the capacity of the subject's immune system to synthesize antibodies, the degree of protection desired and the degree of response to the tagging antigen required. Effective dosages can be readily established by one of ordinary skill in the art through routine trials to establish dose response curves.

Formulation additives

[00101] Typically, vaccine formulations, such as those used in the method of the present invention, are prepared as injectables, either as liquid solutions or suspensions. However, solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes.

The active immunogenic ingredients are often mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants that enhance the effectiveness of the vaccine.

Suitable adjuvants include but are not limited to: aluminium hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion; surfactants, e.g., hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-diocytadecyl-N'-N-bis(2-hydroxyethyl-propane di-amine), methoxyhexadecyl-glycerol, and pluronic polyols; polanions, e.g., pyran, dextran sulfate, poly IC, polyacrylic acid, carbopol; peptides, e.g., muramyl dipeptide, MPL, aminomethylglycine, tuftsin, oil emulsions, alum, and mixtures thereof. Other potential adjuvants include the B peptide subunits of E. coli heat labile toxin or of the cholera toxin. McGhee, J. R., et al., "On vaccine development," Sem. Hematol., 30:3-15 (1993).

Further examples of adjuvants and other agents include aluminium hydroxide, aluminium phosphate, aluminium potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions,
muramyl dipeptide, bacterial endotoxin, lipid X, *Corynebacterium parvum* (*Propionobacterium acnes*), *Bordetella pertussis*, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin, liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.) or Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Michigan).

Typically, adjuvants such as Amphigen (oil-in-water), Alhydrogel (aluminium hydroxide), or a mixture of Amphigen and Alhydrogel are used. Only aluminium hydroxide is approved for human use.

The proportion of immunogen and adjuvant can be varied over a broad range so long as both are present in effective amounts. For example, aluminium hydroxide can be present in an amount of about 0.5% of the vaccine mixture (Al2O3 basis). Conveniently, the vaccines are formulated to contain a final concentration of immunogen in the range of from 0.2 to 200 µg/ml, preferably 5 to 50 µg/ml.

After formulation, the vaccine may be incorporated into a sterile container that is then sealed and stored at a low temperature, for example 4 °C, or it may be freeze-dried. Lyophilisation permits long-term storage in a stabilised form.

Preferably a carrier is also present in the vaccine composition according to the invention. The carrier may be an oil-in-water emulsion, or an aluminium salt, such as aluminium phosphate or aluminium hydroxide. 

In a particularly preferred aspect, the antigens in the vaccine composition according to the invention are combined with the adjuvant 3-de-O-acylated monophosphoryl lipid A (3D-MPL) and the aluminium salt, alum. Typically for human administration, the adjuvants QS21 and 3D-MPL will be present in a vaccine in the range of 1 µg-200 µg, such as 10-100 µg, preferably 10 µg-50 µg per dose.
If the vaccine is delivered in a non-toxic oil-in-water emulsion, the oil-in-water emulsions preferably contain a non-toxic oil, e.g. squalene, squalene and/or alpha tocopherol, an emulsifier, e.g. Tween 80, and an aqueous carrier. The aqueous carrier may be, for example, phosphate buffered saline. Additionally the oil-in-water emulsion may contain span 85 and/or lecithin and/or tricaprylin. Typically, the oil-in-water will comprise from 2 to 10% squalene, from 2 to 10% alpha tocopherol and from 0.3 to 3% Tween 80. Preferably the ratio of squalene:alpha tocopherol is equal to or less than 1 as this provides a more stable emulsion. Span 85 may also be present at a level of 1%. In some cases it may be advantageous that the vaccines of the present invention will further contain a stabiliser. A particularly potent adjuvant formulation involving QS21, 3D-MPL and tocopherol in an oil-in-water emulsion is described in WO95/17210.

The therapeutic regimens and pharmaceutical formulations of the invention may be coadministered with additional immune response enhancers or biological response modifiers including, but not limited to, the cytokines IFN-alpha, IFN-gamma, IL-2, IL-4, IL-6, TNF, or other cytokine-affecting immune cells. In accordance with this aspect of the invention, the formulations of the invention are administered in combination therapy with a therapeutically active amount of one or more of these cytokines. As used herein, the term "cytokine" is meant to mean any secreted polypeptide that influences the function of other cells mediating an immune response. Accordingly, it is contemplated that the the formulations of the invention can be co-administered with a cytokine to enhance the immune response. Preferred cytokines include, but are not limited to, interleukin-1 alpha, (IL-1-\(\alpha\)), interleukin-1 beta (IL-1-\(\beta\)), interleukin-2 (IL-2), interleukin-3 (IL-3), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-7 (IL-7), interleukin-8 (IL-8), interleukin-9 (IL-9), interleukin-10 (IL-10), interleukin-11 (IL-11), interleukin-12 (IL-12), interferon-alpha (IFN-alpha), interferon-beta (IFN-beta), interferon-gamma (IFN-gamma), tumor necrosis factor-alpha (TNF-alpha), tumor necrosis factor-beta (TNF-beta), granulocyte colony stimulating factor (G-CSF), granulocyte/macrophage colony stimulating factor (GM-CSF), and transforming growth factor-beta (TGF-beta).
The first and/or tagging antigen may be formulated into the vaccine as a neutral or salt form. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of protein antigens) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, mandelic, tartaric and maleic. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine and procaine.

**Method of Tagging a Vaccine.**

As indicated above, the formulations of the present invention can be formed by admixing or otherwise combining an existing vaccine with a tagging antigen of the present invention. Thus, the present invention also provides a method of tagging a vaccine against a virus capable of infecting a subject, the method comprising the step of: contacting the vaccine with a tagging antigen that is not derived from an organism capable of naturally infecting the subject, wherein the tagging antigen evokes a detectable immune response in the subject.

Preferably, the tagging antigen is simply added to the vaccine to form the tagged vaccine.

**Vectors, Host Cells etc**

The present invention also provides a vector containing a polynucleotide encoding the tagging antigen or the first antigen. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus, in a further embodiment, the invention provides a method of making polynucleotides encoding the tagging antigen or first antigen by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells.
[00118] Preferably, the vector further comprises a control sequence that is operably linked to and capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" means that the components described are in a relationship permitting them to function in their intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

[00119] The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators.

[00120] Vectors of the invention may be transformed or transfected into a suitable host cell as described below to provide for expression of a protein of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding the protein, and optionally recovering the expressed protein.

[00121] The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin or kanamycin resistance gene for a mammalian vector. Vectors may be used, for example, to transfect or transform a host cell.

[00122] Control sequences operably linked to sequences encoding the protein of the invention include promoters/enhancers and other expression regulation signals. These control sequences may be selected to be compatible with the host cell for which the expression vector is designed to be used in. The term "promoter" is well-known in the art and encompasses nucleic acid regions ranging in size and complexity from minimal promoters to promoters including upstream elements and enhancers.
The promoter is typically selected from promoters which are functional in mammalian cells, although prokaryotic promoters and promoters functional in other eukaryotic cells may be used. The promoter is typically derived from promoter sequences of viral or eukaryotic genes. For example, it may be a promoter derived from the genome of a cell in which expression is to occur.

It may also be advantageous for the promoters to be inducible so that the levels of expression of the heterologous gene can be regulated during the lifetime of the cell. Inducible means that the levels of expression obtained using the promoter can be regulated.

In addition, any of these promoters may be modified by the addition of further regulatory sequences, for example enhancer sequences. Chimeric promoters may also be used comprising sequence elements from two or more different promoters described above.

Vectors of the invention may be introduced into host cells for the purpose of replicating the vectors/polynucleotides and/or expressing the antigens of the invention encoded by the polynucleotides of the invention. Although the antigens of the invention may be produced using prokaryotic cells as host cells, it is preferred to use eukaryotic cells, for example yeast, insect or mammalian cells, in particular mammalian cells.

Preferably, the recombinant construct is in the form of a viral particle able to infect a host cell. Alternatively, the recombinant construct is in the form of a non-replicating plasmid construct or naked DNA vaccine.

Method of Identifying a Tagged Subject

The subjects who are subjected to the method of the present invention or who are administered one of the formulations described herein can be later identified. Thus, the present invention also provides a method of identifying a subject who has been vaccinated with a formulation comprising a first antigen derived from a virus capable of infecting the subject; and a tagging antigen that is not derived from an organism capable of naturally infecting the subject the method
comprising the step of assaying a sample to detect the tagging antigen. The method used to detect the tagging antigen will depend on the nature of the tag. If for example the antigen is merely used to generate antibodies the method would comprise the step of: assaying a sample to detect from the subject antibodies to the tagging antigen, wherein the presence of antibodies to the tagging antigen indicates that they have been immunized with at least the tagging antigen.

[00129] The method may further comprise assaying the sample for the first antigen.

[00130] The presence of antibodies to the first antigen indicates that the subject has been exposed to antigens from the infective virus, either through infection or by vaccination, but antibodies to the tagging antigen in a subject indicates that they have been immunized with at least the tagging antigen, as the antigen is not derived from an organism that infects the subject.

[00131] Preferably, antibodies to the first and/or tagging antigen are detected using enzyme-linked immunosorbent assay (ELISA), in which antibodies are bound to a solid phase and an enzyme-antigen conjugate is used to detect and/or quantify antibody present in the sample. Alternatively, a Western blot assay can be used in which solubilized and separated antigen(s) is bound to nitrocellulose paper. The antibody then is detected by an enzyme or label-conjugated anti-immunoglobulin (Ig), such as horseradish peroxidase-Ig conjugate by incubating the filter paper in the presence of a precipitable or detectable substrate. Western blot assays have the advantage of not requiring purity greater than 50% for the desired antigen(s). Descriptions of ELISA and western blot techniques are found in Chapters 10 and 11 of Ausubel, et al. (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley and Sons (1988). Alternatively, the antibodies can be detected by an immunofluorescent antibody test, such as those well known to the skilled person.
Kits

[00132] The method of immunising a subject according to the present invention and components thereof can be conveniently applied in kit form. Thus, the present invention also provides a kit for immunising a subject comprising:

(a) a first antigen derived from a virus capable of infecting the subject; and
(b) a tagging antigen that is not derived from an organism capable of naturally infecting the subject.

[00133] The method of identifying a tagged subject according to the present invention may also be carried out using a suitable kit. Thus, the present invention also provides a kit comprising: a means for detecting antibodies generated against a tagging antigen that is not derived from an organism capable of infecting the subject.

[00134] Preferably, the kit further comprises a means for detecting antibodies generated against a first antigen derived from a virus capable of infecting the subject.

Examples

[00135] The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these methods in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes.

Example 1: Immunisation of chickens with tetanus toxoid vaccine

Materials and Methods

[00136] Animals and housing conditions: Chickens: 6-7 week old female Hy-Line Brown layer pullets were obtained (Altona Hatchery Pty. Ltd., Forrestfield, WA) and randomly distributed into groups of 5 chickens per pen. Pens were built off the ground and were made of steel wire. All chickens were kept in pens next to each other in identical sized pens.
All animals were housed at the Department of Agriculture Research Station at Medina, Western Australia, following strict animal ethics guidelines (AEC Number: 6-05-55 and 4-06-37).

Vaccination: At weeks 0 and 4, birds were vaccinated subcutaneously (s.c.) by injection using syringes fitted with 21G needles. For Figure 1, birds were vaccinated with 1 mL or 0.5 mL of Equivac™ T (CSL Ltd., Australia) purified, alum-adjuvanted tetanus toxoid (TT) vaccine. For Figure 2, birds were vaccinated with a mixture of a crude, unpurified TT (Pfizer, WA) with an alum adjuvant (1 mg Aluminium Hydroxide will bind 50-200 µg antigen) included. A range of TT doses were used to determine an optimal dose.

Blood collection: Blood samples were collected from the wing veins at 0, 2, 4, 6 weeks post-primary vaccination by venipuncture.

Blood samples from birds were decanted from syringes into glass or serum clot activator-treated plastic vacutainers (Greiner Bio-One, NC, USA) and left to separate overnight at 18-22 °C. Tubes were centrifuged at 1000g for 10 minutes at room temperature and serum was harvested by pipetting. Sera was then stored at 4 °C prior to use in assays before longer term storage at -20 °C (after 1 month) and -80 °C (more than 6 months).

Determination of TT antibody titres: The determination of TT antibody titres in chickens was performed using harvested sera in an indirect ELISA (Example 5). Immunosorbent ELISA plates (Greiner BioOne, Germany) were coated overnight at 4 °C in a humidified chamber with formaldehyde-inactivated, purified TT antigen [0.0125 µg/100 µL] (List Biological Laboratories, Inc., CA, USA) or with unpurified TT antigen [14.58 µg/100 µL] (Pfizer, Western Australia) in 0.05M carbonate buffer, pH 9.6.

Statistical analysis: Statistical analysis on all data was performed using the Students t-test assuming unequal variance between the means. P values less than 0.01 were deemed to be significant.
Results

[00143] Pullets (10 per group) were obtained directly from a commercial hatchery and subcutaneously vaccinated with either 1.0 ml or 0.5 mL of EquivacTM T vaccine. Sera samples were assayed for anti-TT antibodies (see Figure 1). No pre-existing tetanus toxoid antibodies were present in the chickens prior to immunisation. Two weeks after the first vaccination, both groups of vaccinated chickens successfully produced more than 100% of anti-TT antibodies compared to controls. The level of TT antibodies decreased in titre 4 weeks after the initial vaccination, but levels were restored at 6 weeks following a booster immunization given one month after the first vaccination.

[00144] A subsequent experiment involving 50 chickens was performed to assess the optimum dose of TT administered as a vaccine (see Figure 2). In this experiment, TT was obtained from a pharmaceutical company which provided the raw material for the commercial vaccine. This TT preparation was mixed with aluminium hydroxide (alum) in the laboratory. This vaccine is similar to the commercial vaccine used in the previous study. A range of TT doses was used in order to assess the optimum dose which elicited the highest TT antibody levels.

[00145] Figure 2 shows that after 2 vaccinations, antibody levels to TT were up to 70%. There were no significant differences (p>0.01) between levels of TT antibodies obtained in all doses tested. The 0.3 mg TT dose appeared to be as good as the 3 mg TT dose in eliciting and maintaining TT antibody levels.

Example 2: Immunisation of ducks with tetanus toxoid vaccine

Materials and Methods

[00146] Animals and housing conditions: Ducks: 6-8 week old Muscovy ducklings were obtained from local backyard breeders in Western Australia. Ducks were kept in pens with straw strewn on a concrete ground.

[00147] All animals were housed at the Department of Agriculture Research Station at Medina, Western Australia, following strict animal ethics guidelines (AEC Number: 6-05-55 and 4-06-37).
Vaccination: At weeks 0 and 4, birds were vaccinated s.c. by injection using syringes fitted with 21G needles. For Figure 3, birds were vaccinated with a mixture of a crude, unpurified TT (Pfizer, WA) with an alum adjuvant (1mg Aluminium Hydroxide will bind 50-200 µg antigen) included. A range of TT doses were used to determine an optimal dose.

Blood collection: Blood samples were collected from the wing veins at 0, 2, 4, 6 weeks post-primary vaccination by venipuncture.

Blood samples from birds were decanted from syringes into glass or serum clot activator-treated plastic vacutainers (Greiner Bio-One, NC, USA) and left to separate overnight at 18-22 °C. Tubes were centrifuged at 1000g for 10 minutes at room temperature and serum was harvested by pipetting. Sera was then stored at 4 °C prior to use in assays before longer term storage at -20 °C (after 1 month) and -80 °C (more than 6 months).

Determination of TT antibody titres: The determination of TT antibody titres in ducks was performed using harvested sera in an indirect ELISA (Example 5). Immunosorbent ELISA plates (Greiner BioOne, Germany) were coated overnight at 4 °C in a humidified chamber with formaldehyde-inactivated, purified TT antigen [0.0125 µg/100 µL] (List Biological Laboratories, Inc., CA, USA) or with unpurified TT antigen [14.58 µg/100 µL] (Pfizer, Western Australia) in 0.05M carbonate buffer, pH 9.6.

Statistical analysis: Statistical analysis on all data was performed using the Students t-test assuming unequal variance between the means. P values less than 0.01 were deemed to be significant.

Results

An experiment involving 40 ducks was performed to investigate the ability of ducks to produce antibodies to TT following vaccination. Following the same method as that used in the chicken experiment shown in Figure 2, ducks were vaccinated with varying doses of a TT and alum vaccine. Serum samples
collected demonstrated (see Figure 3) that ducks successfully produced antibodies to TT following the booster vaccination. For non-chicken assays, a competitive ELISA was used where inhibition of more than 50% was deemed significant for the presence of anti-TT antibodies.

Example 3: Presence of natural antibody to tetanus toxoid in birds

Materials and Methods

[00154] Animals and housing conditions: Chickens: 6-7 week old female Hy-Line Brown layer pullets were obtained from Altona Hatchery Pty. Ltd., Forrestfield, WA.

[00155] Ducks: 6-8 week old Muscovy ducklings were obtained from local backyard breeders in Western Australia.

[00156] Miscellaneous birds: Blood from Broiler Breeders, Broilers and Layer chickens were collected from two commercial abattoirs in Western Australia. Sentinel backyard chickens from various locations in the Northwest region of Western Australia and wild Plumed Whistling Ducks trapped at Kununurra were also bled to determine if pre-existing tetanus antibodies were present in their sera.

[00157] Blood collection: Blood samples were collected from the wing veins by venipuncture. Blood from abattoir-slaughtered poultry was collected from the jugular vein. Blood samples from the wing vein obtained from birds in the Northwest of Western Australia were collected by Dr Cheryl Johansen's research team at The University of Western Australia.

[00158] Blood samples from birds were decanted from syringes into glass or serum clot activator-treated plastic vacutainers (Greiner Bio-One, NC, USA) and left to separate overnight at 18-22 0C. Tubes were centrifuged at 1000g for 10 minutes at room temperature and serum was harvested by pipetting. Sera was then stored at 4 0C prior to use in assays before longer term storage at -20 °C (after 1 month) and -80 °C (more than 6 months).
Determination of TT antibody titres: The determination of TT antibody titres was performed using harvested sera in an indirect ELISA. Immunosorbent ELISA plates (Greiner BioOne, Germany) were coated overnight at 4 °C in a humidified chamber with formaldehyde-inactivated, purified TT antigen [0.0125 µg/100 µL] (List Biological Laboratories, Inc., CA, USA) or with unpurified TT antigen [14.58 µg/100 µL] (Pfizer, Western Australia) in 0.05M carbonate buffer, pH 9.6.

Statistical analysis: Statistical analysis on all data was performed using the Students t-test assuming unequal variance between the means. P values less than 0.01 were deemed to be significant.

Results

Poultry from various geographical locations and origins were assayed for the presence of TT antibody in the absence of tetanus vaccinations (see Figures 4-10). A summary of the various poultry screened is shown in Table 1.

Table 1: Poultry screened and number positive for tetanus antibodies

<table>
<thead>
<tr>
<th>Poultry</th>
<th>Number tested</th>
<th>Number positive for TT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broiler chickens</td>
<td>294</td>
<td>0</td>
</tr>
<tr>
<td>Broiler Breeder chickens</td>
<td>360</td>
<td>0</td>
</tr>
<tr>
<td>Layer chickens</td>
<td>262</td>
<td>0</td>
</tr>
<tr>
<td>Northwest Australian backyard sentinels</td>
<td>339</td>
<td>0</td>
</tr>
<tr>
<td>Hong Kong/Mainland China chickens</td>
<td>230</td>
<td>0</td>
</tr>
<tr>
<td>Hatchery Birds from Experiments</td>
<td>280</td>
<td>0</td>
</tr>
<tr>
<td>Wild Plumed Whistling ducks (Kununurra)</td>
<td>236</td>
<td>0</td>
</tr>
<tr>
<td>Muscovy ducks (Jandakot farm)</td>
<td>33</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>2034</strong></td>
<td><strong>0</strong></td>
</tr>
</tbody>
</table>

Figures 4-6 show the level of TT antibodies present in the sera of chickens collected from abattoirs in comparison to positive controls from vaccinated chickens (refer Materials and Methods). Broiler birds were found to have a background level of up to 20% of positive control tetanus antibodies compared with less than 40% for the older Breeder and Layer birds. The
Northwest Australian sentinels (Figure 7) also demonstrated background antibody levels of up to 40% of positive control levels. Overall, the background levels were below the positive TT antibody levels observed in Figures 1 and 2, and a background level of 40% was deemed negative for a TT antibody response.

[00163] The background TT antibody level of chickens in another country was also tested as a comparison to our results using regional birds from Western Australia. Assays were performed in a Hong Kong quarantine laboratory using the same methods and reagents as described in the Materials and Methods section. Chicken sera samples (230 samples) were obtained from Hong Kong and mainland China farms. Of these samples, 75 chickens were imported from mainland China farms and had been vaccinated with H5N2; 45 chickens were from Hong Kong farms and had been vaccinated with H5N2 and the remainder were unvaccinated sentinel chickens from Hong Kong farms. All birds were around 90 days old and showed negative levels of tetanus antibodies.

[00164] The presence of natural TT antibodies in ducks was also investigated. Wild ducks from the Kununurra region (Figure 9) and locally farmed Muscovy ducks (Figure 10) were tested and found to be negative for TT antibodies.

Example 4: Combined tetanus toxoid marker and influenza vaccine

Materials and Methods

[00165] Animals and housing conditions: Chickens: 6-7 week old female Hy-Line Brown layer pullets were obtained (Altona Hatchery Pty. Ltd., Forrestfield, WA) and randomly distributed into groups of 5 chickens per pen. Pens were built off the ground and were made of steel wire. All chickens were kept in pens next to each other in identical sized pens.

[00166] Ducks: 6-8 week old Muscovy ducklings were obtained from local backyard breeders in Western Australia. Ducks were kept in pens with straw strewn on a concrete ground.
All animals were housed at the Department of Agriculture Research Station at Medina, Western Australia, following strict animal ethics guidelines (AEC Number: 6-05-55 and 4-06-37).

Preparation of H6N2 Avian Influenza (AI) Virus: A stock of H6N2 AI virus was grown following the standard method provided by the OIE (http://www.oie.int/fr/normes/mmanual/A_00037.htm, accessed on March 22, 2006). Briefly, H6N2 infectious AF was inoculated aseptically into the allantoic sacs of embryonated, SPF chicken eggs (100 µL/egg). Following an incubation of 4 days at 37°C, the infected eggs were cooled to 4°C. The shell tops of each egg were removed and the AF collected aseptically and kept at 4°C. Eggs were randomly tested for HA activity (see below) before pooling the harvested AF. Inactivation of AF was performed by stirring with 0.1% (v/v) formaldehyde (using Analytical Research grade Formalin) for 65 hours at 37°C. The suspension was left at 4°C overnight and tested for HA inactivation using chicken embryo inoculation as per the above standard method.

Vaccination: At weeks 0 and 4, birds were vaccinated s.c. by injection using syringes fitted with 21G needles.

A selection of doses for TT was chosen for Figure 11-14, and mixed with the oil adjuvant, Montanide™ ISA 70 VG (Seppic, France). Administration of an inactivated whole virus H6N2 vaccine prepared in oil was also given in a separate 1mL injection subcutaneously. Vaccine preparations were mixed following the manufacturer's recommended protocols (Seppic, France) using a glass syringe and 21G needle.

Data shown in Figures 15-16 represent an experiment for optimising the dose of TT used and combined both TT and H6N2 vaccine preparations in one injection. The vaccine preparation was mixed by stirring until a water-in-oil homogenous emulsion was obtained.

Blood collection: Blood samples were collected from the wing veins at 0, 2, 4, 6 weeks post-primary vaccination by venipuncture.
Blood samples from birds were decanted from syringes into glass or serum clot activator-treated plastic vacutainers (Greiner Bio-One, NC, USA) and left to separate overnight at 18-22 °C. Tubes were centrifuged at 1000g for 10 minutes at room temperature and serum was harvested by pipetting. Sera was then stored at 4 °C prior to use in assays before longer term storage at -20 °C (after 1 month) and -80 °C (more than 6 months).

Determination of TT antibody titres: The determination of TT antibody titres in chickens was performed using harvested sera in an indirect ELISA (Example 5). Immunosorbent ELISA plates (Greiner BioOne, Germany) were coated overnight at 4 °C in a humidified chamber with formaldehyde-inactivated, purified TT antigen [0.0125 µg/100 µL] (List Biological Laboratories, Inc., CA, USA) or with unpurified TT antigen [14.58 µg/100 µL] (Pfizer, Western Australia) in 0.05M carbonate buffer, pH 9.6.

Statistical analysis: Statistical analysis on all data was performed using the Students t-test assuming unequal variance between the means. P values less than 0.01 were deemed to be significant.

Haemagglutination (HA) Test: Inactivated H6N2 avian influenza was titred following the standard protocol (Manual Diagnostic Tests and Vaccines for Terrestrial Animals, http://www.oie.int/fr/normes/mmanual/A_00037.htm, accessed 7 June 2006). Briefly, the virus suspension was serially diluted in 0.1 M PBS in 25 µL volumes. A further 25 µL PBS was added to the wells to make a final volume of 50 µL. 25 µL of 0.5% (v/v) chicken red blood cells (RBC) were then added to each well and plates were incubated at 4 °C for 60 mins. RBC controls should have settled to a distinct button at the bottom of the well by the end of the incubation period.

The HA titre was determined by tilting the plate at 45 degrees and observing the presence or absence of tear-shaped streaming of the RBC. The highest dilution giving complete HA (no streaming) is the endpoint titre, representing 1 HA unit (HAU).
Determination of Haemagglutination Inhibition (HI) Titres: HI assays were used to determine the levels of HA antibodies in chickens and ducks. Duck antisera to influenza viruses were treated with receptor destroying enzyme (RDE) in order to inactivate non-specific inhibitors. Chicken antisera can be used without RDE treatment (WHO manual on Animal Influenza Diagnosis and Surveillance, WHO/CDS/CSR/NCS/2002.5, p. 31). Lyophilized RDE (Denka Seiken Co. Ltd., Japan) was reconstituted in sterile physiological saline (0.85% NaCl) as per manufacturer's directions. 3 volumes of RDE were then added to 1 volume of serum and incubated overnight in a 37 °C waterbath. The enzyme is then inactivated by heating at 56 °C for 30 mins. After cooling the treated serum, physiological saline was added to yield a final dilution of 1:10.

The HI assay was performed by serially diluting serum in PBS in 25 μL volumes across the plate. Influenza viral antigen (4 HAU in 25 μL) was then added to each well and incubated for 60 mins at 4 °C, after which 25 μL of 0.5% chicken RBC was added and gently mixed. After incubating for 60 mins at 4 °C, the HI titre was assessed by determining the highest dilution of serum causing complete inhibition of 4 HAU of viral antigen. Serum controls (no viral antigen) were also performed in parallel with serum samples in order to discount the presence of non-specific HI activity. All data is expressed as mean antibody titres ± SEM (standard error of the mean) with the exception of HI titres in ducks, which are represented individually.

Results

The combination of using tetanus toxoid as a biological marker to differentiate vaccinated from unvaccinated birds was next investigated. Two sets of experiments were performed. The first involved co-delivery of the tetanus vaccine and an inactivated H6N2 avian influenza vaccine in separate injections, whereas the second experiment investigated the viability of combining both vaccines together in a single injection. Both vaccines incorporated the use of Montanide™ oil as an adjuvant, which is commonly used in commercial poultry vaccinations.
In the co-delivery experiment, 30 chickens and 30 ducks were used to investigate the efficacy of a subcutaneously delivered vaccine. Tetanus toxoid doses (0.1 mg, 0.3 mg, 1.0 mg) were chosen based on the results shown in Figure 2. The H6 HA titre of avian influenza used was 27. The level of tetanus toxoid antibodies and HI titres are shown in Figures 11-14.

Figure 11 shows the levels of TT antibody elicited by vaccination. Positive TT antibody levels were obtained after the second vaccination, with antibody titres up to 100%. The strong antibody titres were maintained out to 20 weeks after the initial vaccination. There were no significant differences between the different doses, although the 0.1 mg TT dose consistently provided slightly higher TT titres compared to the 1 mg TT dose.

Figure 12 shows the HI titres in co-vaccinated chickens. HI titres were successfully elicited following vaccinations, with all dose groups achieving a titre of 29 after the second vaccination. Interestingly, the titre of the Al control (26) after 2 vaccinations was less than the co-vaccinated groups, indicating the possibility of either extra adjuvant present in delivering the two vaccines or some synergistic activity was responsible for the higher HI titres obtained in the co-vaccinated groups. No interference in the induction of specific antibodies to either TT or Al was observed by co-administration of both TT and Al vaccines.

Figures 13-14 show the TT and Al titres in ducks. Ducks generally are not as immunologically responsive as chickens in their antibody responses. Positive anti-TT antibody titres (above 50% inhibition) were only achieved after the second vaccination in all TT dose groups. Once again, the 0.1 mg TT dose provided the strongest anti-TT antibody response, followed closely by the 1 mg TT dose.

The HA titres elicited in ducks was lower than in chickens. Not all ducks produced an immunological response to the vaccine, but the 0.3 mg TT dose with Al did elicit the highest HA antibody response.

The strategy of combining both TT and Al vaccines into one vaccine delivery was next investigated. Having established that the two vaccines did not interfere with each other in terms of eliciting positive antibody responses, the two
vaccines were mixed in oil and the one formulation administered subcutaneously into chickens (10 per group).

[00187] An optimal TT dose of 0.3 mg was chosen based on results shown in Figure 11. This dose was halved (0.15 mg) and doubled (0.6 mg) to compare the difference in TT antibody responses elicited. Control groups receiving the AI vaccine only and 0.3 mg TT only were also included as background comparisons. The TT levels in vaccinated chickens are shown in Figure 15.

[00188] TT antibody titres were detected in all TT vaccinated groups. Antibody levels were greatly increased following the second vaccination, with the 0.3mg TT and 0.6mg TT groups exhibiting comparable levels similar to the TT only control. Note that the control group of AI vaccinated chickens that were not immunised with TT vaccine did not show detectable anti-TT antibodies in the ELISA as expected. No significant (P>0.01) differences were observed between the TT antibody levels in co-vaccinated birds compared to TT only controls.

[00189] Figure 16 shows that the HA titres following vaccinations are similar to those obtained in Figure 12. Note that the control group that received 0.3 mg TT vaccine only without the AI vaccine did not show detectable HI titres specific for H6N2 influenza virus as expected. No significant differences (p>0.01) in HI titres in all co-vaccinated and AI only control groups were observed.

Example 5: ELISA Diagnostic

In this TT marker system, the quantitation of an antibody- response to TT in chickens is readily performed by an indirect ELISA approach, which comprises an enzyme-conjugated antibody, which recognises and binds to chicken immunoglobulins for anti-TT antibody detection in chicken blood (eg rabbit anti-chicken IgG conjugated to horseradish peroxidase). However, this approach cannot be used to detect antibodies from other avian species other than chicken (eg duck) as the conjugated antibody is species-specific and has limited cross-reactivity with heterologous antibody species. Thus the indirect ELISA is limited for antibody detection in chickens only. As other species of birds can be infected
with HPAI strains, another approach is required to detect and monitor TT antibodies in our marker vaccination strategy (eg for surveillance in domestic poultry; duck, turkey, geese, quail or recreational wildlife; storks, flamingos).

5 An alternative approach to the indirect ELISA is utilisation of a competitive ELISA, which contains a modification in the antibody steps. In the competitive ELISA, the avian sera is allowed to bind to the TT antigen coated to the plate prior to the addition of commercially prepared goat sera, with specific antibodies against TT. The two antibody types compete in the binding to the common TT antigen. If specific TT antibodies in the bird serum (positive sera) bind to the TT antigen, then binding of the goat TT antibodies is blocked and competition has occurred. Conversely, if the bird serum does not contain TT-specific antibodies (negative sera), then binding of the goat TT-specific antibodies occurs without competition. The source of sera from bird species is unlimited in this type of universal assay.

10 Determination of TT-specific antibodies in chicken sera obtained from the indirect ELISA is expressed as % of the mean OD TT antibodies in test sera (duplicates) compared to that of the mean OD of positive controls (TT-vaccinated chickens bled at week 6) corrected with subtraction of mean OD negative controls for each sample (corresponding pre-vaccination bleeds at week 0).

\[
\% \text{ Positive} = \frac{(\text{Mean OD Test} - \text{Mean OD Negative control})}{(\text{Mean OD Positive control} - \text{Mean OD Negative control})} \times 100
\]

25 Determination of TT-specific antibodies in bird sera (not limited to chicken species) obtained from the competitive ELISA is expressed as % inhibition by mean OD TT antibodies in test sera (duplicates) compared to that of mean OD negative controls (pre-bleeds at week 0).

\[
\% \text{ Inhibition} = 100 - (100 \times \frac{\text{Mean OD Test}}{\text{Mean OD Negative control}})
\]

Sera samples with an inhibition of more than 50% were deemed to be positive for TT antibodies.
Materials and Methods

Indirect TT ELISA:

Immunosorbent ELISA plates (Greiner BioOne, Germany) were coated overnight at 4 °C in a humidified chamber with formaldehyde-inactivated, purified TT antigen [0.0125 μg/100 μL] (List Biological Laboratories, Inc., CA, USA) or with unpurified TT antigen [14.58 μg/100 μL] (Pfizer, Western Australia) in 0.05M carbonate buffer, pH 9.6. Plates were washed 6 times with PBS pH 7.6/0.05% Tween 20 (PBST) using a 12-well Immuno™ Wash instrument (Nunc International, Australia). Chicken serum was diluted 1/200 in PBST/4% skim milk and 100 μL per well was added in duplicate. Plates were then incubated at 37 °C for 1 hour before washing 6 times in PBST. Rabbit anti-chicken IgG conjugated to Horseradish Peroxidase (HRP) (Chemicon International Inc., Australia) was used at 1/40 000 dilution in PBST/4% skim milk and added to each well in 100 μL volumes, before incubation for 1 hour at 37 °C. Plates were then washed with PBST before adding 100 μL of substrate solution (TMB One solution, Promega Corp., USA). The reaction was stopped after 5 minutes with the addition of 50 μL 2M sulphuric acid. After 15 minutes, plates were read at 450 nm with 630 nm as reference wavelength using a microplate reader (Bio-Rad Model 680, CA, USA).

Competitive TT ELISA:

Plates were similarly coated with TT overnight as described for indirect ELISA and washed 6 times in PBST. Primary antibody (chicken or duck sera, 1/10 dilution, 100 μL/well) was added in duplicate and incubated for 1 hour at 37 °C. Plates were washed 6 times with PBST before addition of goat anti-TT IgG antibody (1/3200 dilution) (Accurate Chemical & Scientific Corp., NY, USA) for one hour at 37 °C. After washing wells 6 times with PBST, chicken anti-goat IgG conjugated to HRP was added to each well and left for another hour at 37 °C. Plates were then washed in PBST again before addition of TMB One Solution. Sulphuric acid (2M) was added 5 minutes later to stop the reaction and the plates were read at 450nm using 630 nm as reference wavelength.
We Claim:

1. A method of tagging a subject to which at least a first antigen has been administered or will be administered, wherein the antigen is derived from an agent capable of infecting the subject and is able to either generate or assist in generating a therapeutic effect in the subject, the method comprising the step of: administering to the subject a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen.

2. A method of tagging a subject that has received a first antigen derived from an infectious agent capable of infecting the subject, which antigen is capable of generating in the subject or at least assisting in the generation of a therapeutic response, the method comprising the step of: administering to the subject a tagging antigen that is detectable in the subject and which is capable of distinguishing treated from untreated subjects, which antigen is expressed exogenously from the first antigen.

3. A method of tagging a subject that has been administered a first antigen derived from a virus capable of infecting the subject, the method comprising the step of:

   (a) administering to the subject a tagging antigen that is not derived from an organism capable of naturally infecting the subject; and

   (b) wherein the tagging antigen evokes a detectable response in the subject.

4. A method of immunising a subject against an infectious agent, said method comprising the steps of:

   (a) administering a first antigen to the subject, wherein the first antigen is able to either generate or assist in generating a therapeutic effect in the subject, and

   (b) administering to the subject a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen identified in step (a).
5. A method of immunising a subject against a virus, comprising the steps of:
   (a) administering to the subject at least a first antigen derived from a virus capable of infecting the subject that delivers or generates a therapeutic benefit;
   (b) simultaneously administering to the subject a tagging antigen that is not derived from an organism capable of naturally infecting the subject, wherein the tagging antigen evokes a detectable immune response in the subject.

6. A method of immunising a subject against a virus, the method comprising the step of: administering to the subject a single dosage form comprising (a) a first antigen derived from a virus capable of infecting the subject and (b) a tagging antigen that is not derived from an organism capable of naturally infecting the subject, wherein the tagging antigen evokes a detectable immune response in the subject.

7. A pharmaceutically acceptable formulation, comprising:
   (a) a first antigen which is able to either generate or assist in generating a therapeutic effect in the subject, and
   (b) a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen identified in step (a).

8. A pharmaceutically acceptable formulation comprises: a first antigen derived from a virus capable of infecting the subject; and a tagging antigen that is not derived from an organism capable of naturally infecting the subject, wherein the tagging antigen evokes a detectable immune response in the subject.

9. A method of tagging a vaccine against a virus capable of infecting a subject, the method comprising the step of: combining a first antigen which is able to either generate or assist in generating a therapeutic effect in the subject, and a marker or reporter that generates a detectable signal in the subject and is capable of identifying the subject as having received the first antigen.
10. A method of tagging a vaccine, comprises the step of:
   (a) contacting the vaccine with a tagging antigen that is not derived from
       an organism capable of naturally infecting the subject; and
   (b) wherein the tagging antigen evokes a detectable immune response in
       the subject.

11. A method of identifying a subject who has been vaccinated with a formulation
    as herein described, the method comprising the step of: assaying a sample from
    the subject to detect the tag.

12. A kit for immunising a subject, comprising: (a) a first antigen derived from a
    virus capable of infecting the subject; and (b) a tagging antigen that is not derived
    from an organism capable of naturally infecting the subject.

13. A kit comprising: a means for detecting antibodies generated against a tagging
    antigen that is not derived from an organism capable of infecting the subject.
Figure 2
Figure 3
Figure 5
Figure 7
Figure 8
Figure 10
Figure 11
Figure 12
Figure 13
**Figure 14: Duck HI titres following co-delivery of TT and AI vaccines.**

<table>
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<th>4 week bleed</th>
<th>2nd Vaccination</th>
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<td>0 (n=10)</td>
<td>20, 20, 40, 0 (n=7)</td>
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<td>0.3 mg TT + H6N2</td>
<td>0 (n=10)</td>
<td>10, 0 (n=6)</td>
<td>0 (n=7)</td>
<td>320, 160, 80, 80, 40, 20, 0</td>
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<td>0 (n=9)</td>
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**Figure 14**
Figure 15
Figure 16
### INTERNATIONAL SEARCH REPORT

**International application No.**

PCT/AU20.06/001682

#### A. CLASSIFICATION OF SUBJECT MATTER

**A61K 39/295, A61K 39/21, GOIN 33/532, C12Q 1/24**

(2007-01)

According to International Patent Classification (IPC) or to both national classification and IPC.

#### B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, MEDLINE, DWPI. Keywords: DIVA or differentiating infected vaccinated animals, tagg, marker, vaccine, exogenous, heterologous

#### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>U S 2006/0024320 A (Meyers), 2 February 2006 Whole document</td>
<td>1, 4, 7, 9, 11-13</td>
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**X** Further documents are listed in the continuation of Box C  **X** See patent family annex

- **"A"** document defining the general state of the art which is not considered to be of particular relevance
- **"E"** earlier application or patent but published on or after the international filing date
- **"L"** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- **"O"** document referring to an oral disclosure, use, exhibition or other means
- **"P"** document published prior to the international filing date but later than the priority date claimed

**"T"** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

**"X"** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

**"Y"** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

**"G"** document member of the same patent family

**Date of the actual completion of the international search**

17 JANUARY 2007

**Date of mailing of the international search report**

7 FEB 2007

**Name and mailing address of the ISA/AU**

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<td>Capua I et al., &quot;Development of a DIVA (Differentiating Infected from Vaccinated Animals) strategy using a vaccine containing a heterologous neuraminidase for the control of avian influenza&quot;. Avian Pathology, February 2003, voh32(l):47-55 Whole document</td>
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INTERNATIONAL SEARCH REPORT

**Box No. II** Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **Claims Nos.:**
   because they relate to subject matter not required to be searched by this Authority, namely:

2. **Claims Nos.:**
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. **Claims Nos.:**
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

**Box No. III** Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See supplemental sheet

1. **As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.**

2. **X** As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. **As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:**

4. **No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:**

**Remark on Protest**

- **The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.**

- **The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.**

- **No protest accompanied the payment of additional search fees.**

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Form PCT/ISA/210 (continuation of first sheet (2)) (April 2005)
Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Claims 1, 2, 4, 7, 9, 11 (in part) are directed to a method of tagging a subject that has been administered or will be administered an antigen that is capable of infecting the subject and eliciting a therapeutic response, through the administration of a marker or reporter that generates a detectable signal and is capable of identifying the subject as having received the first antigen. It is considered that this comprises a first distinguishing feature.

Claims 3, 5, 6, 8, 10, 11 (in part), 12, 13 are directed to a method of tagging a subject that has been administered or will be administered an antigen that is capable of infecting the subject and eliciting a therapeutic response, through the administration of a marker or reporter that generates a detectable signal and is capable of identifying the subject as having received the first antigen wherein the tagging antigen is not derived from an organism capable of naturally infecting the subject. It is considered that the feature of the tagging antigen being not derived from an organism capable of naturally infecting the subject comprises a second distinguishing feature.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

The only feature common to all of the claims is the use of a tagging antigen. However, this concept is not novel in the light of: US 2006/0024320 A and Comerci, DJ et al. This means that the common feature can not constitute a special technical feature within the meaning of PCT Rule 13.2, second sentence, since it makes no contribution over the prior art.

Because the common feature does not satisfy the requirement for being a special technical feature it follows that it cannot provide the necessary technical relationship between the identified inventions. Therefore the claims do not satisfy the requirement of unity of invention a posteriori.
This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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<td>AU 43692/99 BR 9911619 CA 2330241</td>
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US 2006204976 WO 2006041978

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX