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(54) **Title**: RECOMBINANT PROTEINS AS VACCINES FOR PROTECTION AGAINST DISEASE INDUCED BY INFECTION WITH MINK ASTROVIRUS

(57) **Abstract**: Pre-weaning diarrhoea, has been a problem in mink farms for many years. The "greasy kits" syndrome is a condition of newborn mink characterised by diarrhoea accompanied by skin swelling and redness, skin exudates and blackness of the nails. The present invention relates to polynucleotides and polypeptides of the capsid protein of a novel mink astrovirus strain denoted DK7627. Such polynucleotides and polypeptides may be used for the production of vaccines against mink astrovirus which may induce pre-weaning diarrhoea in minks. The invention furthermore relates to vectors, host cells, compositions and detection methods.

RECOMBINANT PROTEINS AS VACCINES FOR PROTECTION AGAINST DISEASE INDUCED BY INFECTION WITH MINK ASTROVIRUS

Technical field of the invention

The present invention relates to polynucleotides for use in the production of vaccines. In addition the invention relates to polypeptides for use as vaccines and pharmaceutical compositions. In particular the vaccines are against mink astrovirus infections.

Background of the invention

Pre-weaning diarrhoea, also referred to as "wet kits", "sticky kits" or "greasy kits" syndrome, has been a problem in mink farms for many years. The "greasy kits" syndrome is a condition of newborn mink characterised by diarrhoea accompanied by skin swelling and redness, skin exudates and blackness of the nails. The skin appearance originates from excessive secretion from the apocrine glands that lends the skin wet and greasy, particularly on the neck (Clausen & Dietz, 2004; Czifra et al., 2004). In a case-control study, astrovirus and caliciviruses were detected in faecal samples of diseased mink kits by electron microscopy (EM). The presence of these viruses was epidemiologically linked to the disease and found to represent a significant risk factor both on farm and mink kit level (Englund et al. 2002).

Astrovirus infections usually cause gastroenteritis among young individuals of different species except turkeys and ducks where they are correlated with nephritis and hepatitis. Caliciviruses are cause of gastroenteric disease in different host species, including cattle, sheep, swine and mink. Human caliciviruses have been associated with foodborne outbreaks of gastroenteritis. In mink, calicivirus has been found in cases of pre-weaning diarrhoea, in association with astrovirus. In infection with these viruses, besides the losses resulting from the need of treatment to control secondary bacterial infections, losses from poor skin quality represent the major economic losses in the mink industry.

The genome of astrovirus is 6.7 Kb in length and comprises three open reading frames (ORF), ORF1a, ORF1b, and ORF2. Of these, ORF2 encodes the viral capsid protein that is the outer protein of mink astrovirus and shown to carry the antigenic determinants of the virus (Baule et al., 2007). The genome of mink

astrovirus has been sequenced and subsequently compared to other known non-mink astroviruses (Mittelholzer et al., 2003a and 2003b). The comparisons have shown that the mink astrovirus is distinct from astroviruses of other species. Genetic analysis of the ORF2 gene of mink astroviruses found in faecal samples shows that there are different variants within the known strain of mink astrovirus This indicates that the spectrum of diversity of astrovirus strains needs to be taken into account for effective control of infections by means of vaccination.

The genome of mink caliciviruses comprises two ORFs, ORF1and ORF2, of which ORF2 encodes for a capsid protein. No full-length sequence of the mink calicivirus has been generated. Caliciviruses in general are not readily cultivable, therefore virus isolates of different variants of mink caliciviruses are not available for studies and for vaccine development. Determination of the complete genomic sequence of mink calicivirus is therefore relevant to enable new approaches for vaccine design, diagnostics and virus recovery, ex: from infectious cDNA clones.

Hence, an improved method for vaccinating against mink astrovirus would be advantageous, and in particular a more efficient and/or reliable vaccine against multiple virus strains would be advantageous.

Summary of the invention

The present invention relates to vaccines that prevent clinical manifestations of disease caused by mink astrovirus by inducing specific antibodies and cellular responses that are a correlate of protection. The invention includes related materials and tools that are used in connection with its production and application. It also includes methods associated to the application of the invention.

The present inventors have characterized a novel strain of astrovirus (in here denoted DK7627) which is considered pathogenic to minks (see example 9). The characterization of the capsid proteins from the strain may be useful for the production of vaccines against astrovirus infection of said strain in minks. Though variants within a mink astrovirus exist, it is surprising that a completely new strain has been discovered (DK7627). The definition of a new strain may be

stated as a variant of a virus that bears genotypic or phenotypic characteristics different from the ones known of that particular virus

The novel strain DK7627 described within this text clearly fulfil this definition since it is genetically different by 40% in the capsid protein from the described mink astrovirus DK4265. In challenge experiments the DK7627 caused severe disease in mink, readily transmitted to in-contact peers showing the pathogenic potential. The disease is characterized by diarrhoea with shedding of virus in faeces from 2 to 29 days after infection.

The vaccines are based on the recombinant capsid protein or active derivatives thereof of mink astrovirus DK7627 e.g. expressed from stably transfected cells of e.g. mammalian origin such as mink origin.

Thus, an object of the present invention relates to producing vaccines against a novel strain of mink astrovirus. In particular, it is an object of the present invention to provide a vaccine which solves the above mentioned problems of the prior art with protection against multiple strains of viruses causing pre-weaning diarrhoea.

Isolated polynucleotides

To be able to produce the proteins/peptides which may be used as vaccines it may be necessary to know the polynucleotides encoding the proteins. Thus, one aspect of the invention relates to an isolated polynucleotide which has at least 85% sequence identity to

a) a polynucleotide as set out in SEQ ID NO: 1, 13 or 15; and/or
b) a polynucleotide encoding a polypeptide according to SEQ ID NO: 2, 14 or 16; and/or
c) a fragment of (a) or (b), wherein said fragment has a minimum length of 100 nucleotides,

wherein said isolated polynucleotide comprises nucleic acids encoding for one or more B-cell epitopes and/or one or more T-cell epitopes.

The polynucleotide sequence set out in SEQ ID NO: 1 encodes for the capsid protein of the novel mink astrovirus strain denoted DK7627.

Vector

It may be advantageously to have the polynucleotides according to the invention positioned in a vector. Thus another aspect of the present invention relates to a recombinant vector comprising an isolated polynucleotide according to the invention and a promoter which is operably linked to said polynucleotide. Such vector may be used for amplification, storage and expression of the relevant proteins expressed from the polynucleotide.

Recombinant polypeptide

The vaccines according to the invention are in the form of proteins or peptides. Thus yet another aspect of the present invention relates to a recombinant polypeptide expressed by any polynucleotide according to the invention. The proteins or peptides according to the invention may have the potential to provide an immune response in a subject such as a mink, and species infected with an astrovirus having a sequence highly identical to the sequence of the present invention.

Host cell

It may also be advantageously to have the vector positioned in a host cell. Thus in still another aspect of the present invention is to provide a recombinant host cell expressing a polypeptide according to the invention. Such host cells may be used for amplification, storage and expression from the vectors according to the invention. A host cell may both have bacterial, yeast and mammalian origin, however conventional proteins expressed in bacterial cell hosts have drawbacks such as difficulty to purify and loss of function due to lack of post-translational modifications in the bacterial system. Conventional mammalian systems on the other hand, offer these advantages but the yield of protein is rather low compared to expression in bacteria. Protocols to increase protein yield in mammalian expression exist, based on use of plasmids with strong promoters, high efficiency transfection of mammalian cells and inducible vectors. Mostly, the procedures rely on transient transfection of currently used laboratory cell types. However the polynucleotides according to the invention may also be stably transfected into a host cell.

Method for producing a recombinant polypeptide

It may also be considered advantageous to have a method for producing a recombinant polypeptide according to the invention. Thus, in another aspect the invention relates to a method of producing a recombinant polypeptide according to the invention, comprising the steps of:

a) contacting a host cell line with a vector according to the invention,
b) cultivating said cells under conditions suitable for the production of the polypeptide.
c) purifying said polypeptide.

The method may be used to generate different types of vaccines according the polypeptides of the invention.

Composition

In yet a further aspect the invention relates to a composition comprising a recombinant polypeptide according to the invention. Similarly the invention relates to a composition, for use as a vaccine in mink, mice and/or humans. Similarly, the invention relates to a composition, for use as a vaccine in mink, mice and/or humans against an infection of at least one mink astrovirus strain. Since multiple strains of astrovirus exist it would be advantageously to have multiple vaccines/compositions available. The vaccines according to the invention may provide protection against the astrovirus strain denoted DK7627. One infection caused by astrovirus in minks is pre-weaning diarrhoea. Thus, the invention also relates to a vaccine against pre-weaning diarrhoea and conditions coming as sequelae of astrovirus infection. Thus, the present invention may be a pharmaceutical composition in the form of a vaccine.

Method for detecting mink astrovirus

In a final aspect the invention relates to a method for the detection of mink astrovirus in a sample comprising the steps:

I. contacting physiological material with a probe wherein said probe is selected from:
    a) a polynucleotide as set out in SEQ ID NO 1, 13 or 15;
    b) a polynucleotide that is capable of hybridising to SEQ ID NOs 1, 13 or 15 under stringent conditions,

c) a polynucleotide which has at least 85% sequence identity to a polynucleotide as set out in any one of (a) to (b),

d) a fragment of (a) (b) or (c), wherein said fragment has a length of minimum 10 nucleotides, and

II. detecting a successful binding event between the probe and at least one component of the sample.

The described detection method may be helpful in assisting in determining whether e.g. a mink or population of minks are infected by a specific virus or strain of viruses such as astrovirus or more specifically mink astrovirus strain DK7627.

Brief description of the figures

Figure 1

Figure 1 shows schematics of the cloning of ORF2 of mink astrovirus based on the published sequence of DK4265 (SEQ ID NO: 3) (full-length and truncated constructs). The genome of mink astrovirus is represented. The amplified full-length ORF2 gene coding for the complete capsid protein is indicated as C1. The N-terminal and C-terminal truncated ORF2 gene, encoding truncated capsid proteins as indicated as C2 (SEQ ID NO: 17) and C4 (SEQ ID NO: 19), respectively. The location of the primers used for amplification of each strain, listed in Table 1, is indicated in the drawing.

Figure 2

Figure 2 shows stably transfected mink foetal cells expressing the capsid protein of a mink astrovirus (DK5790) highly identical to strain DK4265 as source of protein. Expression demonstrated by immunofluorescence, as described under host cell.

Figure 3

Figure 3 shows the expression of the capsid protein of mink astrovirus DK5790, highly identical to DK4265 (SEQ ID NO: 4) from stably transfected mink foetal cells. The position of the 87-kDa polyprotein is indicated by the arrow in the right side. The molecular weight standard (M) is shown in the left. A) Detection of the

capsid protein in cells transiently transfected with clones of DK5790 and DK7627. Transient transfected cells are used for selection of clonal cells to continue into stable transfection.

B) Detection of the capsid protein in stably transfected cells, showing increase in protein production upon cell propagation during selection. Proteins purified through affinity column.

Figure 4

Figure 4 shows stability of the OFR2 gene of a mink astrovirus highly identical to strain DK4265 (SEQ ID NO: 3) (full-length and truncated forms) inserted in the transfected cells, as detected by PCR.

Figure 5

Figure 5 shows the expression of the capsid protein of mink astrovirus strain DK7627 (SEQ ID NO: 2) from transiently transfected mink foetal cells. The position of the 87-kDa polyprotein is indicated by the arrow in the right side. The molecular weight standard (M) is shown in the left. A) Transient transfected cells are used for selection of clonal cells to continue into stable transfection.

Figure 6

Figure 6 shows the expression of the capsid protein of mink astrovirus DK7627 capsid proteins C1, C2 and C4 from stably transfected mink foetal cells.

Figure 7

Figure 7 shows antibodies to the capsid protein of mink astrovirus in mice sera 3 weeks after first and second injection with the C1, C2 and C4 proteins of DK7627. The sera were tested with an indirect ELISA (COD> 0.5). The mean value and (±) SD of each group is illustrated as bars. Asterisk indicates a significant difference between mean value of immunized and the control group using Student t-test ($P < 0.005$). The C2 polypeptide induced higher levels of serum antibodies, while the C4 showed a response similar to that of the full-length capsid protein, C1.

Figure 8

Figure 8 shows proliferation ability of splenocytes from immunized and non-immunized mice after stimulation with different concentrations of the corresponding proteins. The data are mean OD readings of triplicate wells from one representative experiment. Letters indicate significant difference at the given protein concentration, between immunized and non-immunized mice ($P < 0.005$). A) C1, B) C2 and C) C4 proteins of strain DK7627.

Figures 9-14

Figures 9-14 show cytokine production in supernatants from splenocytes of mice immunized (top panel) and naïve (bottom panel) after stimulation with different concentrations of the corresponding proteins C1, C2 and C4 of strain DK7627. The data are mean fluorescence intensity (MFI) readings of duplicate wells from one representative experiment. Data are presented for IFN-γ, IL-2, IL-4, IL-5, IL-10 and IL-12 in figures 9-14 respectively.

Figure 15

Figure 15 shows the predicted protein model and folding based on the deduced amino acid sequences of the full-length capsid proteins of DK4265 and DK7627. A) DK4265, B) DK7627, and C) Superimposement of both models.

The present invention will now be described in more detail in the following.

Detailed description of the invention

Definitions

Prior to discussing the present invention in further details, the following terms and conventions will first be defined:

Biological sample

The (biological) sample or physiological material of the invention refers to a sample previously obtained from a subject such as a mink. The sample may be a biopsy or a body fluid sample such as a blood sample or a faecal sample. The

sample may be processed before subjecting the sample to any of the methods of the invention.

Conservative substitution

As used herein the term "conservative substitution" refers to a gene product wherein a substitution of one amino acid with another with generally similar properties (size, hydrophobicity, etc) likely does not seriously affect the overall function of the protein.

Thus, conservative amino acid substitution as used herein relates to the substitution of one amino acid (within a predetermined group of amino acids) for another amino acid (within the same group), wherein the amino acids exhibit similar or substantially similar characteristics.

Within the meaning of the term "conservative amino acid substitution" as applied herein, one amino acid may be substituted for another within the groups of amino acids indicated herein below:

- Amino acids having polar side chains (Asp, Glu, Lys, Arg, His, Asn, Gln, Ser, Thr, Tyr, and Cys,)
- Amino acids having non-polar side chains (Gly, Ala, Val, Leu, Ile, Phe, Trp, Pro, and Met)
- Amino acids having aliphatic side chains (Gly, Ala Val, Leu, Ile)
- Amino acids having cyclic side chains (Phe, Tyr, Trp, His, Pro)
- Amino acids having aromatic side chains (Phe, Tyr, Trp)
- Amino acids having acidic side chains (Asp, Glu)
- Amino acids having basic side chains (Lys, Arg, His)
- Amino acids having amide side chains (Asn, Gln)
- Amino acids having hydroxy side chains (Ser, Thr)
- Amino acids having sulphor-containing side chains (Cys, Met),
- Neutral, weakly hydrophobic amino acids (Pro, Ala, Gly, Ser, Thr)
- Hydrophilic, acidic amino acids (Gln, Asn, Glu, Asp), and
- Hydrophobic amino acids (Leu, Ile, Val)

Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

Accordingly, a gene product according to the invention may comprise, within the same gene product, or among different gene products, at least one substitution, such as a plurality of substitutions introduced independently of one another.

It is clear from the above outline that the same gene product may comprise more than one conservative amino acid substitution from more than one group of conservative amino acids as defined herein above.

Operably linked

The term "operably linked" refers to the connection of elements being a part of a functional unit such as a gene or an open reading frame. Accordingly, by operably linking a promoter to a polynucleotide encoding a polypeptide the two elements becomes part of the functional unit – a gene. The linking of the promoter to the polynucleotide enables the transcription of the polynucleotide directed by the promoter. By operably linking two heterologous polynucleotides encoding a polypeptide the sequences becomes part of the functional unit – an open reading frame encoding a fusion protein comprising the amino acid sequences encoding by the heterologous polynucleotide. By operably linking two polypeptides, the sequences become part of the same functional unit – a polypeptide. Operably linking two heterologous amino acid sequences generates a hybrid (fusion) polypeptide.

Recombinant

Recombinant gene, promoter, nucleic acid sequence (DNA), amino acid sequences (polypeptide) refers to the products generated by genetic engineering such as the combination or insertion of one or more nucleic acid sequences, thereby combining nucleic acid sequences that would not normally occur together (recombinant nucleic acid sequence, recombinant polynucleotide). Accordingly, recombinant expression refers to the expression of a RNA transcript and/or polypeptide from a coding region directed by a heterologous promoter or a

promoter comprising heterologous promoter/enhancer elements. A recombinant polynucleotide or protein/polypeptide, may also be considered as an isolated unit.

Sequence identity

The term "sequence identity" indicates a quantitative measure of the degree of homology between two amino acid sequences or between two nucleic acid sequences of equal length. If the two sequences to be compared are not of equal length, they must be aligned to give the best possible fit, allowing the insertion of gaps or, alternatively, truncation at the ends of the polypeptide sequences or nucleotide sequences. The sequence identity can be calculated as $\frac{(N_{ref}-N_{dif})100}{N_{ref}}$, wherein Ndif is the total number of non-identical residues in the two sequences when aligned and wherein Nref is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC (Ndif=2 and Nref=8). A gap is counted as non-identity of the specific residue(s), i.e. the DNA sequence AGTGTC will have a sequence identity of 75% with the DNA sequence AGTCAGTC (Ndif=2 and Nref=8).
With respect all embodiments of the invention relating to nucleotide sequences, the percentage of sequence identity between one or more sequences may also be based on alignments using the clustalW software (http:/www.ebi.ac.uk/clustalW/index.html) with default settings. For nucleotide sequence alignments these settings are: Alignment=3Dfull, Gap Open 10.00, Gap Ext. 0.20, Gap separation Dist. 4, DNA weight matrix: identity (IUB).
Alternatively, and as illustrated in the examples, nucleotide sequences may be analysed using programme DNASIS Max and the comparison of the sequences may be done at http://www.paralign.org/. This service is based on the two comparison algorithms called Smith-Waterman (SW) and ParAlign. The first algorithm was published by Smith and Waterman (1981) and is a well established method that finds the optimal local alignment of two sequences. The other algorithm, ParAlign, is a heuristic method for sequence alignment. Default settings for score matrix and Gap penalties as well as E-values were used.

Vector

The term "vector" refers to a DNA molecule used as a vehicle to transfer recombinant genetic material into a host cell. The four major types of vectors are

plasmids, bacteriophages and other viruses, cosmids, and artifical chromosomes. The vector itself is generally a DNA polynucleotide that consists of an insert (a heterologous polynucleotide, transgene) and a larger polynucleotide that serves as the "backbone" of the vector. The purpose of a vector which transfers genetic information to the host is typically to isolate, multiply, or express the insert in the target cell. Vectors called expression vectors (expression constructs) are specifically adapted for the expression of the heterologous polynucleotidein the target cell, and generally have a promoter that drives expression of the heterologous polypeptide. Simpler vectors called transcription vectors are only capable of being transcribed but not translated: they can be replicated in a target cell but not expressed, unlike expression vectors. Transcription vectors are used to amplify the inserted heterologous sequences. The transcripts may subsequently be isolated and used in as templates suitable in vitro translations systems.

Active derivative thereof

The term "active derivative thereof" relates in the present context to a polynucleotide or polypeptides which pertains substantially the same functional activity as polynucleotide or polypeptide to which it is compared (the sequence from which it is derived). The same functional activity (or functional equivalent) may be an activity of at least 25%, such as at least 40%, such as at least 60 %, such as at least 80% of the sequence to which it is compared. It is of course to be understood that a functional equivalent may have a higher activity such as at the most 400%, such as at the most 350%, such as at the most 300%, such as at the most 200%, such as at the most 175%, such as at the most 150%, or such as at the most 125% when compared to a sequence. In the present context the same functional activity of the polypeptides can measured by comparing the evoked immune response in a subject such as a mink or mice. It is to be understood that "active derivative thereof" will be shorter that the sequence itself by at least one amino acid. Thus, an active derivative refers to a sequence of amino acids that comprise a part of a longer sequence of amino acids (e.g., polypeptide). In the present context "active derivative thereof" may be considered to be the presence of a B-cell epitope and/or at least one T-cell epitope in said derivative. Thus, in a particularly preferred embodiment the "active derivative thereof" comprises at least one B-cell epitope and/or at least one T-cell epitope.

Antigen

In the present context the term "antigen" refers to a substance that when introduced into the body stimulates the production of an antibody. Similarly the term "antigenic" refers to a substance which may function as an antigen.

Vaccine

A vaccine is a biological preparation that improves immunity to a particular disease. A vaccine typically contains an agent that resembles a disease-causing microorganism. The agent stimulates the body's immune system to recognize the agent as foreign, destroy it, and "recognize" it, so that the immune system can more easily recognize and destroy any of these microorganisms that it later encounters. Vaccines can be prophylactic (e.g. to prevent or ameliorate the effects of a future infection).

Effective amount

The term 'effective amount' refers to an amount or concentration of a substance such as a polypeptide, a polynucleotide or an antibody which is effective to produce a protective prophylactic or therapeutic response with respect to the disease in question. In general, an effective amount of the substance, which is administered to a subject, such as a mink, will vary depending upon a number of factors associated with that subject, including whether the subject has previously been exposed to the disease (virus). The person of ordinary skill in the art can determine an effective amount of the substance by varying the dosage of the product and measuring the resulting cellular and humoral immune and/or therapeutic responses subsequent to administration. In particular, the concentration range of an immunogenic substance is chosen so as to enhance the likelihood of eliciting an immunogenic response e.g. vaccinating the recipient for a long period of time, without causing an infection in the vaccine recipient. In an embodiment according to the invention said composition or vaccine is provided in an effective amount.

B-cell epitope

A 'B-cell epitope' is defined as an antigenic determinant, which functionally is the portion of an antigen, which combines with the antibody paratope. B-cell epitopes are usually composed of approximately 6 amino acids and are expected to be

located at the surface of the protein and surface probability programs and hydrophobicity plots can therefore help defining areas with B-cell epitopes. With respect to the present invention the Protean 4.0 software in the DNAstar package may be used with default settings when defining such areas. Specific B-cell epitopes should preferably be determined experimentally, which can be done by methods well known to the person of ordinary skill in the art.

T cell epitope

The term 'T cell epitope' refers to a sequence of about ten amino acids that are part of a much longer, folded chain of amino acids and can lead to activation of a T-cell when presented on the surface of a cell in complex with Major Histocompatibility Complex (MHC) II and/or 1. Probability values for putative T-cell epitopes within a polypeptide may be obtained with the use of computers, neural networks and prediction servers such as SYFPEITHI server at Centre for Biological Sequence Analysis BioCentrum-DTU, Technical University of Denmark (http://syfpeithi.bmi-heidelberg.com/Scripts/MHCServer.dll/EpPredict.htm) which is used with default unchangeable settings.

Immune response

In the present context, the term 'immune response' is used in its broadest meaning referring to the response that occurs in the body of the subject (e.g. mink or mice) as reaction to its contact with a foreign substance. An immune response comprises the activation of B-lymphocytes and/or T-cells. Activation of B-lymphocytes can result in production of antibodies that can target an antigen. T-cells can be CD8+ or CD4+ or CD8-/CD4-. Activation of an immune response also comprises the activation of macrophages and/or the production of specific T and B memory cells.

Isolated polynucleotides

To be able to produce the proteins/peptides which may be used as vaccines it is necessary to know the nucleic acid sequence of the proteins. Thus, one aspect of the invention relates to an isolated polynucleotide which has at least 85% sequence identity to

a) a polynucleotide as set out in SEQ ID NO: 1, 13 or 15; and/or

b) a polynucleotide encoding a polypeptide according to SEQ ID NO: 2, 14 or 16; and/or

c) a fragment of (a) or (b), wherein said fragment has a minimum length of 100 nucleotides,

wherein said isolated polynucleotide comprises nucleic acids encoding for one or more B-cell epitopes and/or one or more T-cell epitopes.

The polynucleotides according to the invention (e.g. SEQ ID NO: 1 which encode for the capsid protein of the astrovirus strain denoted DK7627, may have a certain degree of variation. Since mutations may exist which e.g. result in conservative substitutions on the resulting polypeptide, one embodiment according to the invention relates to polynucleotides according to the invention having a sequence identity of at least 90% such as at least 95% such as at least 99% or such as 100%. It is to be understood that these sequence identities may also form part of all of the polynucleotides under a)-c).

The polynucleotides that are capable of hybridising to SEQ ID NO: 1 under stringent conditions may be used as e.g. detection probes or amplification probes during production or detection assays.

The polynucleotides encoding a polypeptide according to SEQ ID NO: 2 may vary in sequence due to the degenerative nature of the genetic code. Thus, different polynucleotides may encode for the same polypeptide. The person skilled in the art would know which sequences will encode a polypeptide according to SEQ ID NO: 2.

The disclosed fragments encoding for a polypeptide able to induce an immune response (such as displaying antigenic or immunogenic properties) should preferably have a minimum length of at least 15 amino acids, such as at least 40 amino acids, such as at least 50 amino acids, such as at least 60 amino acids, such as at least 100 amino acids, such as at least 150 amino acids, such as at least 200 amino acids, such as at least 400 amino acids, such as at least 500 amino acids, such as at least 600 amino acids, or such as at least 700 amino acids. The person skilled in the art can transfer these numbers into nucleic acid lengths.

Examples of fragments which may induce an immune response are: Peptides that used to make anti-peptide sera:

MC033 – RNQGPGKRWNSKKGR (SEQ ID NO: 21);
MC307 – SDASERSPVNEGSVG (SEQ ID NO: 22), and
MC452 – YNTGDSNVKSHGKFD (SEQ ID NO: 23).
Polynucleotides encoding truncated proteins:
Truncated fragment C2 of strain DK4625 (SEQ ID NO: 17);
Truncated fragment C2 of strain DK7627 (SEQ ID NO: 13);
Truncated fragment C4 of strain DK4625 (SEQ ID NO: 19);
Truncated fragment C4 of strain DK7627 (SEQ ID NO: 15).
It is to be understood that these polynucleotides also forms part of the invention. The fragments from DK4625 may code for polypeptides which may form part of a composition together with polypeptides according to the invention.

Thus, in an embodiment said fragment is selected from SEQ ID NO's 13 and 15.
In a further aspect of the invention relates to an isolated polynucleotide which has at least 85% sequence identity to

a) a polynucleotide as set out in SEQ ID NO: 1, 13 or 15.
b) a polynucleotide that is capable of hybridising to SEQ ID NO: 1, 13 or 15 under stringent conditions;

In another embodiment the invention relates to a polynucleotide encoding a polypeptide selected from SEQ ID NO 2, 14, 16, 18, 20, 21, 22, or 23.

In another embodiment said fragment is selected from a region within position 481-1890 in SEQ ID NO 1. This region encompasses amino acids 161 to 630 of the capsid protein.

Since the capsid protein in it's naturally position in the virus particle comprises a region which is presented on the outside of the virus particle, it may be this region which will provide the immune-response. Thus, in an embodiment said fragment encodes for a part of the capsid protein externally present on the virus, when naturally present in the virus.

However, since fragments of SEQ ID NO: 1 or the complementary polynucleotides may be used as e.g. primers fragments could also have a length of at least 10 nucleotides, such as at least 15 nucleotides or such as at least 25 nucleotides.

Primers are likely to have a higher sequence identity such as at least 90% such as at least 95% such as at least 99% or such as 100%.

Examples of primers are shown in table 1. Thus in an embodiment said fragment is selected from SEQ ID NO's 24-29. SEQ ID NO's 24-29 are primers which may be uses to amplify strains of astrovirus as indicated in table 1. E.g. primers SMC3FN and SMC4RN are specific for strain DK7627. Such primers may be used in a detection method according to the invention

Vector

It may be advantageously to have the polynucleotide according to the invention positioned in a vector. Thus another aspect of the present invention relates to vector comprising an isolated polynucleotide according to the invention and a promoter which is operably linked to said polynucleotide. Such vector may be used for amplification, storage and expression of the relevant proteins expressed from the polynucleotides. In an embodiment the vector is selected from the group consisting of plasmids, PCR products, cDNA construct, virus particle and infectious cDNA clones.

Recombinant polypeptide

The vaccines or pharmaceutical compositions according to the invention comprise proteins or peptides. Thus, yet another aspect the present invention relates to a recombinant polypeptide or active derivative thereof expressed by any polynucleotide according to the invention, wherein said recombinant polypeptide and active derivative thereof comprises at least one B-cell epitope and/or at least one T-cell epitope. The proteins or peptides according to the invention may have the potential to provide an immune response in a subject, such as a mink.

In a similar aspect the present invention relates to a recombinant polypeptide or active derivative thereof, having at least 85% sequence identity to SEQ ID NO: 2, 14 or 16, wherein said recombinant polypeptide and active derivative thereof comprises at least one B-cell epitope and/or at least one T-cell epitope.

In an embodiment the recombinant polypeptides or an active derivative thereof have at least 85% sequence identity to SEQ ID NO: 2, such as at least 90% sequence identity, such as at least 95% sequence identity such as at least 99% sequence identity or such as 100% sequence identity to SEQ ID NO: 2 or an active derivative thereof.

In another embodiment said "active derivative thereof" has a length of at least 33 amino acids, such as at least 50 amino acids, such as at least 100 amino acids, such as at least 200 amino acids, such as at least 300 amino acids, such as at least 400 amino acids, such as at least 500 amino acids, or such as at least 600 amino acids.

In the present context the term "an active derivative thereof" relates to polypeptides having substantially the same functional activity as SEQ ID NO: 2. The same functional activity of the polypeptides may measured by comparing the evoked immune response in a subject. In a particularly preferred embodiment the "active derivative thereof" comprises at least one B-cell epitope and/or at least one T-cell epitope. In another embodiment said active derivative thereof is selected from a region within position 161 to 630 in SEQ ID NO 2.

Examples of active derivatives are polypeptides corresponding to amino acid sequences of DK7627 (SEQ ID NO: 2):

- C2 of strain DK7627: position 161-778 (SEQ ID NO: 14)
- C4 of strain DK7627: position 1-630 (SEQ ID NO: 16)
- Position 161 to 630 of the capsid protein of DK7627.

To increase solubility, expression level, improve purification or detect the polypeptides, it may be advantageously that the polypeptides are operably linked to a tag. Thus, in an embodiment the recombinant polypeptides are operably linked to a tag. Examples of tags are BCCP-tag, c-myc-tag, calmodulin-tag, FLAG-tag HA-tag His-tag), Maltose binding protein-tag Nus-tag Glutathione-S-transferase-tag Green fluorescent protein-tag Thioredoxin-tag S-tag Strep-tag human protein C tag, Chitin binding protein tag T7-tag, Myc-tag, V5-tag, VSV-tag, Avi.tag, BioEase-tag, SNAP-tag, FlaSH-tag, Nus A-tag, and DsbA-tag. Such tags may subsequently be cleaved off if necessary.

Host cell

Mink astroviruses and mink caliciviruses are not readily isolated or do not grow to high titers in cell cultures. This limits the use of classical methods to produce a vaccine against infection with these pathogens, found in association with pre-weaning diarrhoea.

Conventional proteins expressed in bacterial host cells have drawbacks such as difficulty to purify and loss of function due to lack of post-translational modifications in the bacterial system. Conventional mammalian systems on the other hand, offer these advantages but the yield of protein is rather low compared to expression in bacteria. Protocols to increase protein yield in mammalian expression exist, based on use of plasmids with strong promoters, high efficiency transfection of mammalian cells and inducible vectors. Mostly, the procedures rely on transient transfection of currently used laboratory cell types.

The system of this invention uses the integration of the coding sequence of the capsid proteins of mink astrovirus and/or mink calicivirus in the genomic DNA of the cells by creating stably transfected cells. These are selected cells that express the inserted genes, enabling the target protein to be expressed along with regular cellular proteins. Through this selection, cells expressing the protein can be targeted for propagation, and by this means the level of expression is increased. In transient transfections only few cells express the protein, which results in that the amount of protein produced in mammalian cells under those conditions is not high. In addition, in contrast to transient transfections where cells have to be transfected anew each time the protein is required, the expressing cells from this system are a permanent source of antigen. The generated cells can be preserved frozen at ultra-low temperatures, and can thereafter be de-frozen for propagation and production of the protein on demand. The cells express increased levels of protein upon propagation, which makes the system amenable for improvement of yield for mass production purposes. The procedure for creation of stably transfected cells is known and used in research laboratories. However, the approach has not been applied as means to create a source of production of antigens for vaccines and diagnostic purposes.

Thus, an aspect of the invention relates a recombinant host cell expressing a polypeptide or active derivative thereof according to the invention. The host cell may be bacteria, yeast or mammalian cell lines. Although in certain instances bacterial expressed proteins may provide a sufficient immune response, it is often considered more useful to have the proteins expressed in e.g. mammalian systems. Thus, in one embodiment the host cell is a mammalian cell line selected from the group consisting of mink cell lines, human cell lines, dog cell lines, rodent cell lines (such as mink cells), hamster cell lines and rabbit cell lines. Though many different cell lines may be used, for protection in minks a mink cell line would normally be considered preferred. Examples of mink cell lines are mink foetal cells and mink intestinal cells.

In a more specific embodiment the at least one encoded polypeptide encoded by the recombinant host cell is SEQ ID NO: 2 or an active derivative thereof. SEQ ID NO: 2 is the discovered full length capsid protein from mink astrovirus strain DK7627. Active derivatives thereof may be truncated versions of the proteins or synthetic peptides which may still provide an immune response when used as a vaccine. Examples of derivatives are synthetic peptides and any truncated forms of said protein, in particular the said C2 and C4 variants. C2 of strain DK7627: position 161-778; C4 of strain DK7627: position 1-630.

Polypeptide obtained from a host cell

As mentioned above, conventional proteins expressed in bacterial cell hosts have drawbacks such as difficulty to purify and loss of function due to lack of post-translational modifications in the bacterial system. Conventional mammalian systems on the other hand, offer these advantages but the yield of protein is rather low compared to expression in bacteria. Protocols to increase protein yield in mammalian expression exist, based on use of plasmids with strong promoters, high efficiency transfection of mammalian cells and inducible vectors. Thus, in a further embodiment the invention relates to a recombinant polypeptide or active derivative thereof according to the invention, obtainable from a host cell according to the invention.

When the proteins are obtained from a host cell, such as a yeast or mammalian cell, it will likely result in a protein comprising modifications not present when the proteins/polypeptides are obtained by in vitro translations or from bacterial expression systems.

Antigenic

The polypeptides according to the invention should preferably be able to elicit an immune response in a subject such as a mink. Thus, in a further embodiment the recombinant polypeptides or active derivative thereof, is antigenic.

By inducing an immune response in e.g. minks, protection from a later infection causing e.g. pre-weaning diarrhoea may be avoided. Antigenic may also be understood as displaying antigenic or immunogenic properties. Thus, in a particularly preferred embodiment the recombinant polypeptides or active derivative thereof comprises at least one B-cell epitope and/or at least one T-cell epitope.

Antibody

It may be an advantage if antibodies against SEQ ID NO: 2 or part of SEQ ID NO: 2 were available. Thus in an embodiment the invention relates to a recombinant polyclonal antibody wherein said polyclonal antibody has binding specificity to a polypeptide according to the invention. In another embodiment the invention relates to a recombinant monoclonal antibody wherein said monoclonal antibody has binding specificity to a polypeptide according to the invention. The person skilled in the art would know how to produce such antibodies.

Method for producing a recombinant polypeptide

It may also be considered advantageous to have a method for producing a recombinant polypeptide according to the invention. Thus, in another aspect the invention relates to a method of producing a recombinant polypeptide or active derivative thereof according to the invention, comprising the steps of:

a) contacting a host cell line with a vector according to the invention,
b) cultivating said cells under conditions suitable for the production of the polypeptide.
c) purifying said recombinant polypeptide.

The method may be used to generate different types of vaccines according the polypeptides of the invention.

Composition

In yet a further aspect the invention relates to a composition comprising a recombinant polypeptide or active derivative thereof according to the invention. The compositions according to the invention may be used as a vaccine or prophylaxis treatment. Thus in an embodiment the invention relates to a composition comprising a recombinant polypeptide or active derivative thereof according to the invention, for use as a vaccine in mink, mice and/or humans. The composition according to the invention may be a pharmaceutical composition such as a vaccine. In an aspect the invention therefore also relates to a composition comprising a polypeptide selected from the group consisting of SEQ ID NO: 2, 14 and 16 or active derivatives thereof. Example 9 shows that the strain DK7627 is indeed pathogenic, indicating the relevance for a vaccine against such infection. Example 10 shows that C1, C2 and C4 polypeptides derived from DK7627 can indeed elicit a cellular immune response in a test mammal, though the response elicited by C1 was less pronounced.

In a further embodiment, the invention relates to a composition, for use as a vaccine in mammals, such as mink, mice and/or humans, against an infection of at least one mink astrovirus strain, such as DK7627. One infection caused by astrovirus in minks is pre-weaning diarrhoea. Thus, the invention also relates to a vaccine for use against pre-weaning diarrhoea and/or conditions coming as sequelae of astrovirus infection, such as DK7627.

This could also be formulated as use of a composition according to invention for the manufacture of a vaccine for protection against an infection of at least one mink astrovirus strain, such as DK7627.

Similar, it may be formulated as a composition according to the invention for use in the prophylactic treatment of mammals, such as mink, mice and/or humans, against an infection of at least one mink astrovirus strain, such as DK7627.

Pharmaceutical compositions may comprise further components for sustaining the compositions. Thus, in a further embodiment the composition comprises at least one recombinant polypeptide or derivative thereof according to the invention, and at least one pharmaceutically acceptable carrier, diluent, or excipient.

In figure 7 it can be seen that the immune response is much higher after the second vaccination cycle. Thus, in an aspect of the present invention said composition is to be applied with a dosage regime comprising at least to cycles of immunisation such as 2-10 cycles of immunisation, such as 2-5 cycles of immunisation, such as 2-4 cycles of immunisation, such as 2-3 cycles of immunisation or such as 2 cycles of immunisation. The period between said cycles may be from 1 day to several weeks or month. Thus, in an embodiment said immunisation cycles are separated with a period of 1 day to 1 year, such as 1 day to 6 month, such as 1 day to 4 month, such as 1 day to 2 month, such as 1 day to 1 month, such as 1 day to 2 weeks, or such as 1-7 days.

Proteins may be expressed from the ORF2 of different variants of mink astrovirus and mink calicivirus in order to ensure coverage for diverse variants. Since it has now been determined that multiple strains of astrovirus exist it would be advantageously to have a vaccine providing protection against more than one strain of viruses. The vaccine or vaccine system according to the invention may therefore provide protection against the astrovirus strain denoted DK7627 + additional astrovirus strains. In an additional embodiment the composition may also confer protection against others types of virus, such as calicivirus.
Since multiple strains of virus may cause e.g. pre-weaning diarrhoea, it would be advantageously to have vaccines comprising further polypeptides inducing an immune response against other strains of mink astrovirus and mink calicivirus as relevant pathogens in pre-weaning diarrhoea. Thus, in a further embodiment the invention relates to a composition according to the invention, comprising at least one further recombinant polypeptide or active derivative thereof selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, and SEQ ID NO: 12, wherein said at least one further recombinant polypeptide or active derivative thereof comprises at least one B-cell epitope and/or at least one T-cell epitope.
SEQ ID NO: 4 is the polypeptide sequence of a known mink astrovirus sequence capsid protein DK4265 (GenBank AY179509.1) (SEQ ID NO: 3 is the corresponding polynucleotide sequence).
SEQ ID NO: 6 is the polypeptide sequence of the minor basic protein of DK08-52/414-2 from calicivirus (SEQ ID NO: 5 is the corresponding nucleotide sequence).

SEQ ID NO: 8 is the polypeptide sequence of the minor basic protein of DK08-52/415-2 from calicivirus (SEQ ID NO: 7 is the corresponding nucleotide sequence).

SEQ ID NO: 10 is the polypeptide sequence of the capsid of Mink enteric calicivirus strain Canada 151A (Sapovirus) (GenBank accession AY144337) (SEQ ID NO: 9 is the corresponding nucleotide sequence).

SEQ ID NO: 12 is the polypeptide sequence of the minor basic protein of Sapovirus Hu/Ehime475/2004/JP (GenBank accession DQ366344) (SEQ ID NO: 11 is the corresponding nucleotide sequence). Thus, the composition may comprise at least two further polypeptides such as three and such as four further polypeptides. Since both astrovirus and calicivirus may cause disease in e.g. minks in might be advantageously to provide a vaccine which provides protection against strains from both viruses.

As mentioned before, "active derivative thereof" relates to polypeptides having substantially the same activity as the full length or truncated polypeptides. Examples of such polypeptides which may be comprised in the composition according to the invention are:

Truncated fragment C2 of strain DK4625 (SEQ ID NO: 18);

Truncated fragment C2 of strain DK7627 (SEQ ID NO: 14);

Truncated fragment C4 of strain DK4625 (SEQ ID NO: 20);

Truncated fragment C4 of strain DK7627 (SEQ ID NO: 16).

Thus, in an embodiment the composition according to the invention further comprises at least one polypeptide selected from the group consisting of SEQ ID NO's: 14, 16, 18 or 20 or an active derivative thereof, wherein said at least one further recombinant polypeptide or active derivative thereof comprises at least one B-cell epitope and/or at least one T-cell epitope.

Methods for detecting mink astrovirus

In an aspect the invention relates to a method for detecting mink astrovirus in a sample comprising the steps:

I. contacting physiological material with a probe wherein said probe is selected from:

   a) a polynucleotide as set out in SEQ ID NO: 1, 13 or 15;

   b) a polynucleotide that is capable of hybridising to SEQ ID NO: 1, 13 or 15 under stringent conditions:

c) a polynucleotide which has at least 85% sequence identity to a polynucleotide as set out in any one of (a) to (b);

d) a fragment of (a) (b) or (c), wherein said fragment has a length of minimum 10 nucleotides, and

II. detecting a successful binding event between the probe and at least one component of the sample.

The polynucleotides under b) which are able to hybridize to SEQ ID NO: 1 under stringent conditions may have a minimum length of at least 10 nucleotides such as at least 15, such as at least 20, such as at least 40, such as at least 80, such as at least 200, or such as at least 400 nucleotides. Shorter polynucleotides may be used for PCR amplifications or arrays, whereas longer polynucleotides may be used for in situ hybridizations, such as FISH.

One embodiment according to the invention relates to polynucleotides according to the invention having a sequence identity of at least 90% such as at least 95% such as at least 99% or such as 100%.

PCR amplifications may be a preferred method to detect the presence of mink astrovirus. Thus, in an embodiment said polynucleotide capable of hybridizing to SEQ ID NO: 1 or its complementary sequence under stringent conditions, are selected from SEQ ID NO's 20-29. E.g. primers SMC3FN (SEQ ID NO: 28) and SMC4RN (SEQ ID NO: 29) are specific for strain DK7627.

The person skilled in the art would know different types of PCR reactions such as a standard PCR or real-time PCR such as Q-PCR.

In an embodiment said detected mink astrovirus mink astrovirus strain is DK7627.

An infection or a previous infection may also be detected. Thus, in an aspect the invention related to a method for detecting antibodies against mink astrovirus capsid proteins, comprising the
steps:

a) contacting a sample of physiological material with at least one polypeptide according to the invention,

b) incubating the physiological material with said one or more polypeptides so that antibodies able to bind to said polypeptides will form a complex,
c) detecting the presence of antibodies in said sample, indicating that antibodies against said polypeptides are present.

Such an assay may be performed in the form of an ELISA assay or similar type of assay. The person skilled in the art would know how to perform such assays using e.g. antibodies recognizing antibodies against the target antibodies from the relevant organism. Such binding my subsequently be detected by fluorescence.
In the present context physiologically material also referred to as a biological sample may be previously provided from a mink e.g. in the form a faecal sample, a blood sample or processed blood sample. However, in certain instance other subjects may also be infected by mink astrovirus, thus the sample may also be previously obtained from e.g. human, rodents, mice and dogs.
In an embodiment said mink astrovirus strain is DK7627.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention.

All patent and non-patent references cited in the present application, are hereby incorporated by reference in their entirety.

The invention will now be described in further details in the following non-limiting examples.

Examples

Example 1

Cloning of the full-length and truncated forms of the ORF2 gene of mink astrovirus

For the development of the expression system for the full-length and truncated forms of the capsid protein of mink astrovirus, a sample DK5790 containing an astrovirus highly identical and therefore of the same genotype as the published sequence DK4265 was used.

The diagram in Figure 1 shows the strategy used to clone the full-length and truncated forms of ORF 2 of mink astrovirus. The pDual-GC expression plasmid was selected due to the versatility of allowing expression both in bacterial and in mammalian systems. In addition, the plasmid vector contains His and c-Myc tags to allow detection and affinity purification of the expressed proteins. The PCR products generated for ORF2 of mink astrovirus and the vector were cleaved with Eam1104I restriction enzyme, purified from agarose gels and then ligated with T4 DNA ligase. The ligation reaction was used to transform XL1-Blue MRF' cells by electroporation, according to the manufacturer's instructions. The bacteria were spread in LB media containing Kanamycin and incubated at 37ºC overnight. Screening for recombinants was done by PCR. Thereafter positive colonies were propagated and used to purify the plasmid DNA, using Wizard® Plus SV Miniprep kit (Promega). The mini-preps were checked for expression of protein using the in vitro transcription-translation system (TNT) from Promega. The clones giving positive signal for a band at the expected size were selected as the right expression clones.

TABLE 1. Primer pairs used for amplification of the full-length and truncated fragments of the ORF 2 gene of mink astrovirus, selected based on the published sequence DK4265 and on the determined sequence of strain DK7627.

ORF2 gene	Primers	Sequence of primer	Position in genome	Product size (bp)	Predicted Protein size
C1 of strains DK4265 and DK7627	SMC1F SMC2R	5'- *TAC TCT TCA* ATG GCG TCC GCC AAT CAG - 3' 5'- *TAC TCT TCG AAG* GTT CTT TGA GGA AAT TGC - 3'	4178-4192 6499-6482	2322	84.1 KDa
C2 of strain DK4265	SMC3F SMC2R	5'- *TAC TCT TCG ATG* TCA TTG AAT TTG ACA - 3'	4658-4672	1842	66.5 KDa
C4 of strain DK4265	SMC1F SMC4R	5'- *TAC TCT TCT AAG* CAT AAA GGT GCC ACT ACG - 3'	6037-6020	1860	67.5 KDa
C2 of strain DK7627	SMC3FN SMC2R	*5´- TAC TCT TCG ATG* ATC TCC CTT AAC CTC - 3'		1857	66.6 KDa
C4 of	SMC1F			1890	67.8

strain DK7627	SMC4RN	5' - *TAC TCT TCT AAG* GTT GTA GAC AAG CAA GTA - 3'			KDa

F indicates the forward primer. A seamless tail was added to the 5' end of these primers to create an Eam1104I recognition site at the 5´ ends of the amplicon: *TAC TCT TCA ATG* for SMC1F (SEQ ID NO: 24) and *TAC TCT TCG ATG* for SMC3F (SEQ ID NO: 26) and SMC3FN (SEQ ID NO: 28).

R indicates the reverse primer. A seamless tail was added to the 5' end of the primers to create an Eam1104I recognition site at the 3´ ends of amplicon: *TAC TCT TCG AAG* for SMC2R (SEQ ID NO: 25) and *TAC TCT TCT AAG* and for SMC4R (SEQ ID NO: 27) and SMC4RN (SEQ ID NO: 29). (see also figure 1).

It us to be understood that the primers listed in table 1 also forms part of the present invention. Primers SMC3FN and SMC4RN are specific for strain DK7627.

Example 2

Establishment of stably transfected mink foetal cells – strain of DK5790*, and evaluation of expression by immunofluorescence*

The plasmid pDual-GC carries a neomycin resistant gene that confers resistance of successfully transfected cells to Geneticin treatment. The mink foetal cells were obtained from the DTU cell bank. They were propagated in Dulbeco's EMEM medium supplemented with 10% FCS, 1% of L- Glutamine, 100µg/ml of penicillin and 50µg/ml of streptomycin. The cells were incubated at 37°C, in a humidified incubator with 5% CO_2**.** For transfections, the cells (1.5 x 10^5 cells/ml) were grown in 6-well plates and subsequently transfected with 1 µg of the corresponding expression clones, using liposome-mediated transfection. Transfected cells were then subcultured in the presence of 100-250 µg/ml of Geneticin (Invitrogen) in order to select cells where the ORF2 gene has been integrated. The medium in the cells was changed regularly with media containing Geneticin to continue selection. When clonal cells have been established, the cells were checked for the presence of the cloned gene by PCR. This procedure was repeated upon cell passaging, to evaluate the stability of the inserted genes. Cells showing a positive PCR reaction when then propagated and analysed for expression of the protein. Cells found to be producing protein were further propagated and then frozen at -134°C.

The stably transfected cells were grown on 8-well chamber slides and at the day after the cells were fixed with 4% paraformaldehyde for 30 minutes. The cells were permeabilized by incubation with 0.1% of Triton X-100 for 10 minutes, and then incubated with polyclonal serum from mink infected with astrovirus, diluted 1:100 as primary antibody. The primary antibody was removed and the cells were washed three times with PBS without Ca^{++} and Mg^{++} containing 10 mM glycine. An anti-mustelid antibody raised in mouse diluted 1:10 was added to the cells and incubated at 37°C for 60 minutes. The cells were washed and before and incubated as before with an anti-mouse antibody labelled with Cy3 as revealing antibody, dilted 1:500. The nuclei were stained by adding Hoechst 33342 (Molecular Probes), diluted 1:100 for contrast. The cells were washed as before and observed in a fluorescence microscope (Figure 2).

Example 3

SDS-PAGE and Western blot analysis of expressed C1 proteins

The transfected cells were washed with phosphate-buffered saline and lysed in NP40 lysis buffer (20 mM Tris-HCl [pH 7.6], 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing a complete protease inhibitor cocktail (Boehringer-Mannheim, Mannheim, Germany). The cell extracts were centrifuged at 5000 rpm for 10 min to separate the supernatant from the pellet. Then pellet and supernatant were mixed respectively with 1× and 4× SDS sample buffer and separated by SDS-PAGE. The proteins were then transferred to a PVDV membrane and analyzed by Western blotting by incubation successively with a polyclonal antiserum for mink astrovirus as primary antibody, a mouse anti-mustelid IgG and a rabbit anti-mouse IgG conjugated to horseradish peroxidase. The incubations were done for 1 hour each step, with three times 10 minutes washes in PBS-Tween in between. Development was done using diamino-benzydine (DAB). Figure 3A shows the results from a Western blot with non-purified protein lysates of the full-length capsid proteins of mink astrovirus DK5790 and DK7627. The expressed proteins are indicated by an arrow. Figure 3B shows and increasing and then stabilised level of expression at cell passages P1, P3, P7 and P9 (here represented with DK5790). DK5790 is of the same genotype as DK4265 (96% identity).

Example 4

Stability of the OFR2 gene of mink astrovirus (full-length and truncated forms) inserted in the transfected cells, as detected by PCR.

The PCR was performed from cells transfected with C1, C2 and C4 at different passages and consistently shows the presence of the inserted ORF2 gene in stably transfected cells. See figure 1 for background and figure 4 for the data.

Example 5

Determination of antibodies against the C protein in mink sera

Sera from immunised animals are tested for presence of specific antibodies against the mink astrovirus capsid proteins with the indirect enzyme linked immunosorbent assay (ELISA). Briefly, 96 wells plates were coated at 4°C over night with 500 ng/well of the recombinant capsid protein diluted in carbonate buffer (pH 9.6). After 4 times washing with PBS-T (PBS containing 0.05% Tween20) sera diluted 1:100 in blocking solution (PBS/Tween 20 with 5% skimmed milk) were added and incubated at 37°C for 1 hour. The horseradish peroxidase-labeled mouse anti-mustelid IgG secondary antibody diluted 1:1600 in blocking solution was added after washing and then incubated at 37°C for 1 hour. After washing and 10 minutes incubation with substrate solution (tetramethylbenzidine) the reactions were stopped by adding of 2M H_2SO_4. The optical density (OD) is measured at 450 nm with an ELISA microplate reader.

Example 6

Sequencing of ORF2 of calicivirus from mink

Clinical samples positive for calicivirus by EM, were used to extract RNA following the TRIZOL protocol (Invitrogen). The cDNA was synthesized using an anchored oligo d(T) and Superscript III reverse transcriptase enzyme, according to the manufacturer´s instructions. For PCR amplification the primer set 5'- GAT TAC TCC AAG TGG GAC TCC AC- 3' targeting a conserved sequence in the RNA-dependent RNA polymerase, and 5' - TGG GCG ATG GGC TTC CCT TTG located in the 3' end was used. The reactions were set with PFU Ultra high fidelity DNA

polymerase, in a mix containing 1xbuffer, 200 µm of dNTP mix, 10 picomole of each primer, 2.5 U of enzyme and 1 µl of cDNA. The PCR- positive samples were used to prepare sequencing reactions, using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequences were edited and analysed with multiple programs of the Lasergene package (DNASTAR, Inc).

Example 7

Purification of the C1 protein expressed from stably transfected mink foetal cells

Purification was done in affinity columns through a nickel matrix. Typically, cells were lysed on ice with phosphate buffer containing 20 mM immidazole, 1% v/v of Triton X-100, 5 µg/ml of DNAse I and RNase A, as lysis and membrane solubilization buffer. The lysates equilibrated in phosphate buffer, pH 7.4 were then run through the affinity columns. The columns were subject to stringent washing to remove unbound material. Following the washes the proteins were eluted in phosphate buffer containing 500 mM immidazole. The proteins were run through PD10 columns or dialyzed against PBS for buffer exchange to remove toxic substances used in the purification procedure. Western blot detection of protein was done as described in example 3, and is exemplified for the capsid protein of DK7627 in figure 5.

Example 8

Stable expression of proteins/peptides from mink cells.

Data on the cells expressing the peptides DK7627 from which are being (stable) expressed from the cells. The methodology used to create the stable transfected cells expressing proteins of strain DK7627 was the same as described in example 2. Stable transfected mink cells were produced and the cells were shown to express the corresponding proteins C1, C2 and C4 by immunofuoresncence (performed as described in example 2) and by Western blotting (performed as described in example 3). The mink cells stable expressing the C1, C2 and C4 of DK7627 are shown in figure 6.

Example 9

Pathogenity of DK7627

Example showing that the new virus strain (DK7627) is indeed infectious (pathogenic). Strain DK7627 was used in challenge experiments and caused severe disease in mink, readily transmitted to in-contact peers showing the pathogenic potential. The mink have shown fever, profuse diarrhoea up to 10 days, wet skin due to excessive excretion of the apocrine glands and dehydration, with shedding of virus from 2 to 29 days after infection. Gross pathological findings were not exclusive for astrovirus infection, but histopathological findings were consistent with those of astroviral enteric infections in juvenile animals of other species, where the virus causes an osmotic diarrhoea.

These results clearly show that the min astrovirus strain DK7627 is indeed pathogenic.

Example 10

Evoked immune response in mice

In the present example the antibody response, proliferative ability and induction of cytokines were determined following immunization of mice with full-length (SEQ ID NO 2), an N-terminally truncated form (SEQ ID NO 14) and a C- terminally truncated forms (SEQ ID NO: 16) of the capsid protein from DK7627.

The period when vaccines can be tested for challenge in mink is limited to the reproduction period, which is only once a year. Therefore mice are also valuable to speed up the testing.

The reason for using mice is that mice are a well established laboratory model for testing of vaccines to evaluate their ability to induce immune responses. Mink are used in the next stage in order to evaluate protection from the vaccine.

Materials and Methods

Construction and expression of the full-length and truncated capsid proteins

The construction and expression of the full-length and truncated capsid proteins of mink astrovirus have been described before. C1 refers to the full-length protein (SEQ ID NO 2); C2 refers to an N-terminal truncated protein (SEQ ID NO 14); C4 is a C-terminal truncated protein (SEQ ID NO 16). The proteins have been

expressed from stably transfected cells and were purified by affinity in a nickel resin.

Immunization of mice with the C1, C2 and C4 proteins

Four groups, each containing 8 Balb c mice were used in this study. Three groups were injected subcutaneously with 0.2 ml of a mixture of 5 µg of C1-, C2-, or C4 proteins in PBS with 10 µg of AbISCO-100 (Isconova, Uppsala, Sweden). The control was injected with 0.2 ml of PBS containing10 µg of AbISCO-100. Four weeks after the first immunization, the mice received a second injection of protein or control media applied as before. Blood for sera were collected three weeks after each immunization. Two animals in each group were sacrificed at weekly, and spleens were harvested.

Determination of antibodies in an ELISA

To detect antibodies specific to the capsid protei of mink astrovirus, high-binding ELISA plates (MaxiSorp, Nunc) were coated overnight at 4°C with 500 ng/well of a recombinant capsid protein expressed in bacteria, diluted in carbonate buffer (pH 9.6). The plates were washed three times with PBS-T (PBS with 0.05 % Tween 20) and blocked with PBS-T containing 5% of skimmed milk for 1 hour. The sera were added at a dilution of 1:100 made in blocking buffer, and the plates and incubated for 1 hour. After washing with PBS-T, horseradish peroxidase-labeled rabbit anti-mouse IgG diluted 1:2000 was added, and the plates were incubated for another 1 hour. After three washes as before, the substrate solution (tetramethylbenzidine) was added and the reaction was stopped by adding H2SO4. All incubation steps were performed at room temperature. The optical density (OD) was measured at 450 nm in an ELISA microplate reader.

Stimulation of lymphocytes for in vitro proliferation assay and for cytokine determination.

Spleens collected from the mice were processed for isolation of splenocytes. The final splenocytes suspensions were counted and plated out in 96-well plates at a density of 2×105 cells/well in RPMI medium supplemented with 5% of foetal calf serum, 0.2% of L-Glutamin, 100 Units/ml of Penicillin, 100µg/ml of Streptomycin, and 0.1% of 2-mercaptoethanol. The cells were stimulated with 1, 2, 4 and 8 µg/ml of the corresponding proteins, in triplicate wells for each protein

concentration. Cells from the control mice were also stimulated in the same manner. Following incubation for 48 hours at 37°C, 5% CO2, the plates were centrifuged at 1200 rpm for 10 minutes, and the supernatants were removed and stored at -70°C for determination of cytokines.

For evaluation of proliferation, replica plates containing cell suspension were prepared and treated as before. Following incubation for 48 hours at 37°C, 5% CO2, proliferation of specific cells was assessed using a WST-1 rapid Cell Proliferation Kit (Calbiochem, Germany). 10 µl of the WST-1 mixture, a colorimetric indicator of cell viability was added, and absorbance at 450 nm was measured with a spectrophotometer reader 2hours later.

Determination of cytokines in a Luminex assay

Culture supernatants were analysed using a 6-plex kit (Invitrogen) that detects IFN-γ, IL2, IL-4, IL-5, IL-10 and IL-12. Briefly, filter plates were pre-wetted and 50 µl of coated bead suspension was added to each well and washed twice in a Tecan device. The samples and standards (50 µl) were then added in duplicate wells, the plates were sealed and shaken for 30 seconds at 1100 rpm and then incubated for 1hour, shaking at 300 rpm. The plates were washed three times as before, and 25 µl of diluted detection antibody was added to each well. The plates were shaken as before and then incubated for 30 minutes, shaking at 300 rpm, in the dark. After three washes as before, 50 µl of 1× streptavidin-PE was added to every well and the plates were incubated for 10minutes. The plates were washed again and the beads were resuspendeded in 125 µl of the resuspension buffer, mixed and immediately read on the Luminex instrument using software.

Statistical analysis

The mean OD value and standard deviation (SD) of ELISA test of each group of immunized and non-immunized mice were analyzed by using Student's t-test for differences among groups. $P<0.005$ was considered significant. Comparison of secreted cytokine profiles following Luminex was done using GraphPad Prism software version 4.

Results

Antibody responses to the full length, N- and C-terminally truncated astrovirus capsid proteins evaluated in mice

Blood of mice immunized with C1, C2 and C4 polypeptides and of control mice were collected three weeks after the first and second injection. The sera were analyzed with an ELISA for detection of antibodies to the capsid protein of mink astrovirus. The antibody levels after first immunisation were low and without difference between the immunized and non-immunized mice groups. After receiving the booster injection the immunized mice showed an increase in antibody levels ($P < 0.05$) compared to the controls. Interestingly, the antibody levels induced by the C2 protein increased significantly ($P < 0.005$), and were markedly higher than antibodies stimulated by the full-length (C1) or the C-terminal truncated (C4) capsid proteins (Fig. 7). The antibody reactions to the C1 and C4 proteins had similar OD values.

Proliferative responses following immunization of mice with the full length, N- and C-terminally truncated astrovirus capsid proteins

The viability of splenocytes using WST-1 labelling mixture was measured, showing effects of different concentrations of protein concentration on splenocyte proliferation. Readings indicative of lymphocyte proliferation were recorded in supernatants from stimulated splenocytes in the immunized but not in the control mice (Fig 8. A, B, C). The C1 did not induce splenocyte proliferation at concentrations higher than 4 µg/ml. In contrast, the two truncated forms of the capsid protein, in particular the C2, showed an increase in proliferation ability in response to increased protein concentration ($P<0.01$).

Induction of cytokines following immunization of mice with the full length, N- and C-terminally truncated astrovirus capsid proteins

Culture supernatants from stimulated splenocytes were tested for secreted cytokines using a 6-plex bead array that measures IFN- γ, IL-2, IL-4, IL-5, IL-10 and IL-12.

IL-2, IL-10, IL-12 and IFN-γ are representative of cellular response whereas IL-4 and IL-5 are representative for humoral response.

Cellular response (cell-mediated immunity) is an active arm of adaptive immunity which functions against pathogens that survive inside phagocytic cells as well as those which infect non-phagocytic cells. The most important roles of cellular immunity during the course of infection are:

- Eliminating microbe-infected cells (e.g. virus infected cells or intracellular bacteria-infected cells) or even tumour cells by presenting tumour antigens and activating cytotoxic T-cells (CTLs).
- Enhancing the killing ability of macrophages and natural killer (NK) cells.
- Affecting innate and adaptive immune responses by induction of different cells to produce cytokines such as interleukins, interferons, chemokines, etc.

The results are displayed in the array of figures in Figures 9-14, where the top panel represents the immunized, while the bottom panel represents the naïve (control) mice. For IFN- γ, a significant induction was stimulated by the N- and the C-terminally truncated proteins, while the response to the full-length protein was rather low. The naïve mice show reading to the C4 antigen, but the values are below the readings of the immunized mice. For IL-2 a significant readout was found in mice immunized with the C4 protein. For IL-4, the MFI values were in general low, however with a significant difference between the full-length which shows basal signs, and the C2 and C4 proteins. The IL-5 response showed a similar pattern to the IL-2, with higher and significant stimulation induced in mice immunized with the C4 protein. For IL-10, significantly high levels of cytokine were induced by stimulation with the C4 protein, and readings from the C2 protein though not as high they were above the C1 values at increasing amount of protein. For IL-12 there were not differences between the readings in immunized and in non-immunized mice, showing that there is no induction of this cytokine by any of the proteins used.

In general, the production of cytokine increased with the amount of protein used in the stimulation, with significant differences at the amount of 5 µg of protein used in the immunization.

Though inducing low level of antibodies, the C4 is endowed with better capacity for mounting a recall response by means of strong cellular immunity. The C2 protein combines both ability to induce high level of antibodies (humoral response) with capacity to stimulate cytokine production (cellular response) therefore stimulating both arms of the immune response.

Conclusion

These results show that the N-terminally truncated protein (SEQ ID NO 14) induced significantly higher level of serum antibodies after a second immunization, as evaluated by ELISA. The antibody level induced by the full-length (SEQ ID NO 2) and the C-terminal truncated proteins (SEQ ID NO 16) were comparable and higher than the controls. Furthermore, the proliferative responses and cytokine induction stimulated by the truncated proteins were significantly higher than those elicited by the full-length capsid protein.

Example 11

Sequence alignments

The present example illustrates different sequence identities and sequence distances between mink astrovirus strains DK7627 and DK4265.

The sequence differences in the capsid gene of strain DK7627 with regards to strain DK4265 can be appreciated from alignments between the two full length capsid genes (C1), the N-terminally truncated capsid genes (C2), and the C-terminally truncated genes (C4). As previously mentioned C2 of strain DK7627 comprises the amino acid sequence positions 161 to 778 of the capsid protein of this strain and C4 of strain DK7627 comprises the amino acid sequence positions 1 to 630 of the capsid protein of this strain.

Alignment between the different domains gave the following results:

Nucleotide level		
Sequences	Sequence identity	Sequence distance
C1 DK4265 (SEQ ID NO 3) and C1 DK7627 (SEQ ID NO 1)	54.1	47.3%
C2 DK4265 (SEQ ID NO 17) and C2 DK7627 (SEQ ID NO 13)	46.8%	54.1%
C4 DK4265 (SEQ ID NO 19) and C4 DK7627 (SEQ ID NO 15)	53.1%	49.9%
Protein level		
C1 DK4265 (SEQ ID NO 4) and C1 DK7627 (SEQ ID NO 2)	57.5%	51.9%

C2 DK4265 (SEQ ID NO 18) and C2 DK7627 (SEQ ID NO 14)	52.7%	61.2%
C4 DK4265 (SEQ ID NO 20) and C4 DK7627 (SEQ ID NO 16)	58.1%	51.4%

As an RNA virus, the mink astrovirus genome is prone to mutations and, though not yet described, possible recombination with homologous (viral) and heterologous (non-viral sequences). Recombination among astroviruses has been described for example between astroviruses from human and astroviruses from marine mammals. Such events may result in strains with different genomes and characteristics as compared to previously known strains. For the mink astrovirus, recombination events have not yet been studied, therefore the origin and reason for the pronounced genetic difference of strain DK7627 with regards to the known mink astrovirus represented by strain DK4265 has not yet been established. It is also plausible that these astroviruses have co-existed as genetically diverse strains in the mink population, and could only be detected by molecular testing as described in here. On the other hand, as the outer protein of the virus the capsid is subject to an immunological pressure due to the host's response. This pressure can also drive the evolution of new genetic variants, showing as accumulation of acquired mutations in the gene encoding the capsid protein, as means to evade the host's immune defence.

It is surprising that the present inventors have found a novel strain of mink astrovirus with a low sequence similarity, which has also found to be pathogenic. The skilled person may expect to find strains with the same genotype (high sequence similarity), e.g. DK5790. However, the skilled person would not expect to find a completely new strain with another genotype.

Example 12

In silico models of the capsid proteins of strains DK4265 and DK7627.

The predicted protein model and folding was determined based on the deduced amino acid sequences of the full-length capsids of these strains. As shown in Fig 15 there are localised differences in the predicted structures of these proteins (A,

DK4265 and B, DK7627). In the picture superimposing both models (C) there are few regions of overlap between the structures, indicating that they do not have same folding.

Conclusion

These results clearly indicate that there is a surprisingly low homology between DK4265 and DK7627. Furthermore, these results show that there is good evidence for that DK7627 is indeed a novel strain.

References

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Claims

1. An isolated polynucleotide which has at least 85% sequence identity to

a) a polynucleotide as set out in SEQ ID NO: 1, 13 or 15; and/or

b) a polynucleotide encoding a polypeptide according to SEQ ID NO: 2, 14 or 16; and/or

c) a fragment of (a) or (b), wherein said fragment has a minimum length of 100 nucleotides,

wherein said isolated polynucleotide comprises nucleic acids encoding for one or more B-cell epitopes and/or one or more T-cell epitopes.

2. A recombinant vector comprising an isolated polynucleotide according to claim 1 and a promoter which is operably linked to said polynucleotide.

3. A recombinant polypeptide or active derivative thereof expressed by any polynucleotide according to claim 1, wherein said recombinant polypeptide and active derivative thereof comprises at least one B-cell epitope and/or at least one T-cell epitope.

4. A recombinant polypeptide or active derivative thereof, having at least 85% sequence identity to SEQ ID NO: 2, 14 or 16, wherein said recombinant polypeptide and active derivative thereof comprises at least one B-cell epitope and/or at least one T-cell epitope.

5. The recombinant polypeptide or active derivative thereof according to claim 3 or 4, wherein said recombinant polypeptide or active derivative thereof has at least 85% sequence identity to SEQ ID NO: 2, 14 or 16, such as at least 90% sequence identity, such as at least 95% sequence identity such as at least 99% sequence identity or such as 100% sequence identity.

6. The recombinant polypeptide or active derivative thereof according to any of claims 3-5, wherein said polypeptide is operably linked to a tag.

7. A recombinant host cell expressing a polypeptide or active derivative thereof according to any of claims 3-6.

8. A recombinant polypeptide or active derivative thereof according to any of claims 3-6, obtainable from a host cell according to claim 7.

9. A method of producing a recombinant polypeptide according to any of claims 3-6 or 8, comprising the steps of:

a) contacting a host cell line with a vector according to claims 2,
b) cultivating said cells under conditions suitable for the production of the polypeptide.
c) purifying said recombinant polypeptide or active derivative thereof .

10. A composition comprising a recombinant polypeptide or active derivative thereof according to any of claims 3-6 or 8.

11. The composition according to claim 10, for use as a vaccine in mammals such as mink, mice and/or humans.

12. The composition according to any of claims 10 or 11, for use as a vaccine in mammals, such as mink, mice and/or humans, against an infection of at least one mink astrovirus strain, such as DK7627.

13. The composition according to any of claims 10-12, comprising at least one further recombinant polypeptide or active derivative thereof selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18 and SEQ ID NO: 20, wherein said at least one further recombinant polypeptide or active derivative thereof comprises at least one B-cell epitope and/or at least one T-cell epitope.

14. A method for the detection of mink astrovirus in a sample comprising the steps:

I. contacting physiological material with a probe wherein said probe is selected from:
   a) a polynucleotide as set out in SEQ ID NO 1, 13 or 15;
   b) a polynucleotide that is capable of hybridising to SEQ ID NOs 1, 13 or 15 under stringent conditions,

c) a polynucleotide which has at least 85% sequence identity to a polynucleotide as set out in any one of (a) to (b),

d) a fragment of (a) (b) or (c), wherein said fragment has a length of minimum 10 nucleotides, and

II. detecting a successful binding event between the probe and at least one component of the sample.

15. The method according to claim 13, wherein said polynucleotide capable of hybridizing to SEQ ID NO: 1 or its complementary sequence under stringent conditions, is selected from the group consisting of SEQ ID NO's 20-29, such as SEQ ID NO's 28 and 29.

16. A method for detecting antibodies against mink astrovirus capsid proteins, comprising the steps:

a) contacting a sample of physiological material with at least one polypeptides according to any of claims 3-5 and 7,

b) incubating the physiological material with said one or more polypeptides so that antibodies able to bind to said polypeptides will form a complex,

c) detecting the presence of antibodies in said sample, indicating that antibodies against said polypeptides are present.

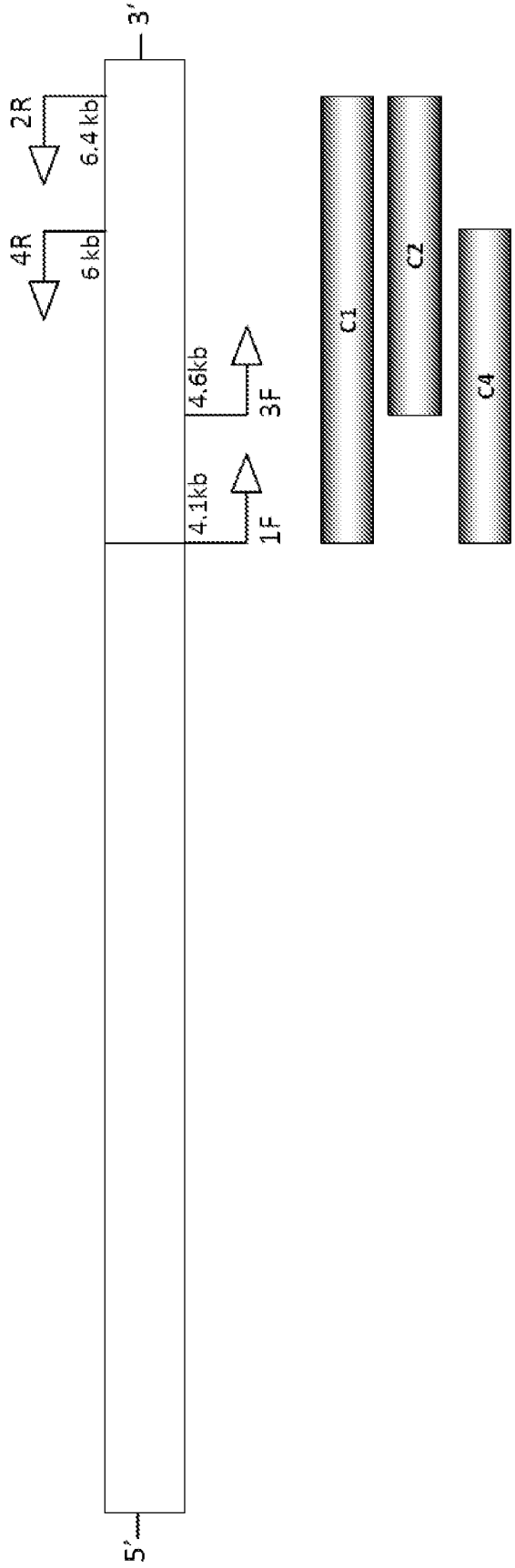


Fig. 1

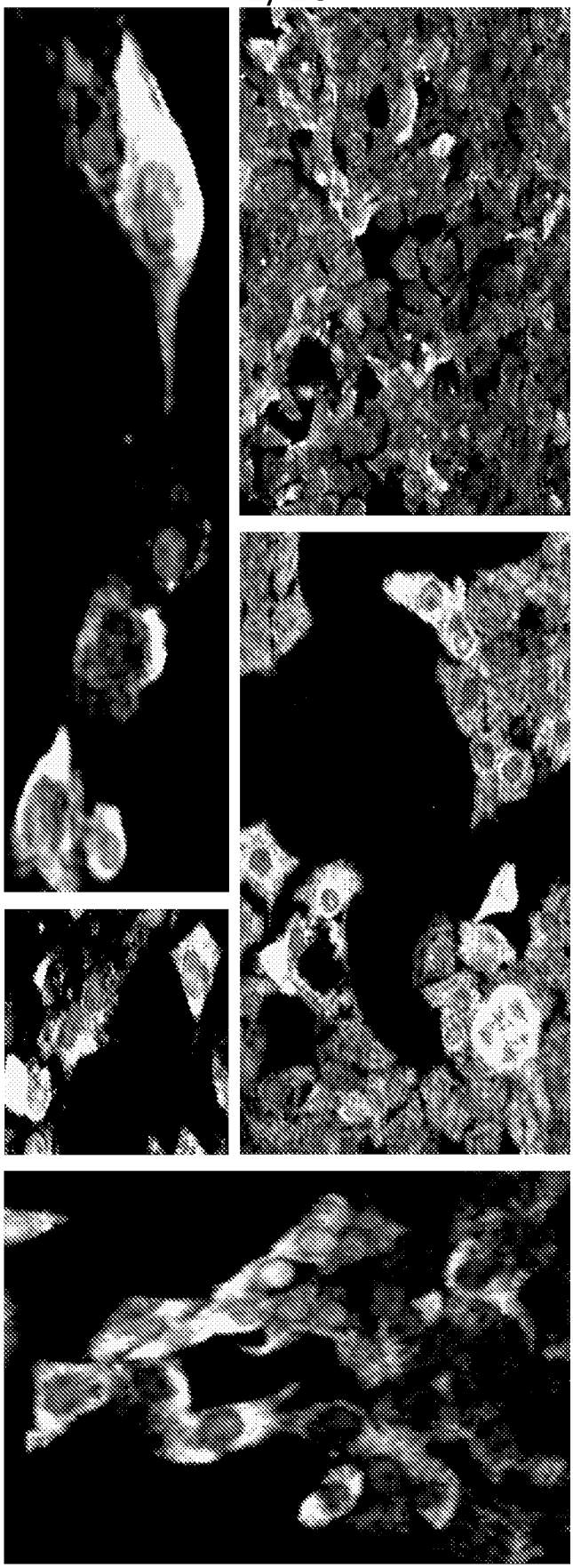


Fig. 2

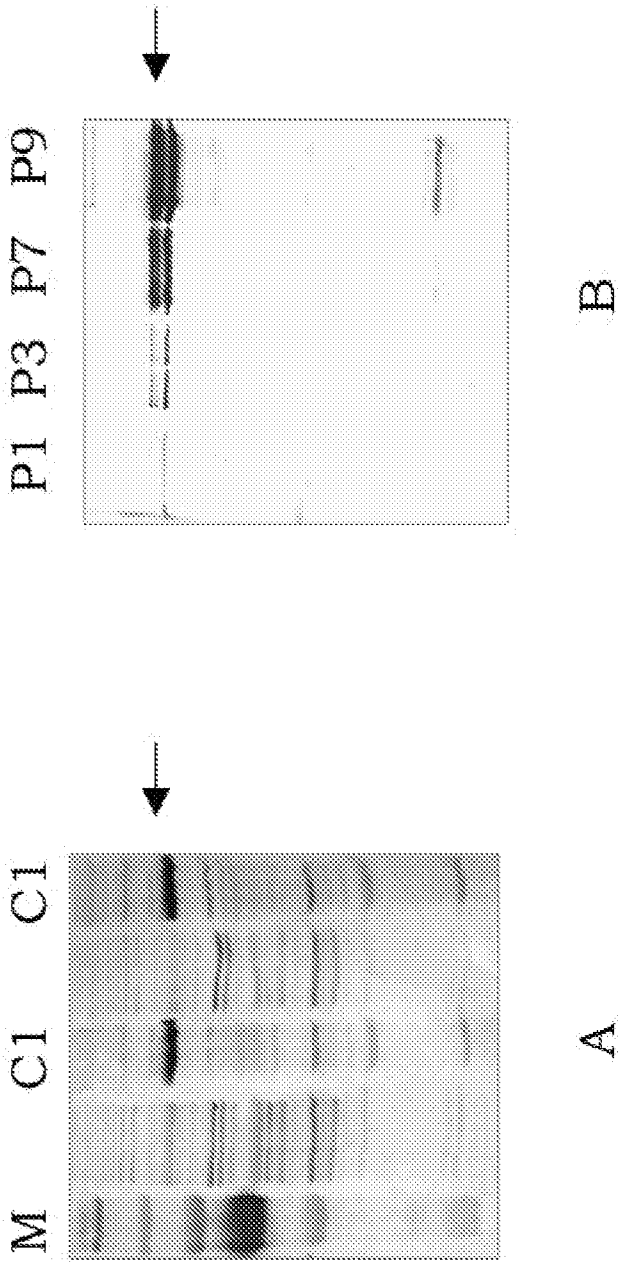


Fig. 3

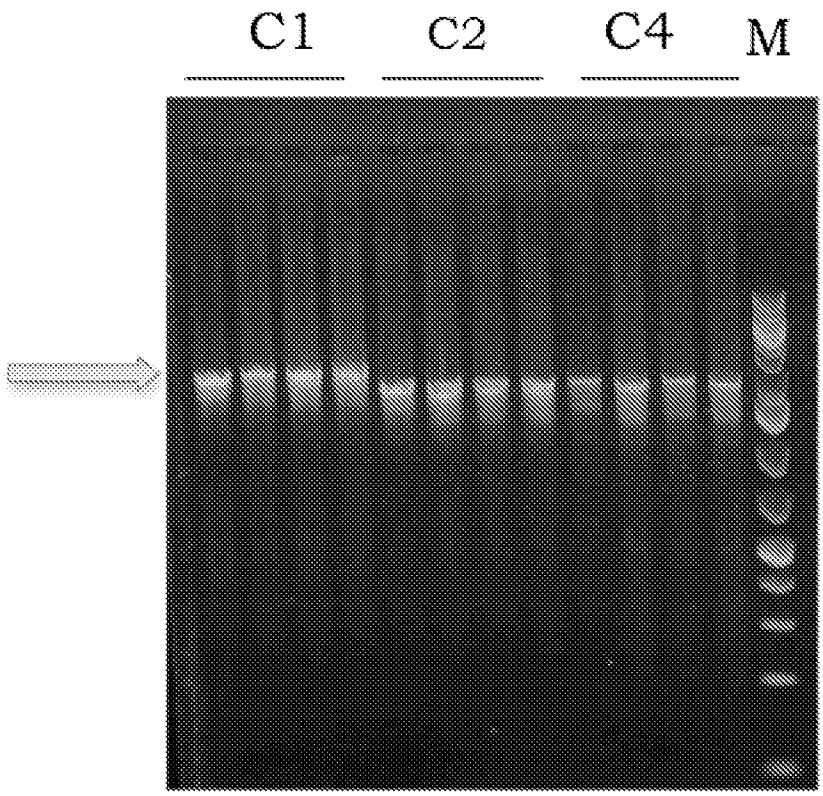


Fig. 4

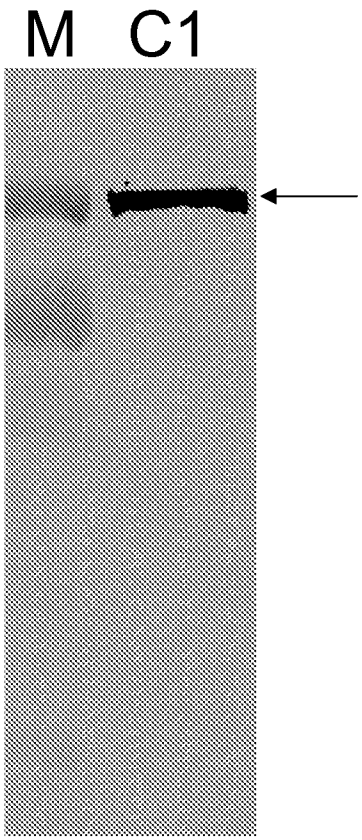


Fig. 5

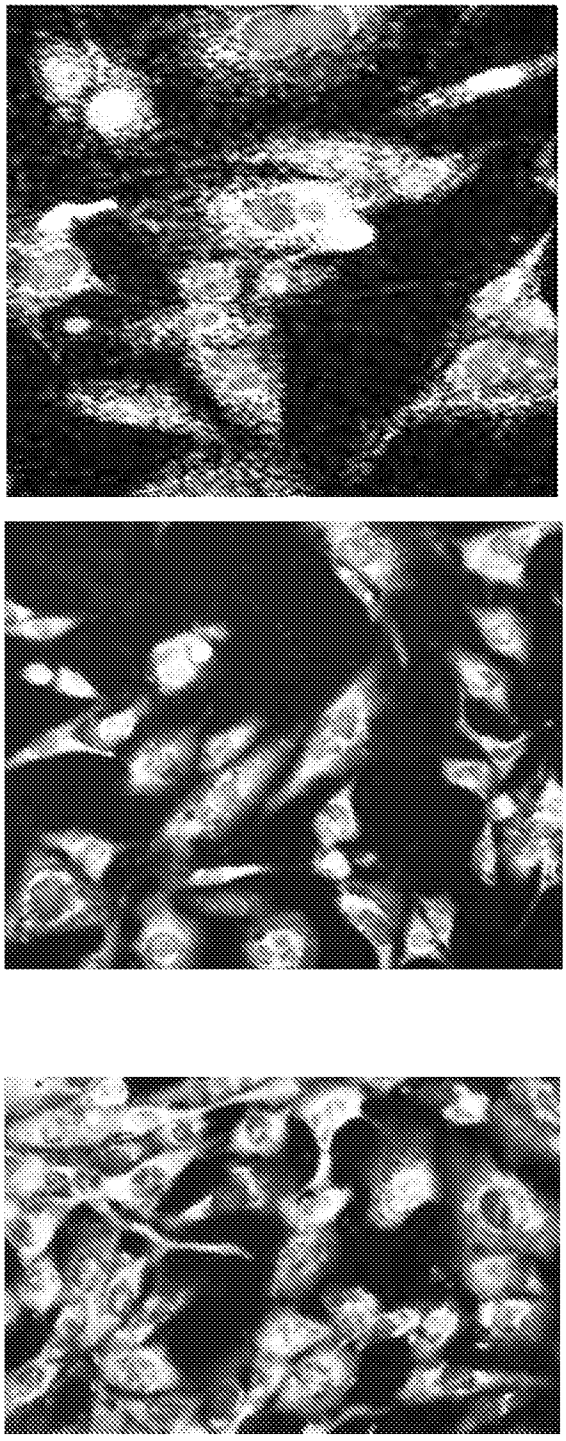


Fig. 6

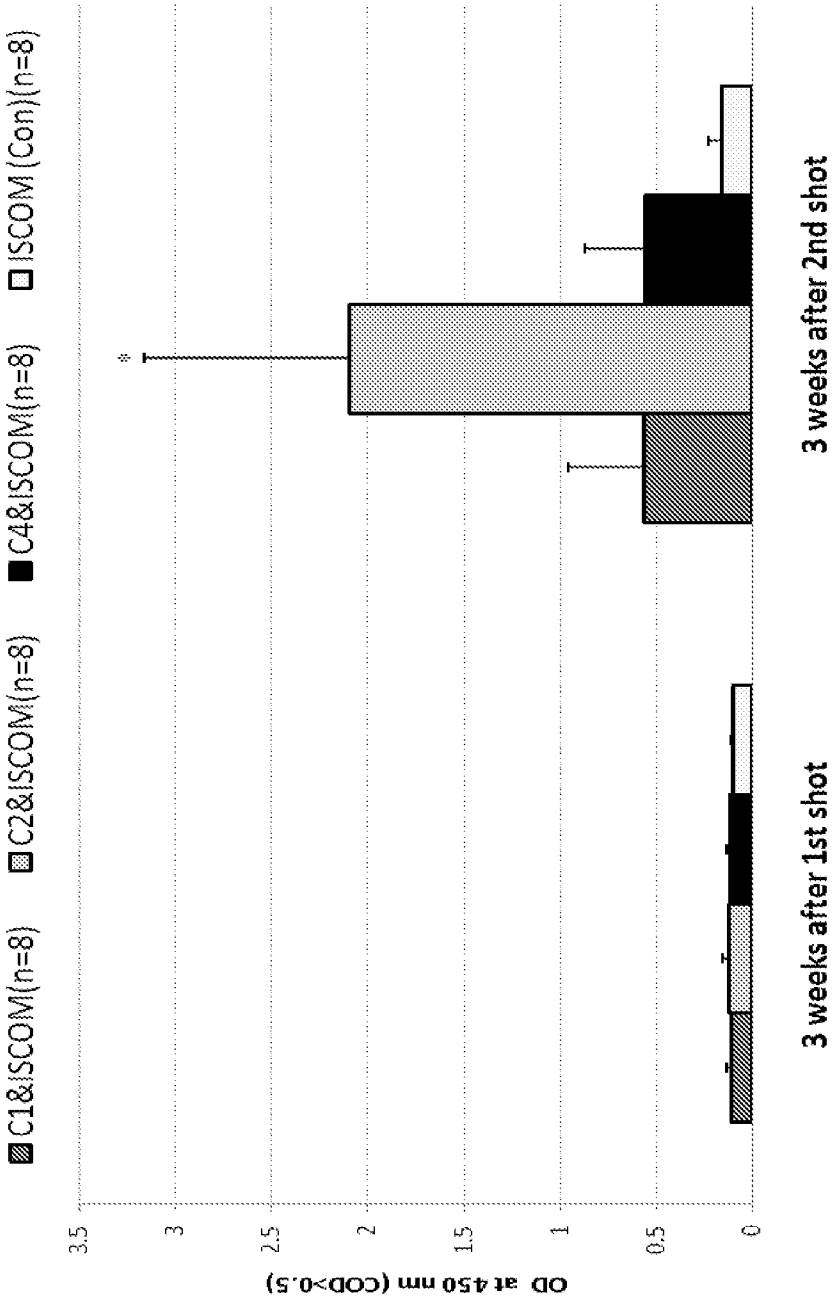
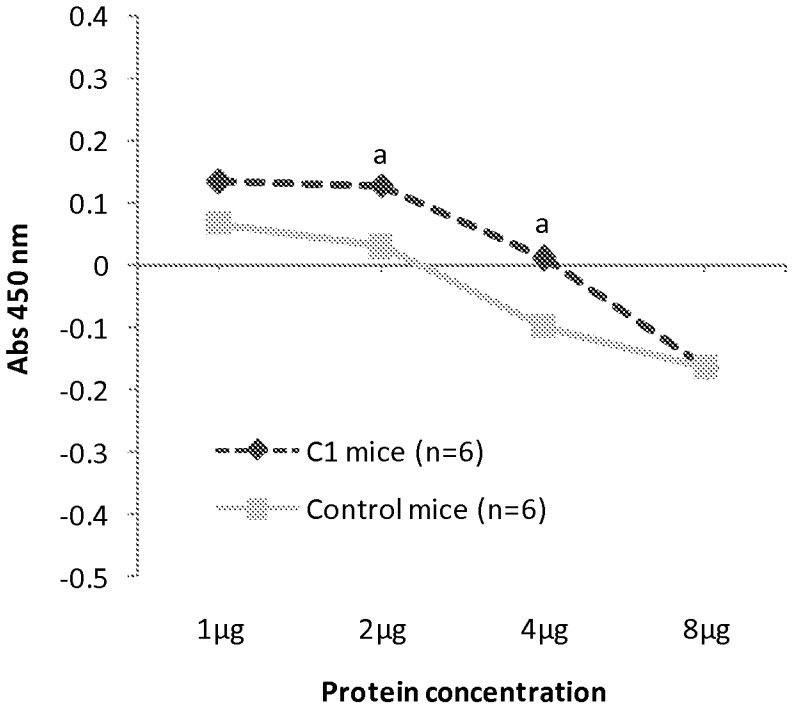

C1&ISCOM(n=8)
C2&ISCOM(n=8)
C4&ISCOM(n=8)
ISCOM (Con)(n=8)
OD at 450 nm (COD>0.5)
3.5
3
2.5
2
1.5
1
0.5
0
*
3 weeks after 1st shot
3 weeks after 2nd shot

Fig. 7

A


0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
Abs 450 nm
a
a
C1 mice (n=6)
Control mice (n=6)
1µg
2µg
4µg
8µg
Protein concentration

B

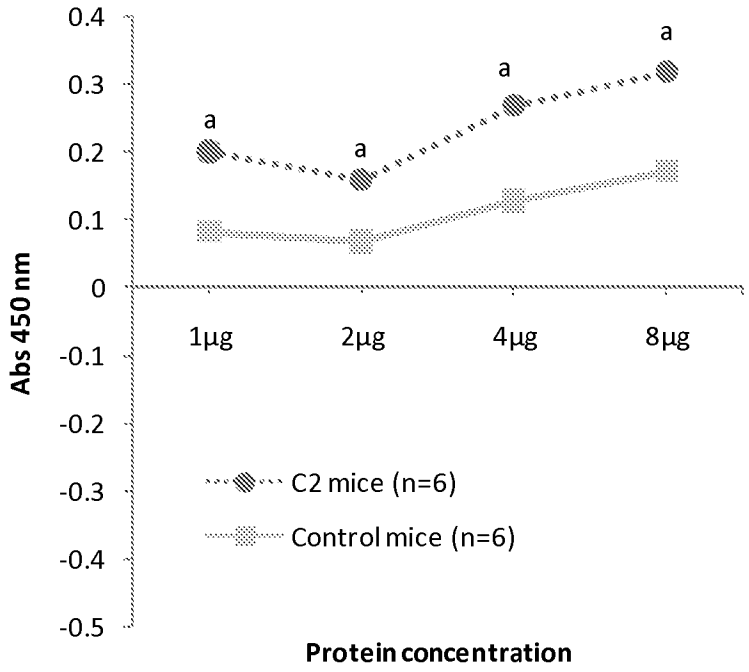
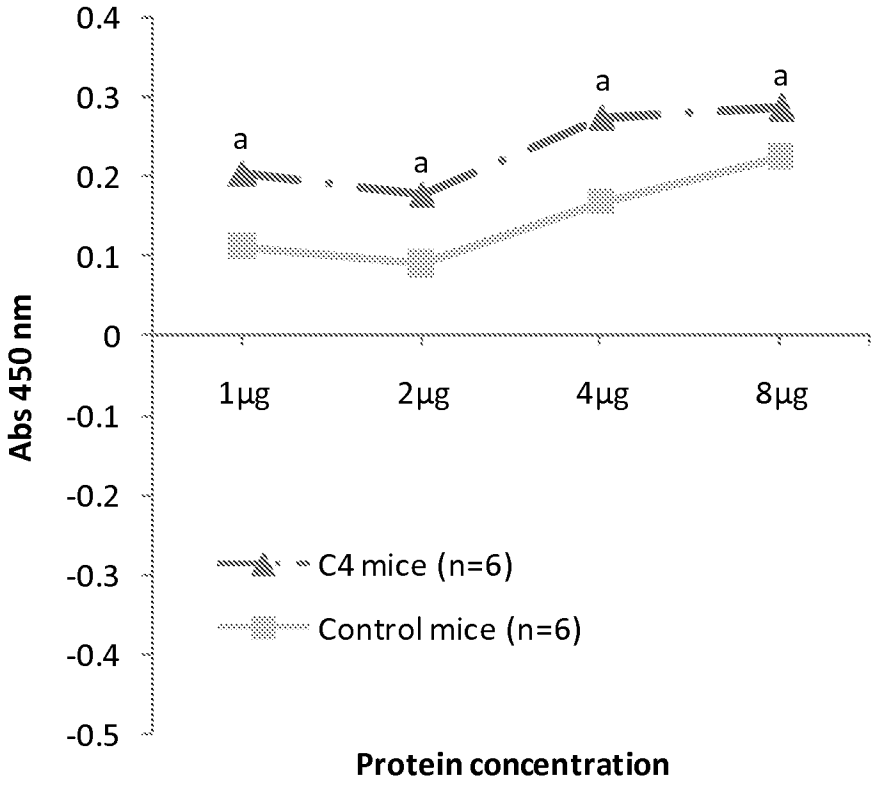

0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
Abs 450 nm
a
a
a
a
C2 mice (n=6)
Control mice (n=6)
1µg
2µg
4µg
8µg
Protein concentration

Fig. 8

C



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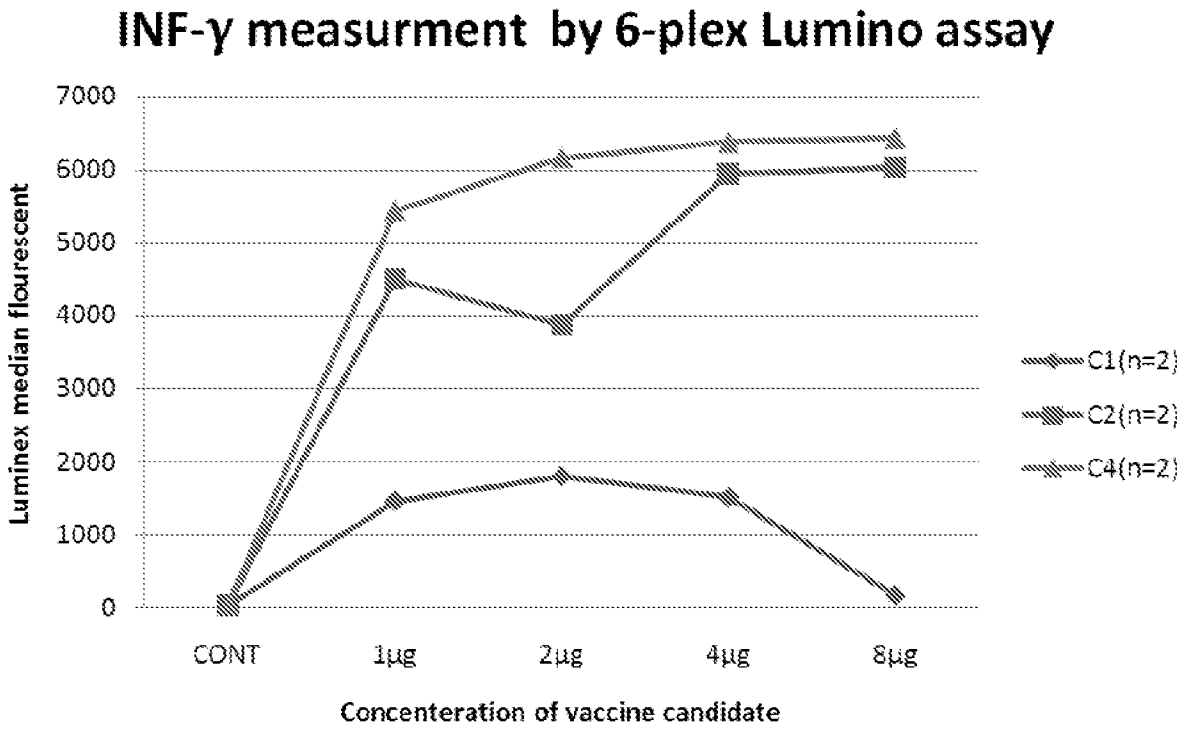

INF-γ measurment by 6-plex Lumino assay
Luminex median flourescent
7000
6000
5000
4000
3000
2000
1000
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
C1(n=2)
C2(n=2)
C4(n=2)

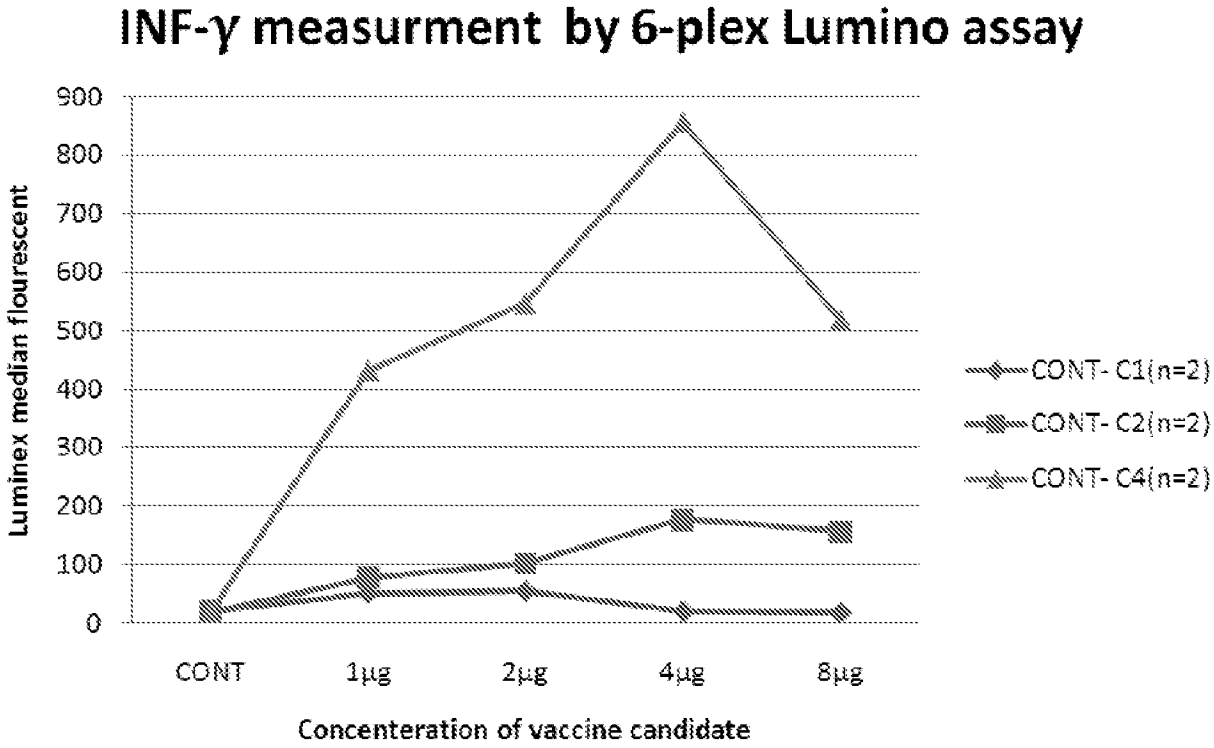
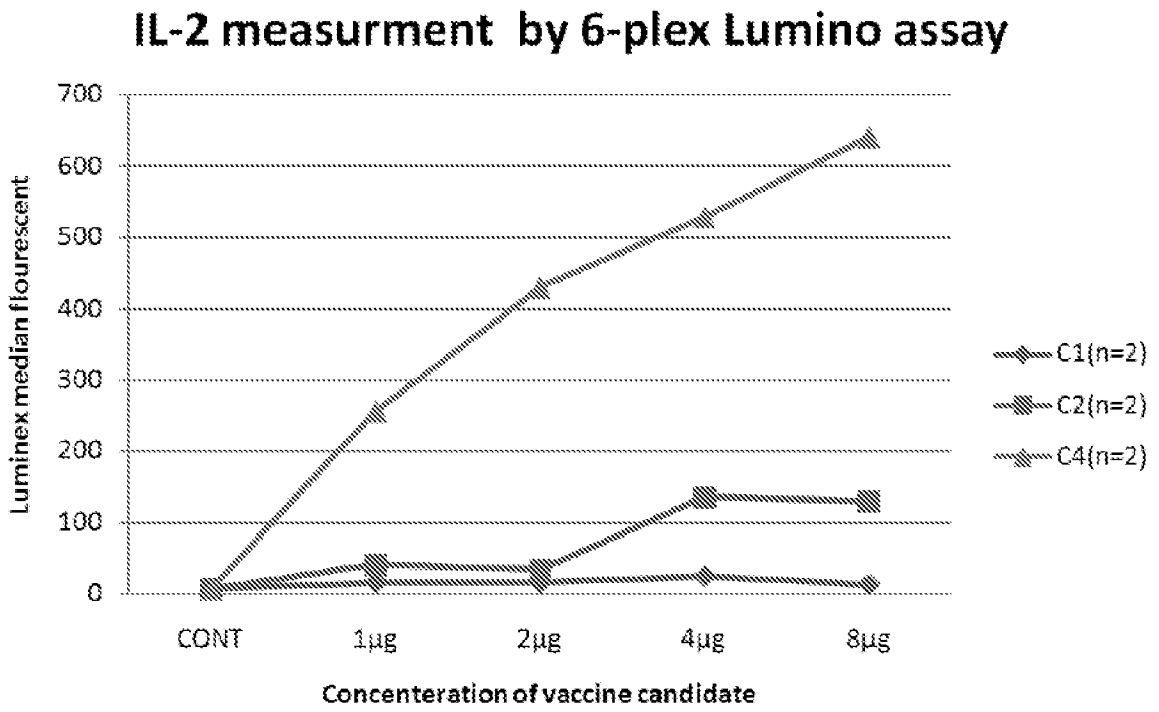

INF-γ measurment by 6-plex Lumino assay
Luminex median flourescent
900
800
700
600
500
400
300
200
100
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
CONT- C1(n=2)
CONT- C2(n=2)
CONT- C4(n=2)

Fig. 9


IL-2 measurment by 6-plex Lumino assay
Luminex median flourescent
700
600
500
400
300
200
100
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
C1(n=2)
C2(n=2)
C4(n=2)

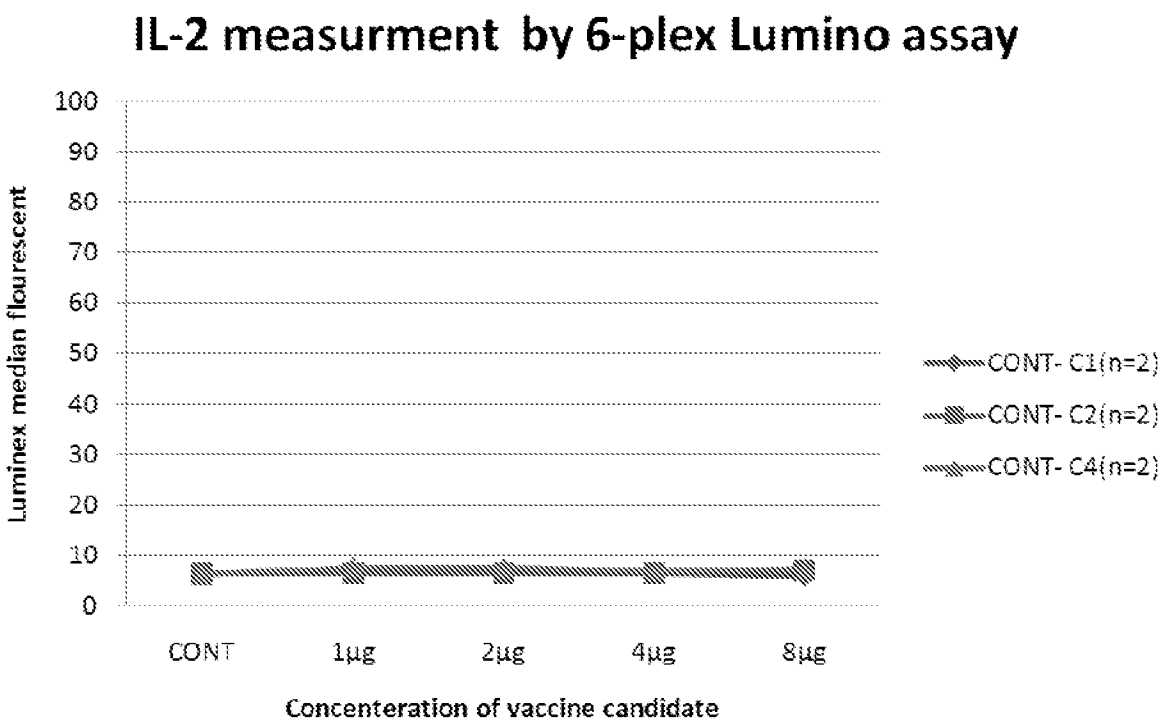
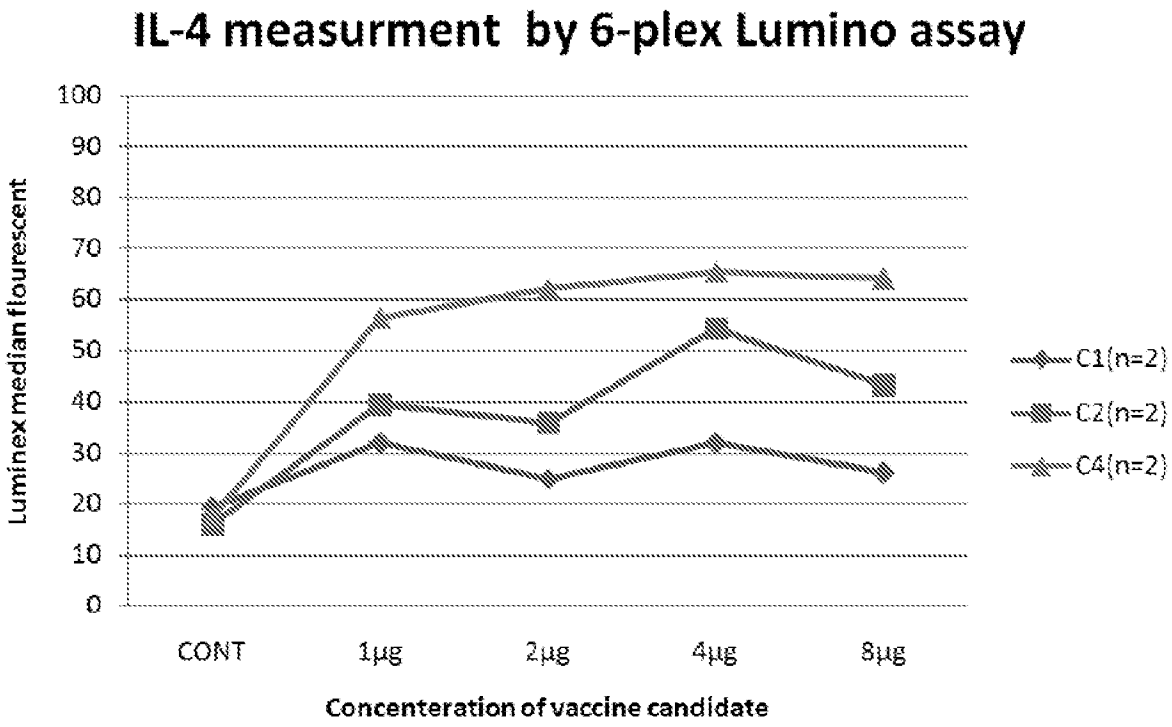

IL-2 measurment by 6-plex Lumino assay
Luminex median flourescent
100
90
80
70
60
50
40
30
20
10
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
CONT- C1(n=2)
CONT- C2(n=2)
CONT- C4(n=2)

Fig. 10


IL-4 measurment by 6-plex Lumino assay
Luminex median flourescent
100
90
80
70
60
50
40
30
20
10
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
C1(n=2)
C2(n=2)
C4(n=2)

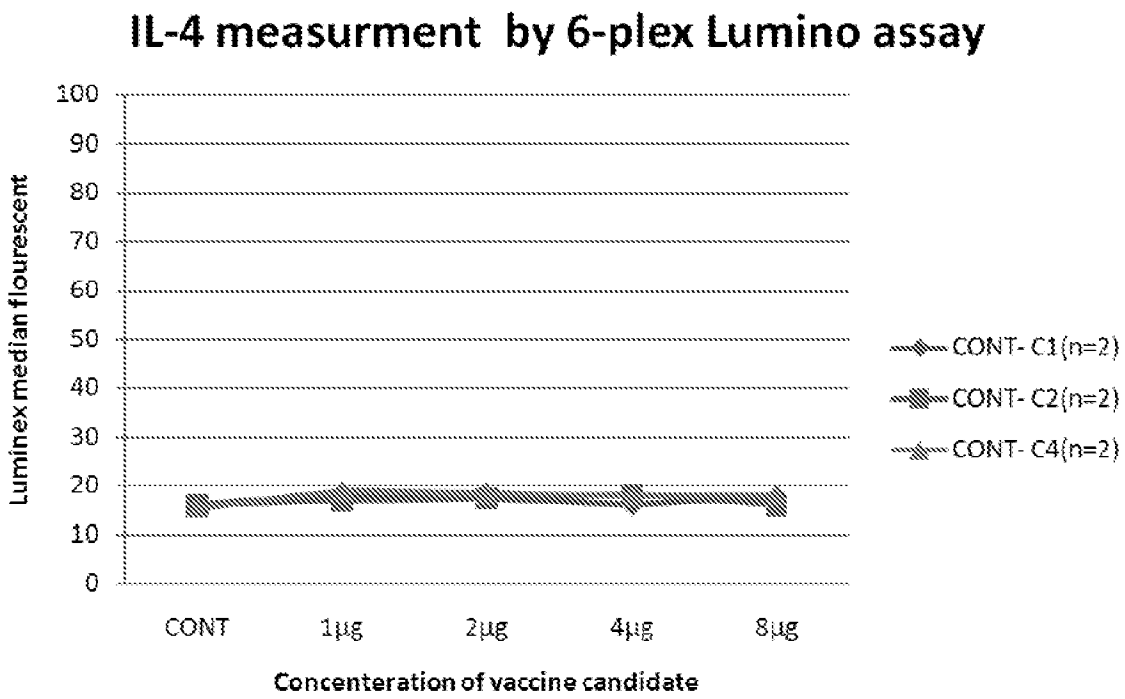
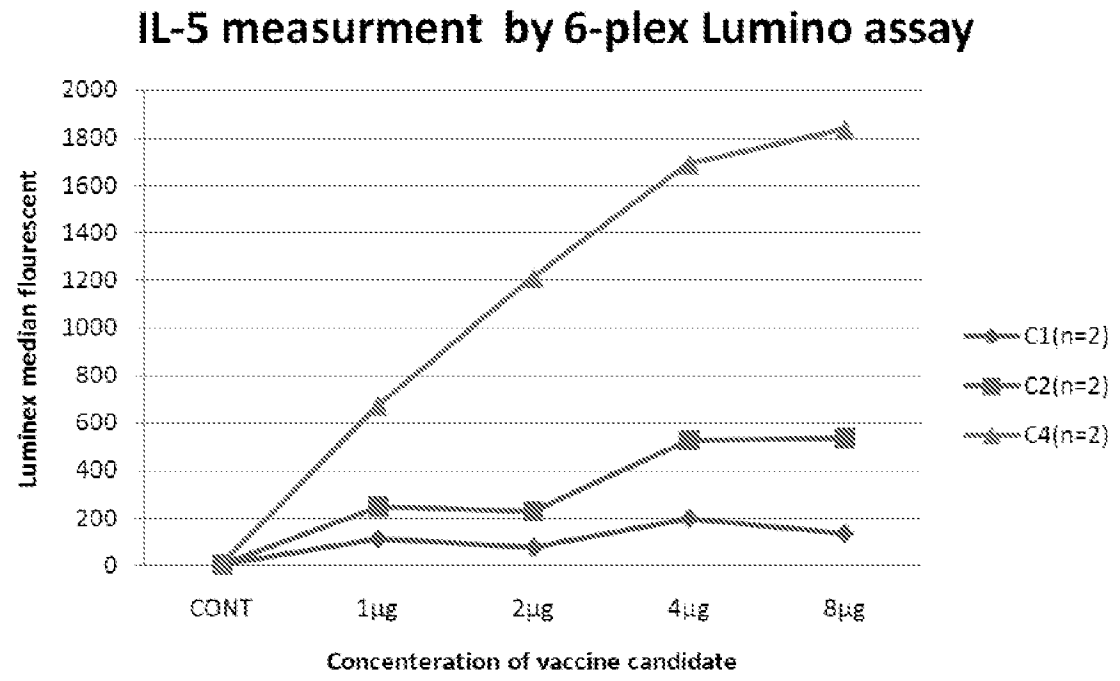

IL-4 measurment by 6-plex Lumino assay
Luminex median flourescent
100
90
80
70
60
50
40
30
20
10
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
CONT- C1(n=2)
CONT- C2(n=2)
CONT- C4(n=2)

Fig. 11


IL-5 measurment by 6-plex Lumino assay
Luminex median flourescent
2000
1800
1600
1400
1200
1000
800
600
400
200
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
C1(n=2)
C2(n=2)
C4(n=2)

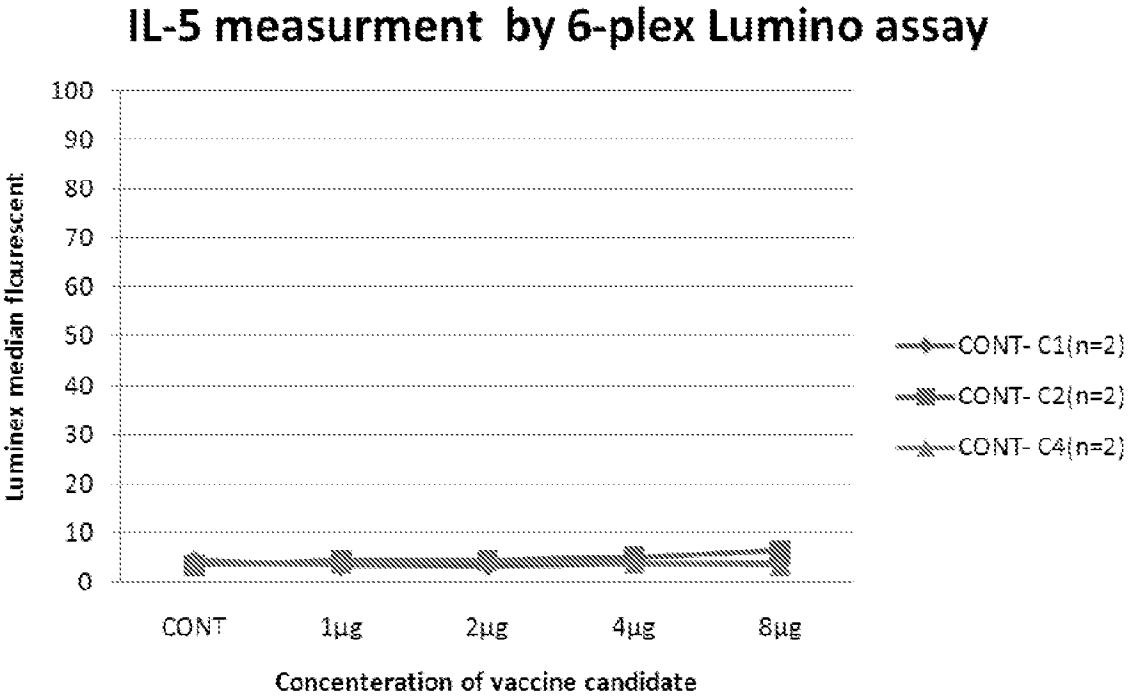
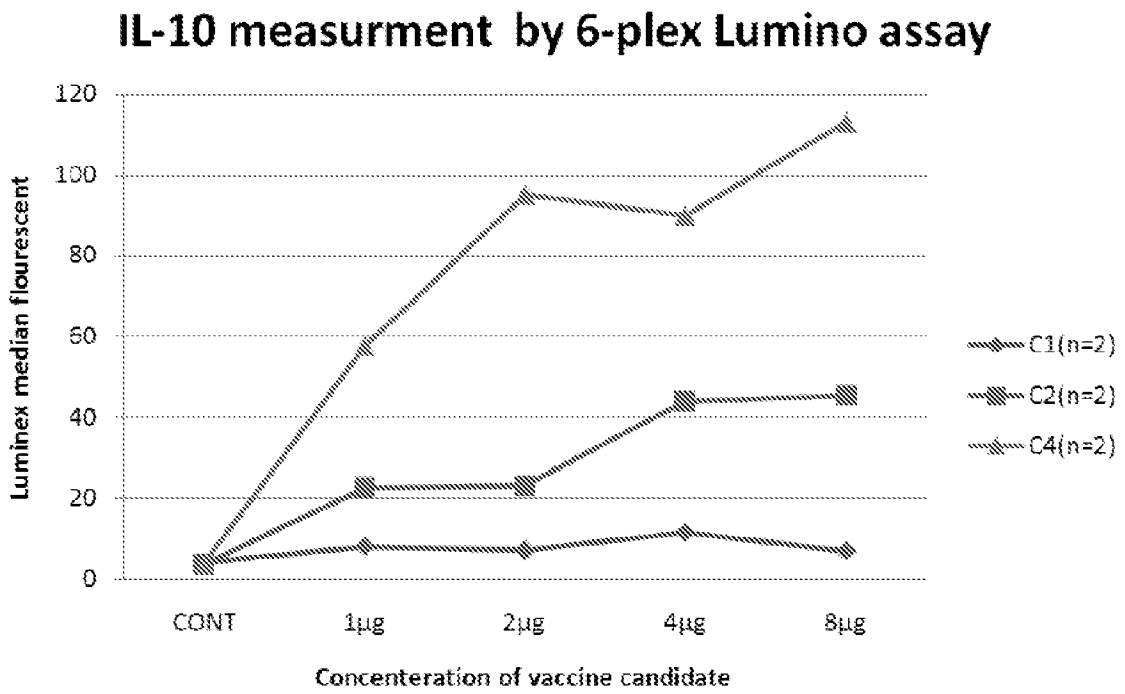

IL-5 measurment by 6-plex Lumino assay
Luminex median flourescent
100
90
80
70
60
50
40
30
20
10
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
CONT- C1(n=2)
CONT- C2(n=2)
CONT- C4(n=2)

Fig. 12


IL-10 measurment by 6-plex Lumino assay
Luminex median flourescent
120
100
80
60
40
20
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
C1(n=2)
C2(n=2)
C4(n=2)

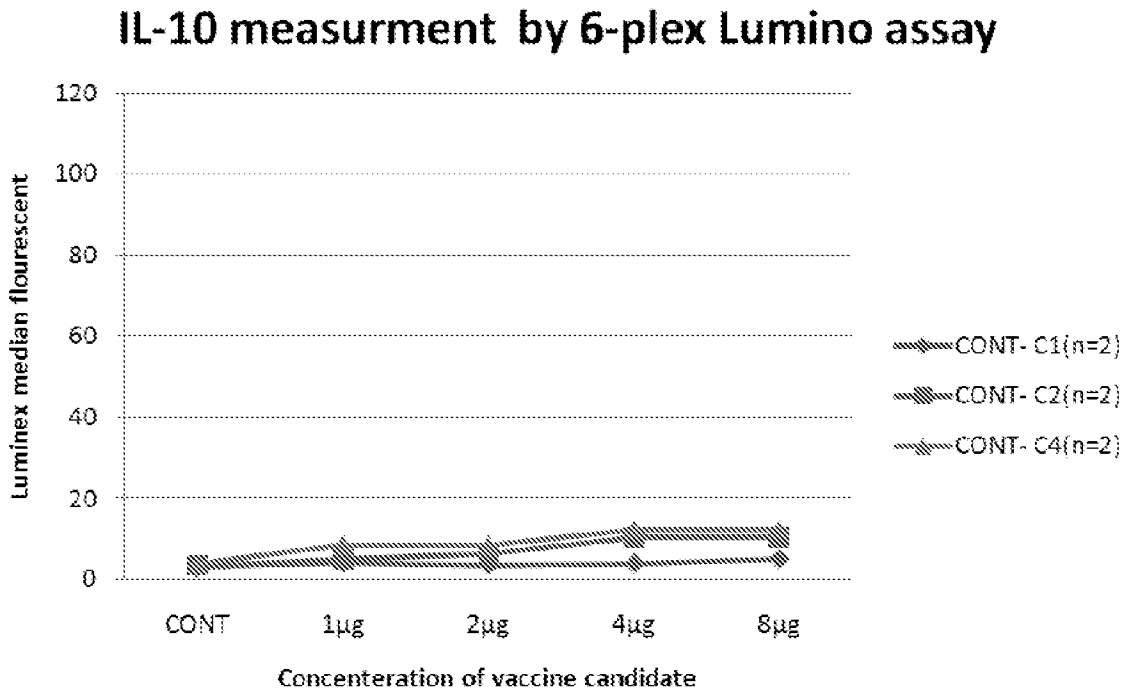
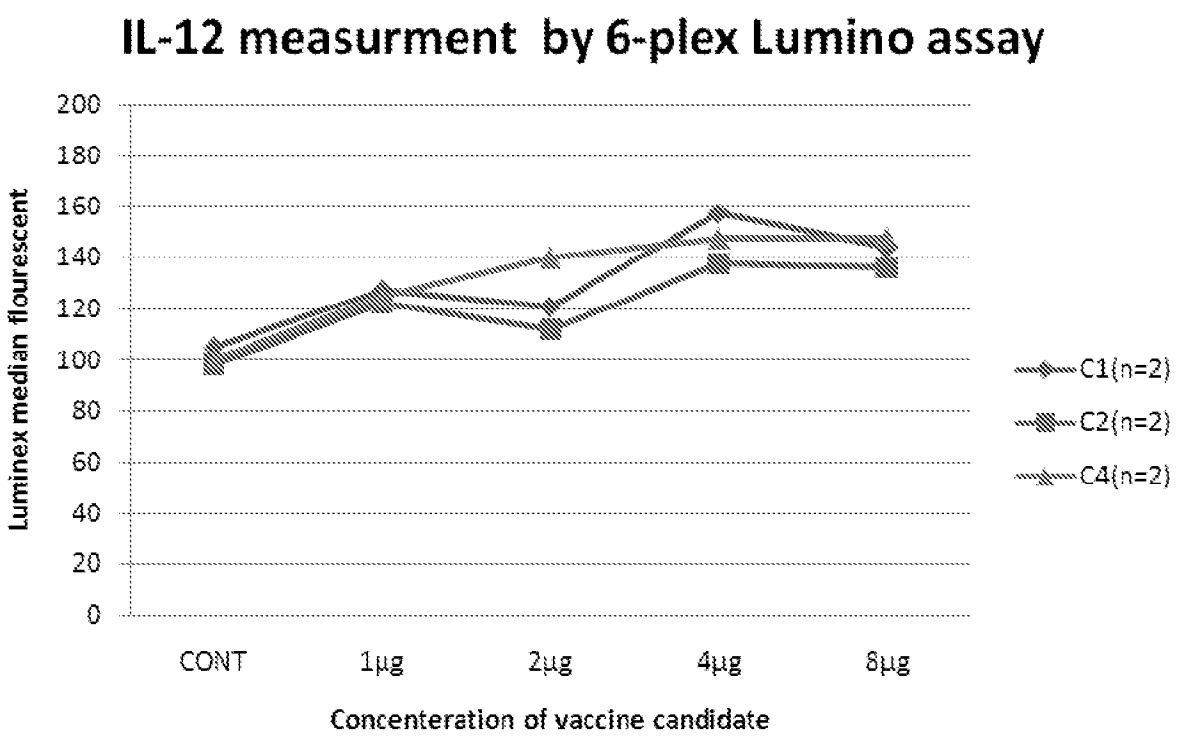

IL-10 measurment by 6-plex Lumino assay
Luminex median flourescent
120
100
80
60
40
20
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
CONT- C1(n=2)
CONT- C2(n=2)
CONT- C4(n=2)

Fig. 13


IL-12 measurment by 6-plex Lumino assay
Luminex median flourescent
200
180
160
140
120
100
80
60
40
20
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
C1(n=2)
C2(n=2)
C4(n=2)

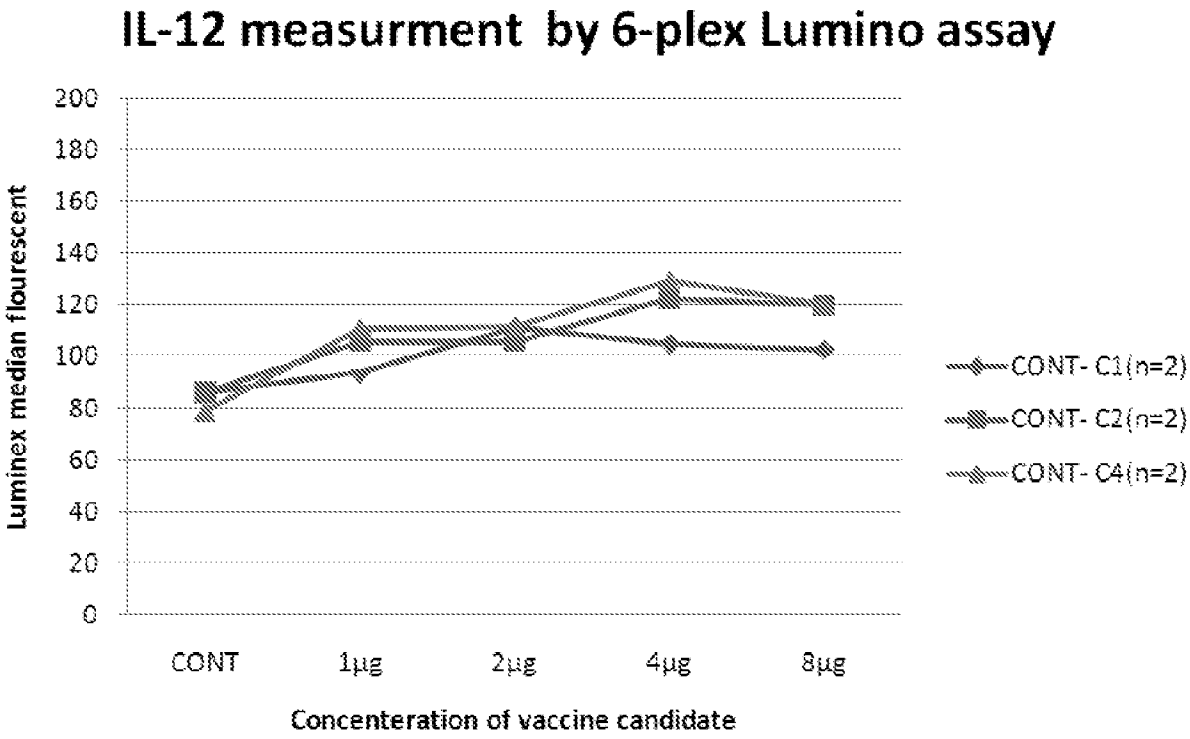

IL-12 measurment by 6-plex Lumino assay
Luminex median flourescent
200
180
160
140
120
100
80
60
40
20
0
CONT
1µg
2µg
4µg
8µg
Concenteration of vaccine candidate
CONT- C1(n=2)
CONT- C2(n=2)
CONT- C4(n=2)

Fig. 14

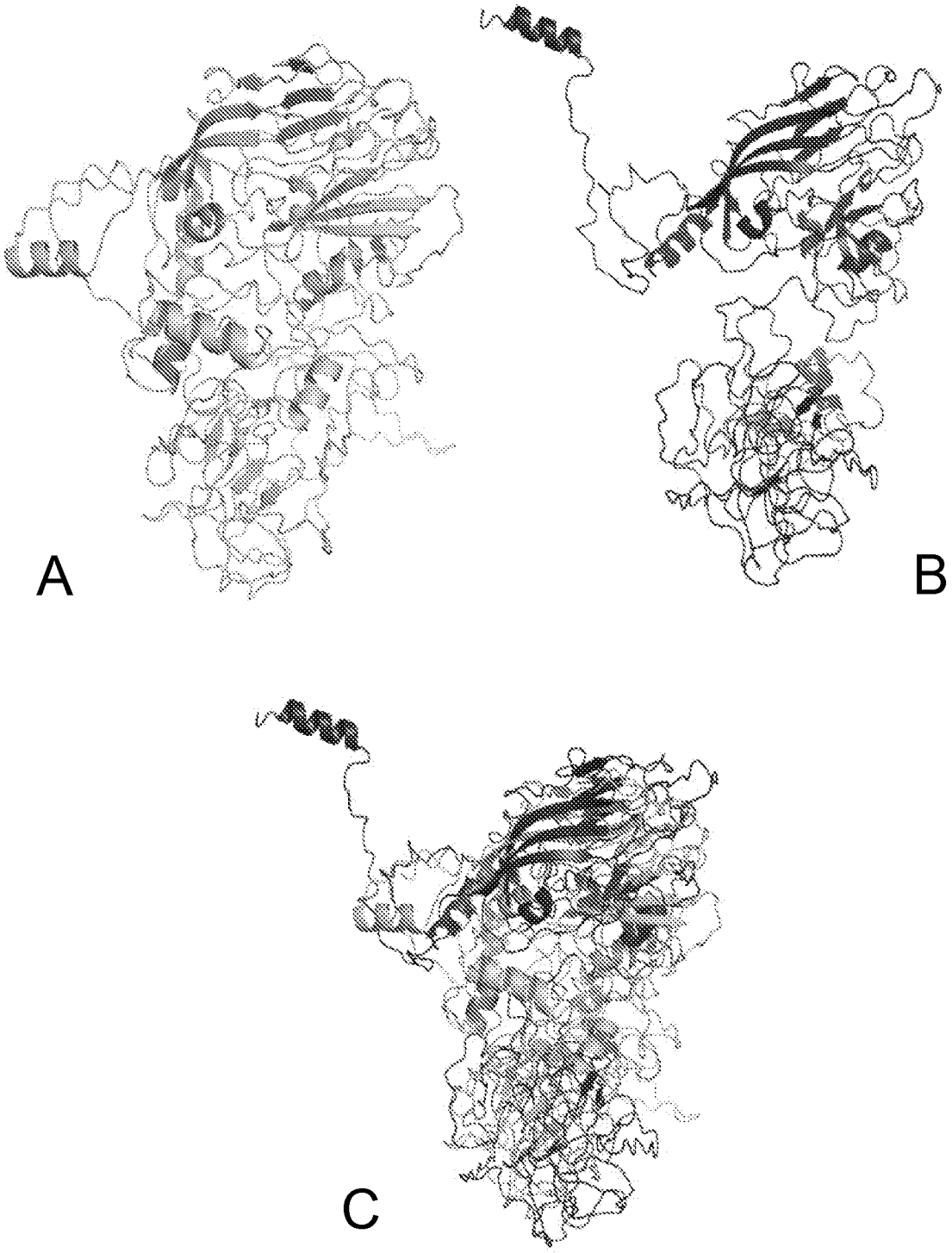


Fig. 15