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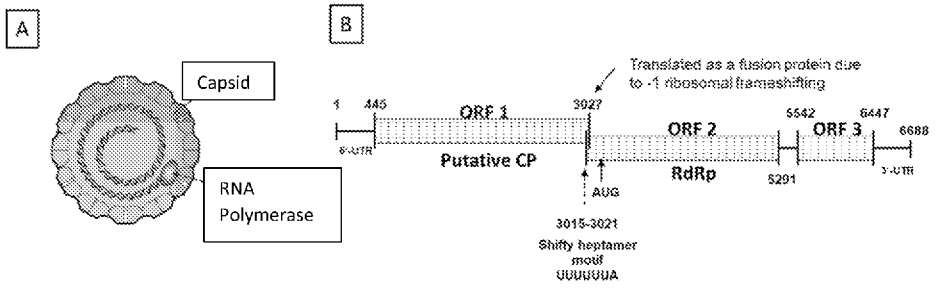
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(54) Title: USE OF INTERFERON AS AN ADJUVANT IN VACCINES

FIG 1.




(57) Abstract: Disclosed are salmon interferon sequences useful as adjuvants in fish vaccines. Also disclosed are fish vaccines utilizing said interferon sequences and methods for prevention of fish infections utilizing these vaccines.

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# USE OF INTERFERON AS AN ADJUVANT IN VACCINES

## FIELD OF THE INVENTION

**[001]** This invention is generally in the field of aquaculture vaccines.

## BACKGROUND

**[002]** Aquaculture has experienced an enormous growth in productive terms, accounting to >527% in the 1990–2018 time frame. In 2018, aquaculture contributed to approximately 46% of the global total production of aquatic organisms (179 M tons) and 52% of seafood for human consumption (fish, crustaceans, mollusks, and other aquatic animals, excluding aquatic mammals, reptiles, seaweeds, and other aquatic plants).

**[003]** Commercial aquaculture is impacted by infectious diseases caused primarily by bacteria, viruses, parasites, and, to a lesser extent, fungi. Bacterial diseases can inflict significant biological, thus economic losses. While these are usually controllable with antibiotics, the indiscriminate use of these pharmaceuticals is ultimately a threat to human health because of the development and transfer of resistance mechanisms among bacterial species, some of which are also human pathogens.

**[004]** Fish vaccination can prevent or mitigate disease spreading with proven effectiveness against many relevant pathogens. One of the obstacles in designing more efficient vaccines is the lack of suitable adjuvants. Most often water-in-oil emulsions are used but this adjuvant type is not suitable for all vaccines. Recently it has been reported that interferon (IFN) showed potent adjuvant activity in combination with a DNA vaccine against infectious salmon anemia virus (ISAV).

**[005]** As in mammals, expression of different salmon type I interferons (IFN-I) in response to virus infection is dependent on their promoters, properties of the virus and the cell's expression of nucleic acid receptors and interferon regulatory factors (IRFs). While IFNa mainly display local antiviral activity, IFNb and IFNc show systemic antiviral activity. In addition, salmon appears to possess several IFN-I receptors, which show selectivity in binding different IFN-I. This complexity in IFN-I and receptors allows for a large variation in functions of the salmon IFN-I.

**[006]** There is a need for further exploration of immunomodulatory effects of type I interferons in order to design more efficient vaccines to combat diseases of Atlantic salmon and other farmed fish.

## SUMMARY OF INVENTION

**[007]** In the first aspect, this application provides a nucleic acid sequence that encodes SEQ ID NO: 6 or SEQ ID NO: 50 or an amino acid sequence that is at least 95% identical to SEQ ID NO: 6 or 50, wherein said nucleic acid sequence is 65-90% identical to SEQ ID NO: 18 or 65-90% identical to SEQ ID NO: 19, and wherein codon frequency is preserved in said nucleic acid sequence. In certain embodiments, said nucleic acid sequence is 70-79% identical to SEQ ID NO: 18 or to SEQ ID NO: 19.

**[008]** In certain embodiments, the frequencies of at least 40% codons in the nucleic acid sequence of the invention do not deviate by more than 30% from the frequencies of the same codons in the genome of the host, and/or the frequencies of at least 30% codons in the nucleic acid sequence of the invention do not deviate by more than 25% from the frequencies of the same codons in the genome of the host, and/or the frequencies of at least 25% codons in the nucleic acid sequence of the invention do not deviate by more than 20% from the frequencies of the same codons in the genome of the host.

**[009]** In certain embodiments, the nucleic acid sequence is at least 90% identical or at least 95% identical or at least 99% identical or 100% identical to SEQ ID NO: 20 or SEQ ID NO: 17.

**[0010]** In certain embodiments, the amino acid sequence encoded by the nucleic acid sequence according to the invention is at least 97% identical to SEQ ID NO: 6 or at least 99% identical to SEQ ID NO: 6. In certain embodiments, at least 50% (or at least 60% or at least 70% or at least 80% or at least 90%) of the amino acids different from the corresponding amino acids in SEQ ID NO: 6 are conservative substitutions.

**[0011]** In certain embodiments, the amino acid sequence encoded by the nucleic acid sequence according to the invention is at least 97% identical to SEQ ID NO: 50 or at least 99% identical to SEQ ID NO: 50. In certain embodiments, at least 50% (or at least 60% or at least 70% or at least 80% or at least 90%) of the amino acids different from the corresponding amino acids in SEQ ID NO: 50 are conservative substitutions.

**[0012]** In the second aspect, provided is an expression cassette, said expression cassette comprising the nucleic acid sequence according any embodiment of the first aspect, under an operative control of a second promoter. In certain embodiments, the second promoter is cytomegalovirus (CMV) immediate-early enhancer and promoter. In certain embodiments, the expression cassette further comprises a nucleic acid sequence encoding an antigen under operative control of a first promoter. In certain embodiments, the first promoter is cytomegalovirus (CMV) immediate-early enhancer and promoter.

**[0013]** In certain embodiments, the antigen is of a viral origin. In certain embodiments, the virus is a non-enveloped virus. In a particular set of embodiments, the virus is Piscine Myocarditis Virus (PMCV) and the antigen is an amino acid sequence encoded by ORF-1 or a fragment said amino acid sequence, wherein said fragment is capable of eliciting the protective immune response.

**[0014]** In certain embodiments, the antigen is SEQ ID NO: 1 or an amino acid sequence that is 95% identical thereto.

**[0015]** In some other embodiments, Piscine Myocarditis Virus (PMCV) ORF-1 antigen comprises, from N- to C- terminus:

a. a sequence that is at least 90% identical, or at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical, or 100% identical to SEQ ID NO: 34 or SEQ ID NO: 35, and

b. an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to any one of SEQ ID NOs: 25-33,

wherein,

i) said Piscine Myocarditis Virus (PMCV) ORF-1 antigen lacks SEQ ID NO: 15 or a sequence at least 90% identical to SEQ ID NO: 15; and/or

ii) compared to SEQ ID NO: 1, said Piscine Myocarditis Virus (PMCV) ORF-1 antigen comprises an internal deletion that is at least four consecutive amino acids long.

**[0016]** In certain embodiments, the Piscine Myocarditis Virus (PMCV) ORF-1 antigen is at least 90% identical to SEQ ID NO: 11 or SEQ ID NO: 12 or SEQ ID NO: 46.

**[0017]** In other embodiments, the antigen is of bacterial origin and comprises a protein present on the outer surface of said bacteria, or a fragment of said protein capable of eliciting protective immune response.

**[0018]** In other embodiments, the antigen is of parasitic origin and is a protein from a gut of a sea louse of family *Caligidae*. In certain embodiments, the antigen comprises SEQ ID NO: 21 or an amino acid sequence at least 90% identical thereto.

**[0019]** In the third aspect, the disclosure provides a vector comprising the expression cassette according to any embodiment of the second aspect of the invention. In certain embodiments, the vector is a viral vector. In other embodiments, the vector is a plasmid vector. In a set of preferred embodiments, the vector is NTC9385R (Nature Technology Corporation) or a variant thereof, as described in the Examples.

**[0020]** In a fourth aspect, the disclosure provides a vaccine comprising the vector according to any embodiment of the third aspect of the invention.

**[0021]** In a fifth aspect, the disclosure provides a method of protecting a salmonid from an infection, the method comprising administering to the salmonid in need thereof the vaccine according to any embodiment of the fourth aspect of the invention.

**[0022]** In certain embodiments, the salmonid is selected from the group consisting of Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), or Coho Salmon (*Oncorhynchus kisutch*). In a set of embodiments, the salmonid is Atlantic salmon.

**[0023]** In certain embodiments, the salmonid weighs between about 25 and about 200 grams, preferably between about 15 and about 200 grams.

**[0024]** In a sixth aspect, the invention provides a method of evaluating the efficiency of an interferon as an adjuvant, wherein the method comprises:

a) transfecting a nucleic acid sequence encoding an interferon into a source cells;
b) culturing said source cells, wherein said interferon is secreted into a source cell media;
c) contacting cultured target cells with at least a portion of said source cell media, wherein said target cells naturally express Mx gene in response to interferon; and

d) measuring Mx gene expression.

**[0025]** In a seventh aspect, the invention provides a method of determining which nucleic acid sequence in a group of sequences encoding the same Type I interferon confers a more efficient expression.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0026]** Fig. 1A is a general virion structure of *Totiviridae*. Fig 1B is an illustration of genome organization of PMCV. The genome includes three large ORFs.

**[0027]** Fig. 2A in an illustration of the strategy for routing vaccine antigen for expression on the cell-surface. Fig. 2B is an illustration of the strategy to facilitate secreted antigen

**[0028]** Fig. 3A is a photograph demonstrating detection of PMCV ORF1 in non-permeabilized cells. Fig. 3B demonstrates the results obtained with different expression cassettes used for routing full length PMCV ORF-1 to the cell surface.

**[0029]** Fig. 4A is an evaluation of fluorescence intensity of the truncated capsid variants by IF-staining of transfected CHH-1 cells. Fig. 4B is an illustration of protein expression by Western blotting detection from the membrane-fraction by immunoprecipitation (IP-Western). The two blots represent two different experiments.

**[0030]** Fig. 5 is an Evaluation of protein expression in five different plasmid backbones by immunoprecipitation from total cell lysate of transfected CHH-1 cells and Western blotting.

## DETAILED DESCRIPTION

**[0031]** In order to better explain the invention, the following definitions are provided.

**[0032]** The term "about" as applied to a reference number refers to the reference number plus or minus 10 percent of said value.

**[0033]** The term "codon frequency is preserved in said nucleic acid sequence" describes a nucleic acid sequence wherein the frequencies of at least 50% of codons in the nucleic acid sequence of the invention do not deviate by more than 40% from the frequencies of the same codons in the genome of the host. When, for example, the frequency of a given codon in the host genome is, say, 20%, then, this codon is counted among the at least 50% of codons whose frequency does not deviate by more than 40% from the frequency of the same codon in the genome of the host

if the frequency of the same codon in the nucleic acid sequence according to the invention is between 12% (40% less than 20%) and 28 % (40% more than 20%).

**[0034]** For example, codon frequencies in the genome of Atlantic Salmon (*Salmo salar*) are provided in Table 1.

**TABLE 1. CODON FREQUENCY IN THE GENOME OF ATLANTIC SALMON**

| AMINO ACID | CODON | Per 1000 | FREQUENCY |
|---|---|---|---|
| GLY | GGG | 15.87 | 21.91 |
| GLY | GGA | 20.24 | 27.94 |
| GLY | GGT | 14.03 | 19.37 |
| GLY | GGC | 22.3 | 30.78 |
| ASP | GAT | 16.76 | 32.69 |
| ASP | GAC | 34.51 | 67.31 |
| GLU | GAG | 45.67 | 76.69 |
| GLU | GAA | 13.88 | 23.31 |
| VAL | GTG | 29.06 | 45.71 |
| VAL | GTA | 6.19 | 9.74 |
| VAL | GTT | 9.77 | 15.37 |
| VAL | GTC | 18.56 | 29.19 |
| ALA | GCG | 7.2 | 11.08 |
| ALA | GCA | 13.42 | 20.65 |
| ALA | GCT | 17.5 | 26.92 |
| ALA | GCC | 26.88 | 41.35 |
| ARG | AGG | 13.4 | 26.85 |
| ARG | AGA | 10.12 | 20.28 |
| ARG | CGG | 6.61 | 13.25 |
| ARG | CGA | 4.01 | 8.036 |
| ARG | CGT | 5.74 | 11.50 |
| ARG | CGC | 10.02 | 20.08 |
| SER | AGT | 11.2 | 13.54 |
| SER | AGC | 21.01 | 25.40 |
| SER | TCG | 5.31 | 6.42 |
| SER | TCA | 10.15 | 12.27 |
| SER | TCT | 13.86 | 16.76 |
| SER | TCC | 21.18 | 25.61 |
| LYS | AAG | 39.68 | 69.87 |
| LYS | AAA | 17.11 | 30.13 |
| ASN | AAT | 11.42 | 28.36 |
| ASN | AAC | 28.85 | 71.64 |
| MET | ATG | 27.27 | 100 |
| ILE | ATA | 6.37 | 13.59 |
| ILE | ATT | 11.34 | 24.19 |

| ILE | ATC | 29.17 | 62.22 |
|---|---|---|---|
| TRP | TGG | 11.53 | 100 |
| CYS | TGT | 11.05 | 46.80 |
| CYS | TGC | 12.56 | 53.20 |
| TYR | TAT | 8.12 | 27.21 |
| TYR | TAC | 21.72 | 72.79 |
| LEU | TTG | 10.16 | 10.97 |
| LEU | TTA | 3.44 | 3.71 |
| LEU | CTG | 45.99 | 49.65 |
| LEU | CTA | 7.18 | 7.75 |
| LEU | CTT | 7.87 | 8.50 |
| LEU | CTC | 17.98 | 19.41 |
| PHE | TTT | 12.55 | 32.54 |
| PHE | TTC | 26.02 | 67.46 |
| GLN | CAG | 35.96 | 81.21 |
| GLN | CAA | 8.32 | 18.79 |
| HIS | CAT | 8.63 | 32.43 |
| HIS | CAC | 17.98 | 67.57 |
| PRO | CCG | 5.93 | 11.09 |
| PRO | CCA | 13.3 | 24.86 |
| PRO | CCT | 13.89 | 25.97 |
| PRO | CCC | 20.37 | 38.08 |
| THR | ACG | 6.91 | 11.85 |
| THR | ACA | 14.69 | 25.18 |
| THR | ACT | 12.76 | 21.88 |
| THR | ACC | 23.97 | 41.09 |
| **AMINO ACID** | **CODON** | **Per 1000** | **FREQUENCY** |

**[0035]** Codon CGC encodes arginine with frequency 20.08% – i.e., 20.08% arginine residues in the genome of *Salmo salar* are encoded by CGC. If in a nucleic acid sequence between 12.048% and 28.112% of arginine residues are encoded by CGC, then codon CGC is counted within among the at least 50% of codons whose frequency does not deviate by more than 40% from the frequency of the same codon in the genome of *Salmo salar*.

**[0036]** "Therapeutically effective amount" refers to an amount of an antigen or vaccine that would induce an immune response in a subject receiving the antigen or vaccine which is adequate to prevent or reduce signs or symptoms of disease, including adverse health effects or complications thereof, caused by infection with a pathogen, such as a virus or a bacterium. Humoral immunity or cell-mediated immunity or both humoral and cell-mediated immunity may be induced. The immunogenic response of an animal to a vaccine may be evaluated, e.g.,

indirectly through measurement of antibody titers, lymphocyte proliferation assays, or directly through monitoring signs and symptoms after challenge with wild type strain. The protective immunity conferred by a vaccine can be evaluated by measuring, e.g., reduction in clinical signs such as mortality, morbidity, temperature number, overall physical condition, and overall health and performance of the subject. The amount of a vaccine that is therapeutically effective may vary depending on the particular adjuvant used, the particular antigen used, or the condition of the subject, and can be determined by one skilled in the art.

**[0037]** "Treating" refers to preventing a disorder, condition, or disease to which such term applies, or to preventing or reducing one or more symptoms of such disorder, condition, or disease.

**[0038]** In the first aspect, the invention provides a nucleic acid sequence that encodes SEQ ID NO: 6 or SEQ ID NO: 50 or a sequence that is at least 95% identical to SEQ ID NO: 6 (Interferon B of *Salmo salar*) or SEQ ID NO: 50 (Interferon B of *Salmo salar*), wherein said nucleic acid sequence is 65-90% identical to SEQ ID NO: 18 or 65-90% identical to SEQ ID NO: 19, and wherein codon frequency is preserved in said nucleic acid sequence.

**[0039]** In certain embodiments, the nucleic acid sequences of the invention encode an amino acid sequence that is 96, 97, 98, 99, or 100% identical to SEQ ID NO: 6 or SEQ ID NO: 50. The differences may be in the form of insertions, deletions, or substitutions. Preferably, the mutations are substitutions, and more preferably, at least some of these substitutions are conservative substitutions.

**[0040]** The skilled person will further acknowledge that alterations of the nucleic acid sequence resulting in modifications of the amino acid sequence of the protein it codes may have little, if any, effect on the resulting three-dimensional structure of the protein. For example, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in the substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a protein with substantially the same functional activity.

**[0041]** The following six groups each contain amino acids that are typical conservative substitutions for one another: [1] Alanine (A), Serine (S), Threonine (T); [2] Aspartic acid (D), Glutamic acid (E); [3] Asparagine (N), Glutamine (Q); [4] Arginine (R), Lysine (K), Histidine (H); [5] Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and [6] Phenylalanine (F), Tyrosine (Y), Tryptophan (W), (see, e.g., US Patent Publication 20100291549).

**[0042]** In certain embodiments, at least 50%, or at least 60%, or at least 70% or at least 80% or at least 90% or at least 95% or all 100% of amino acids differing from the reference sequence are conservative substitutions.

**[0043]** Protein and/or nucleic acid sequence identities can be evaluated using any of the variety of sequence comparison algorithms and programs known in the art. For sequence comparison, typically one sequence acts as a reference sequence (e.g., a sequence disclosed herein), to which test sequences are compared. A sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

**[0044]** The percent identity of two amino acid or two nucleic acid sequences can be determined for example by comparing sequence information using the computer program GAP, *i.e.,* Genetics Computer Group (GCG; Madison, WI) Wisconsin package version 10.0 program, GAP (Devereux *et al.* (1984), Nucleic Acids Res. 12: 387-95). In calculating percent identity, the sequences being compared are typically aligned in a way that gives the largest match between the sequences. The preferred default parameters for the GAP program include: (1) The GCG implementation of a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted amino acid comparison matrix of Gribskov and Burgess, ((1986) Nucleic Acids Res. 14: 6745) as described in *Atlas of Polypeptide Sequence and Structure*, Schwartz and Dayhoff, eds., National Biomedical Research Foundation, pp. 353-358 (1979) or other comparable comparison matrices; (2) a penalty of 8 for each gap and an additional penalty of 2 for each symbol in each gap for amino acid sequences, or a penalty of 50 for each gap and an additional penalty of 3 for each symbol in each gap for nucleotide sequences; (3) no penalty for end gaps; and (4) no maximum penalty for long gaps.

**[0045]** Sequence identity and/or similarity can also be determined by using the local sequence identity algorithm of Smith and Waterman, 1981, *Adv. Appl. Math.* 2:482, the sequence identity alignment algorithm of Needleman and Wunsch, 1970, *J. Mol. Biol.* 48:443, the search for similarity method of Pearson and Lipman, 1988, *Proc. Nat. Acad. Sci. U.S.A.* 85:2444, computerized implementations of these algorithms (BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.).

**[0046]** Another example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, 1987, *J. Mol. Evol.* 35:351-360; the method is similar to that described by Higgins and Sharp, 1989, *CABIOS* 5:151-153. Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

**[0047]** Another example of a useful algorithm is the BLAST algorithm, described in: Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-410; Altschul *et al.*, 1997, *Nucleic Acids Res.* 25:3389-3402; and Karin *et al.*, 1993, *Proc. Natl. Acad. Sci. U.S.A.* 90:5873-5787. A particularly useful BLAST program is the WU-BLAST-2 program obtained from Altschul *et al.*, 1996, *Methods in Enzymology* 266:460-480. WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span=1, overlap fraction=0.125, word threshold (T)=II. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity.

**[0048]** An additional useful algorithm is gapped BLAST as reported by Altschul *et al.*, 1993, *Nucl. Acids Res.* 25:3389-3402. Gapped BLAST uses BLOSUM-62 substitution scores; threshold T parameter set to 9; the two-hit method to trigger ungapped extensions, charges gap lengths of k a cost of 10+k; $X_u$ set to 16, and $X_g$ set to 40 for database search stage and to 67 for the output

stage of the algorithms. Gapped alignments are triggered by a score corresponding to about 22 bits.

**[0049]** The nucleic acid sequences of the invention are 65-90% identical to SEQ ID NO: 18 or SEQ ID NO: 19, with a proviso that codon frequency is preserved in said nucleic acid sequences. In certain embodiments, the amino acids of the invention are 70-79% or 73-77% identical to SEQ ID NO: 18 or SEQ ID NO: 19, with a proviso that codon frequency is preserved in said nucleic acid sequences.

**[0050]** As disclosed above, the frequencies of at least 50% of codons in the nucleic acid sequence of the invention do not deviate by more than 40% from the frequencies of the same codons in the genome of the host. In certain embodiments, the frequencies of at least 40% codons in the nucleic acid sequence of the invention do not deviate by more than 30% from the frequencies of the same codons in the genome of the host, and/or the frequencies of at least 30% codons in the nucleic acid sequence of the invention do not deviate by more than 25% from the frequencies of the same codons in the genome of the host, and/or the frequencies of at least 25% codons in the nucleic acid sequence of the invention do not deviate by more than 20% from the frequencies of the same codons in the genome of the host. If the host is *Salmo salar*, codon frequencies in *Salmo salar* are provided in Table 1.

**[0051]** In certain embodiments of this first aspect of the invention, the nucleic acid sequences comprise SEQ ID NO: 17 or SEQ ID NO: 20 or are at least 90% identical (i.e., over 91% identical, over 92% identical, over 93% identical, over 94% identical, over 95% identical, over 96% identical, over 97% identical, over 98% identical, or over 99% identical) to one of SEQ ID NO: 17 or SEQ ID NO: 20 and said nucleic acid sequences SEQ ID NO: 6 or 50 or an amino acid sequence at least 95% identical to SEQ ID NO: 6 or 50, as described above.

**[0052]** In a second aspect, disclosed is an expression cassette, wherein the cassette comprises the nucleic acid sequence according to any of the embodiments according to the first aspect of the invention, under operative control of a second promoter. Generally, the second promoter should be able to initiate transcription in the host organism. In the embodiments where the host is a salmonid, such as *Salmo salar*, suitable promoters include, without limitations, simian virus 40 early promoter (SV40), cytomegalovirus immediate-early promoter (CMV), human Ubiquitin

C promoter (UBC), human elongation factor 1α promoter (EF1A), mouse phosphoglycerate kinase 1 promoter (PGK), and chicken β-Actin promoter coupled with CMV early enhancer (CAGG). Generally, the expression cassette of the invention also comprises a polyadenylation signal that terminates transcription of the nucleic acid sequence of the invention.

**[0053]** In certain embodiments, the expression cassette according to this second aspect further comprises a nucleic acid sequence encoding an antigen under the operative control of the first promoter. Similarly to the second promoter, the first promoter may be selected from such exemplary promoters as simian virus 40 early promoter (SV40), cytomegalovirus immediate-early promoter (CMV), human Ubiquitin C promoter (UBC), human elongation factor 1α promoter (EF1A), mouse phosphoglycerate kinase 1 promoter (PGK), and chicken β-Actin promoter coupled with CMV early enhancer (CAGG). Generally, the expression cassette of the invention also comprises a polyadenylation signal that terminates transcription of the nucleic acid sequence encoding the antigen.

**[0054]** Multiple antigens may be suitable for the cassette described herein. In general, the antigens may have viral, bacterial, protozoal, or parasitic origin. Suitable viruses include PMCV, ISA, IPNV, ASPV (Atlantic salmon poxvirus), IHNV (Infectious hematopoietic necrosis virus), VHSV (Viral hemorrhagic septicemia virus) and PRV. Antigens from enveloped and non-enveloped viruses may be included. In certain embodiments, the antigen is a viral structural protein or a capsid protein, or an outer surface protein. Fragments of these proteins capable of eliciting protective immune response are also suitable.

**[0055]** In certain embodiments, the antigen is a Piscine Myocarditis Virus (PMCV) ORF-1 antigen, such as a protein comprising SEQ ID NO: 1 or a sequence that it at least 90% identical to SEQ ID NO: 1 , e.g., at least 95% identical, at least 96% identical, at least 97% identical, at least 98% identical or at least 99% identical to SEQ ID NO: 1. The sequence may differ from SEQ ID NO: 1 by deletions, insertions, or substitutions. Preferably, at least some substitutions are conservative substitutions. In certain embodiments, at least 50% (i.e., at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 99%, or 100%) of the substituted amino acids are conservative substitutions.

**[0056]** The inventors have discovered that the N-terminal portion of SEQ ID NO: 1, i.e., amino acids 1 to about 399 of SEQ ID NO: 1, or more preferably, amino acids 1 to 425, or more preferably, amino acids 1 to 450, or more preferably amino acids 1 to 475, or most preferably, amino acids 1-499 of SEQ ID NO: 1 are necessary and sufficient to target the antigen to cell surface.

**[0057]** At the same time, the inventors have surprisingly discovered that the antigen according to the invention elicits a better immune response as measured by viral load and/or histology score if

a) SEQ ID NO: 25 (intDel14, 15) is deleted while SEQ ID NO: 27, SEQ ID NO: 28 or SEQ ID NO: 31 is retained; or

b) SEQ ID NO: 27, SEQ ID NO: 28 or SEQ ID NO: 31 is deleted while SEQ ID NO: 25 is retained; or

c) SEQ ID NO: 14 is deleted while SEQ ID NO: 32 is retained.

**[0058]** It has also been discovered that the presence of SEQ ID NO: 15 was not sufficient to confer the proper immune response if SEQ ID NO: 32 was deleted.

**[0059]** Accordingly, in certain embodiments, the antigen is a Piscine Myocarditis Virus (PMCV) ORF-1 antigen comprising, from N- to C- terminus:

a) a sequence that is at least 90% identical, or at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical, or 100% identical to SEQ ID NO: 34 or SEQ ID NO: 35, and

b) an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to any one of SEQ ID NOs: 25-33,

wherein:

i) said Piscine Myocarditis Virus (PMCV) ORF-1 antigen lacks SEQ ID NO: 15 or a sequence at least 90% identical to SEQ ID NO: 15; and/or

ii) compared to SEQ ID NO: 1, said Piscine Myocarditis Virus (PMCV) ORF-1 antigen comprises an internal deletion that is at least four consecutive amino acids long.

**[0060]** In all of the embodiments, the differences from the reference sequence (such as SEQ ID NOs: 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35) may be deletions, insertions, or substitutions. Preferably, at least some substitutions are conservative substitutions. In certain embodiments, at least 50% (i.e., at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 99%, or 100%) of the substituted amino acids are conservative substitutions.

**[0061]** In the embodiments where the Piscine Myocarditis Virus (PMCV) ORF-1 antigen lacks SEQ ID NO: 15, it should be understood that a larger deletion, e.g., a C-terminal truncation can also be made in the antigen. In certain embodiments, such larger C-terminal truncation comprises, or consists of, SEQ ID NO: 13.

**[0062]** In certain embodiments, the Piscine Myocarditis Virus (PMCV) ORF-1 antigen as described above includes SEQ ID NO: 25 or a sequence at least 95%, or 96%, or 97% or 98% or 99% identical to SEQ ID NO: 25 wherein said PMCV ORF-1 antigen lacks SEQ ID NO: 4, or SEQ ID NO: 5, or SEQ ID NO: 14, or SEQ ID NO: 15 or a sequence that is at least 90% identical to SEQ ID NO: 4, or SEQ ID NO: 5, or SEQ ID NO: 14, or SEQ ID NO: 15.

**[0063]** In other embodiments, the Piscine Myocarditis Virus (PMCV) ORF-1 antigen as described above includes the sequence that is at least 95% identical to SEQ ID NO: 29 (or a sequence at least 95%, or 96%, or 97% or 98% or 99% identical to SEQ ID NO: 29, wherein said PMCV ORF-1 antigen lacks SEQ ID NO: 5, or SEQ ID NO: 14, or SEQ ID NO: 15 or a sequence that is at least 90% identical to SEQ ID NO: 5, or SEQ ID NO: 14, or SEQ ID NO: 15.

**[0064]** In other embodiments, the Piscine Myocarditis Virus (PMCV) ORF-1 antigen as described above includes the sequence that is at least 95% identical to SEQ ID NO: 32 or a sequence at least 95%, or 96%, or 97% or 98% or 99% identical to SEQ ID NO: 32, wherein said PMCV ORF-1 antigen lacks SEQ ID NO: 14 or SEQ ID NO: 15 or a sequence that is at least 90% identical to SEQ ID NO: 14 or SEQ ID NO: 15.

**[0065]** In other embodiments, the Piscine Myocarditis Virus (PMCV) ORF-1 antigen includes SEQ ID NO: 26 or a sequence at least 95%, or 96%, or 97% or 98% or 99% identical to SEQ ID NO: 26 and lacks SEQ ID NO: 5, or SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 24 or a sequence that is at least 90% identical to SEQ ID NO: 5, or SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 24.

**[0066]** In other embodiments, the Piscine Myocarditis Virus (PMCV) ORF-1 antigen includes SEQ ID NO: 30 or a sequence at least 95%, or 96%, or 97% or 98% or 99% identical to SEQ ID NO: 30 and lacks SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 24 or a sequence that is at least 90% identical to SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 24.

**[0067]** In other embodiments, the Piscine Myocarditis Virus (PMCV) ORF-1 antigen includes SEQ ID NO: 33 or a sequence at least 95%, or 96%, or 97% or 98% or 99% identical to SEQ ID NO: 33 and lacks SEQ ID NO: 24 or a sequence that is at least 90% identical to SEQ ID NO: 24.

**[0068]** In other embodiments, the Piscine Myocarditis Virus (PMCV) ORF-1 antigen includes SEQ ID NO: 27 or a sequence at least 95%, or 96%, or 97% or 98% or 99% identical to SEQ ID NO: 27 and lacks SEQ ID NO: 3, or SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 24 or a sequence that is at least 90% identical to SEQ ID NO: 3, or SEQ ID NO: 14, or SEQ ID NO: 15, or SEQ ID NO: 24.

**[0069]** In other embodiments, the Piscine Myocarditis Virus (PMCV) ORF-1 antigen includes SEQ ID NO: 31 or a sequence at least 95%, or 96%, or 97% or 98% or 99% identical to SEQ ID NO: 31 and lacks SEQ ID NO: 3 or SEQ ID NO: 24 or a sequence that is at least 90% identical to SEQ ID NO: 3 or SEQ ID NO: 24.

**[0070]** In other embodiments, the Piscine Myocarditis Virus (PMCV) ORF-1 antigen includes SEQ ID NO: 28 or a sequence at least 95%, or 96%, or 97% or 98% or 99% identical to SEQ ID NO: 28 and lacks SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 14, or a sequence that is at least 90% identical to SEQ ID NO: 3, or SEQ ID NO: 4, or SEQ ID NO: 14.

**[0071]** In other embodiments, the protein includes SEQ ID NO: 15, or a sequence at least 90% identical thereto, and any one of SEQ ID NOs 4, 5, 25, 26 27, 29, 30, 32, (or an amino acid sequence that is at least 90% identical to SEQ ID NO: 4, 5, 25, 26 27, 29, 30, or 32) wherein, compared to SEQ ID NO: 1, said protein comprises an internal deletion that is at least four consecutive amino acids long, and, optionally, wherein said protein comprises a linker in place of said internal deletion.

**[0072]** In yet other embodiments, the protein lacks SEQ ID NO: 13 or a sequence that is at least 90% identical to SEQ ID NO: 13 and contains SEQ ID NO: 25 or a sequence at least 95%, or 96%, or 97% or 98% or 99% identical to SEQ ID NO: 25.

**[0073]** Compared to SEQ ID NO: 1, said Piscine Myocarditis Virus (PMCV) ORF-1 antigen may comprise an internal deletion that is at least four consecutive amino acids long. In general, the internal deletion may be up to 250 amino acids long. Preferably, the internal deletion is about 10 amino acids long or 10 to 250 amino acids long, or 25 to 250 amino acids long, or 50 to 250 amino acids long, or 100 to 250 amino acids long, or 150-250 amino acids long, or 10 to 200 amino acids long, or 25 to 200 amino acids long, or 50 to 200 amino acids long, or 100 to 200 amino acids long, or 150 to 200 amino acids long or about 200 amino acids long, or about 50 amino acids long or 50 to 150 amino acids long, or 50-100 amino acids long or about 100 amino acids long. In certain embodiments, the internal deletion comprises or consists of SEQ ID NO: 2, SEQ ID NO: 3 or SEQ ID NO: 14.

**[0074]** In certain embodiments, the linker may be present in place of said internal deletion. The lengths of the internal deletion and the linker do not need to be the same. For example, the linker may be 3-50 amino acids long, or 3-40 amino acids long, or 3-30 amino acids long, or 3-20 amino acids long, or 3-10 amino acids long, or about 5 amino acids long, or about 10 amino acids long, or about 20 amino acids long. The exact sequence of the linker is not very important. In general, preferable amino acids are polar uncharged or charged residues, which constitute approximately 50% of naturally encoded amino acids. More specifically, Threonine, Serine, and Glycine may provide good flexibility due to their small sizes, and also help maintain stability of the linker structure in the aqueous solvent through formation of hydrogen bonds with water. In certain embodiments the linker is glycine and/or serine rich. In certain embodiments, the linker is 5 amino acids long and may comprise SEQ ID NO: 36. SEQ ID NO: 36 may be encoded by SEQ ID NO: 37.

**[0075]** In other embodiments, the antigen may comprise SEQ ID NO: 11 or SEQ ID NO: 12 or SEQ ID NO: 46 or an amino acid sequence that is at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or 100% identical to SEQ ID NO: 11 or SEQ ID NO: 12 or SEQ ID NO: 46. Preferably, the differences are substitutions and more preferably, at least 50% (or at least 60% or at least 70% or at least 80% or at least 90% or at least 95% or at least 99%) of the substituted amino acids are conservative substitutions.

**[0076]** Suitable bacteria include, without limitations, *Aeromonas salmonicida* subs. *Salmonicida, Vibrio (Listonella) anguillarum, Vibrio salmonicida, Moritella viscosa,* and *Yersinia ruckeri*. Again, proteins present on the outer surface of the bacteria are preferred antigens, as well as these proteins capable of eliciting protective immune response.

**[0077]** Suitable parasites include sea lice (family *Caligidae*, preferably genera *Lepeophtheirus* or *Caligus*). Proteins originating from sea lice and capable of eliciting protective immune response have been described and include gut peptides or fragments thereof including without limitations SEQ ID NO: 21 or a sequence at least 90% identical thereto.

**[0078]** In certain embodiments, the antigen according to the invention may be engineered to target cell surface. Thus, such engineered antigens will advantageously localize to cell surface where they can be recognized by the host's immune system and thus protective immune response would be generated.

**[0079]** In certain embodiments, the antigen may be presented as a fusion protein comprising, from N-terminus to C-terminus:

a) N-terminal secretion signal sequence of a secreted or a first membrane-bound protein;

b) an antigen from a non-enveloped virus, preferably one of such viruses that infects fish, such as, for example, PMCV, including the PMCV ORF-1 antigen according to any of the embodiments described above;

c) a transmembrane domain of a second membrane-bound protein.

**[0080]** In certain embodiments, said first protein may be selected from the group consisting of Intereferon a, Interferon b, Interferon c, Interferon d, Interferon gamma, interleukine-2, interleukine 4, interleukine-12. The first and the second protein may be identical or different from each other. In some embodiments, the first and/or the second protein may be the fusion protein of Atlantic Salmon Paramyxovirus whose secretion signaling and transmembrane domain sequences are SEQ ID NOs: 38 and 39, respectively. In some embodiments, the first and the second protein may be the Hemagglutinin (HE) protein of Infectious salmon anemia virus (ISAV) whose secretion signaling and transmembrane domain sequences are SEQ ID NOs: 40 and 41, respectively.

**[0081]** In some embodiments, the first membrane protein is identical to the second membrane protein and is viral hemorrhagic septicemia virus G-protein (VHSV-G) whose secretion signaling peptide sequence and the transmembrane domain sequences are SEQ ID NOs 7 and 8, respectively.

**[0082]** Thus, in some embodiments, said N-terminal secretion signal sequence is at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or 100% identical to SEQ ID NO: 7.

**[0083]** As described above, preferably, the mutations leading to the differences from SEQ ID NO: 7 are substitutions. In certain advantageous embodiments, at least half (or at least 60% or at least 70% or at least 80%, or at least 90%) of differing amino acids in said N-terminal secretion signal are conservative substitutions.

**[0084]** In the embodiments, when VHSV-G protein is the source of both the N-terminal secretion signal and the transmembrane domain, said transmembrane domain is at least 90%, or at least 91%, or at least 92%, or at least 93%, or at least 94%, or at least 95%, or at least 96%, or at least 97%, or at least 98%, or at least 99%, or 100% identical to SEQ ID NO: 8.

**[0085]** As described above, preferably, the mutations leading to the differences from SEQ ID NO: 7 and 8 are substitutions. In certain advantageous embodiments, at least half (or at least 60% or at least 70% or at least 80%, or at least 90%) of differing amino acids in said transmembrane domain are conservative substitutions.

**[0086]** Additional fragments of the first and/or second protein may also be included. Thus, in certain embodiments, SEQ ID NO: 7 or a protein that is at least 90% identical to SEQ ID NO: 7 may be included within SEQ ID NO: 48 or an amino acid sequence that is at least 90% identical to SEQ ID NO: 48 or a fragment thereof. It should be understood that said fragment would include SEQ ID NO: 7 or a sequence that is at least 90% identical to SEQ ID NO: 7.

**[0087]** Independently, SEQ ID NO: 8 or a protein that is at least 90% identical to SEQ ID NO: 8 may be included within SEQ ID NO: 49 or an amino acid sequence that is at least 90% identical to SEQ ID NO: 49 or a fragment thereof. It should be understood that said fragment would include SEQ ID NO: 8 or a sequence that is at least 90% identical to SEQ ID NO: 8.

**[0088]** Similarly to SEQ ID NOs 7 and 8, the mutations leading to the differences from SEQ ID NOs 48 and 49 are substitutions. In certain advantageous embodiments, at least half (or at least 60% or at least 70% or at least 80%, or at least 90%) of differing amino acids in said N-terminal secretion signaling sequence and/or in the transmembrane domain are conservative substitutions.

**[0089]** In a third aspect, the application discloses a vector comprising the cassette according to any of the embodiments of the second aspect of the invention.

**[0090]** Different vectors suitable for the invention are known in the art, including both plasmid vectors and viral vectors. Suitable plasmids include, without limitations pUC-based vectors, pVAX-vectors, pcDNA-vectors, NTC-vectors. In a set of preferred embodiments, the vector is NTC9385R (Nature Technology Corporation) or a variant thereof, as described in the Examples.

**[0091]** In other embodiments, a relatively new "doggybone" or DBDNA™ plasmid may be used as a vector. DBDNA™ plasmids as well as the process of making these plasmids have been described at least in WO2018033730, WO2016034849, WO2019193361, WO2012017210 and WO2021161051. The advantage of this approach is that the vector can be synthesized in a cell-free process thus improving manufacturing efficiency. The cell-free process preferably involves amplification of the template via strand displacement replication. This synthesis releases a single stranded DNA, which may in turn be copied into double stranded-DNA, using a polymerase. Alternatively, strand displacement can be achieved by supplying a DNA polymerase and a separate helicase. Replicative helicases may open the duplex DNA and facilitate the advancement of the leading-strand polymerase. The resulting double-stranded DNA concatemer is enzymatically cut and ligated thus forming the doggybone-like shape DNA construct.

**[0092]** Suitable viral vectors include, without limitations, alphaviruses such as SAV, rhabdoviruses such as VHSV and IHNV, paramyxoviruses such as ASP, adenoviruses, poxviruses such as Salmon gill poxvirus, and the like. These viruses can be genetically modified to remove the parts of the viral genomes responsible for replication. Thus, the resulting viruses would be infectious to fish cells and suitable for production of the antigen, but not be pathogenic.

**[0093]** In certain embodiments, the vector may contain the expression cassette as described above and an additional antigen, e.g. a salmonid alphavirus antigen. It is also possible that the

vector would contain an additional nucleic acid sequence encoding a different variant of interferon

**[0094]** A person of ordinary skill in the art will appreciate that multiple methods exist for making the nucleic acids, the expression cassettes and the vectors as described in the first, second, and third aspects of the invention, respectively. For example, the nucleic acid sequences may be designed using software tools, e.g., CLC Main Workbench, and synthesized artificially or generated using targeted mutagenesis. These sequences may be subcloned into expression cassettes and vectors using genetic engineering techniques widely available to one skilled in the art. See, e.g., *Molecular cloning: a laboratory manual* (Sambrook & Russell: 2000, Cold Spring Harbor Laboratory Press; ISBN: 0879695773), and: *Current protocols in molecular biology* (Ausubel et al., 1988+ updates, Greene Publishing Assoc., New York; ISBN: 0471625949).

**[0095]** In a fourth aspect, the application discloses a vaccine comprising the vector according to any of the embodiments of the third aspect of the invention. The vaccine may be monovalent or multivalent. Generally, one dose of the monovalent vaccine may contain at least 1 μg of the vector according to any of the embodiments of the third aspect. In certain embodiments, one dose of the vaccine may contain between 1 μg and about 25 μg of the vector. In other embodiments, one dose of the vaccine may contain between 1 and about 20 μg of the vector, or between about 5 and about 20 μg of the vector or between about 5 and about 15 μg of the vector or between about 10 and about 20 μg of the vector or between about 5 and about 10 μg of the vector.

**[0096]** Multiple antigens suitable for the vaccine according to the invention have been described above. These antigens may be administered in the form of DNA vaccines, including vectors and expression cassettes described above. In other embodiments, these antigens may be administered in forms of subunits (i.e., purified or partially purified proteins). In yet other embodiments, the antigens may comprise inactivated or attenuated organisms.

**[0097]** In addition to the antigens, the vaccine of the invention may further comprise adjuvants, i.e., the substances that enhance or modulate immune response, in addition to the nucleic acid sequence encoding interferon of SEQ ID NO: 6 or SEQ ID NO: 50 or an amino acid sequence that is 95% identical to SEQ ID NO: 6 or 50. Suitable adjuvants are well known in the art. Suitable

non-limiting adjuvants include saponins (e.g., Quil A), alum, CpG oligonucleotides, poly I:C, oligoribonucleotides, cytokines, glycolipids such as BAY®1005, quaternary amines such as dimethyl dioctadecyl ammonium bromide (hereinafter, "DDA"). Complexes comprising the saponin, the sterol (e.g., cholesterol), and, optionally, a phospholipid, have been described in the art. Combinations of CpG oligonucleotides and Saponin, CpG and cholesterol, and CpG and alum have been reported to elicit synergistic effects.

**[0098]** In addition to the antigens and optional additional adjuvants, the vaccine may also comprise a suitable pharmaceutical carrier. The appropriate carrier is evident to those skilled in the art and will depend in large part upon the route of administration. Additional components that may be present in this invention are adjuvants, preservatives, surface active agents, chemical stabilizers, suspending or dispersing agents. Typically, stabilizers, adjuvants and preservatives are optimized to determine the best formulation for efficacy in the target subject.

**[0099]** In certain embodiments, the vaccine may include liposomal adjuvant and/or carrier to facilitate the transport of the vector across the cell membrane and thus result in an increased expression of the antigen and/or the molecular immunomodulator. A suitable non-limiting example of such liposomal adjuvant/carrier system is described, for example, in the US patent 10,456,459.

**[00100]** Accordingly, in a fifth aspect, the application discloses a method of protecting a salmonid against an infection, the method comprising administering to the salmonid in need thereof the vaccine according to any of the embodiments of the fourth aspect of the invention. Thus, the disclosure also provides the vaccine according to any of the embodiments of the fourth aspect for use in protecting a salmonid against an infection.

**[00101]** It should be clear to one of ordinary skill in the art that the infection that the antigen(s) present in the vaccine dictate what invention the vaccine would protect against. Thus, for example and without limitation, if the vaccine comprises a nucleic acid sequence encoding a PMCV antigen, then the vaccine would be used against infection caused by PMCV.

**[00102]** The vaccine according to the invention may be administered to the salmonid by a variety of routes, including, without limitation, intramuscularly. Preferably, the vaccine is administered

in a microdose such that the volume of one dose is under 500 µl, or under 400 µl, or under 300 µl or under 200 µl or about 100 µl or under 100 µl, or about 50 µl or about 25 µl.

**[00103]** The vaccine disclosed herein may be used in protecting multiple salmonid species against an infection. Suitable salmonids include, without limitations, Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*), rainbow trout (*Oncorhynchus mykiss*), sockeye salmon (*Oncorhynchus nerka*), Chinook salmon (*Oncorhynchus tshawytscha*) and other species.

**[00104]** Salmonids of different ages (or weights) may be vaccinated according to the invention. In certain embodiments, the salmonid weighs between about 15 and about 200 grams at the time of vaccination. Thus, the weight of the salmonid at the time of the vaccination may be between about 15 and about 200 grams or between about 40 and about 110 grams or between about 50 and about 100 grams.

**[00105]** In a sixth aspect, the invention provides a method of evaluating the efficiency of a Type I interferon as an adjuvant. The phrase "evaluating the efficiency of a Type I interferon as an adjuvant" refers both to the level of expression of the amino acid for the interferon protein from a given nucleic acid sequence and to the adjuvanting activity of the interferon. The principle of this test is to quantify Mx-expression (IFN-induced gene) by RT-qPCR. Mx proteins belong to the superfamily of large GTPases with antiviral activity against a wide range of RNA viruses. *In vivo*, the expression of Mx genes is tightly regulated by the presence of type I interferons (IFNs), and their induction has been described during several viral infections.

**[00106]** In general, the method comprises:

a) transfecting a nucleic acid sequence encoding an interferon into a source cells;
b) culturing said source cells, wherein said interferon is secreted into a source cell media;
c) contacting cultured target cells with at least a portion of said source cell media, wherein said target cells naturally express Mx gene in response to interferon; and
d) measuring Mx gene expression.

**[00107]**In this method, a higher Mx gene expression indicates a greater efficiency of the tested Type I interferon as an adjuvant. Multiple Type I interferons are suitable to be tested in this method, including without limitations Interferons A (also known as alpha interferons) and Interferons B (also known as beta interferons), and Inteferons C. In Atlantic salmon eleven IFN genes were identified in the same genomic region encoding two IFNa (IFNal and IFNa3), four IFNb (IFNb1-b4) and five IFNc (IFNc1-c4) genes. See, e.g., EP2950816B.

**[00108]**In certain embodiment, the Type 1 interferon is an IFNb interferon, including, without limitations, the interferons encoded by the nucleic acid sequences according to the first aspect of the invention.

**[00109]**The nucleic acid sequences encoding said Type 1 interferon may be cloned into an expression cassette or a vector, such as a plasmid vector or a viral vector generally described above, under control of a promoter that allows expression in the source cells. Such promoters have also been described above and include, without limitations, CMV early late promoter. The transfection techniques are also well known and include, without limitations, electroporation, calcium phosphate and lipid-based compounds such as, for example, LIPOFECTAMINE ®.

**[00110]**This method is particularly suitable in situations where the target cells cannot be easily transfected or where the source cells do not express Mx in response to the stimulation with the Type I interferon, or both.

**[00111]**In certain embodiments, particularly suitable for testing adjuvanting potential of interferons in the context of aquaculture vaccines, the source cells are fish cardiovascular CHH-1 cells and the target cells are TO-cells as CHH-1 cells do not express Mx in response to IFNb stimulation and TO-cells not easily can be transfected. Both TO-cells and CHH-1 cells have been described in the literature. CHH-1 cells are commercially available from Sigma-Aldrich. Other cell lines can also be used in this assay. Suitable non-limiting example of source cells are CHSE cells and suitable non-limiting examples of the target cells are SHK and ASK cells.

**[00112]**The expression of the Mx gene can be measured in multiple ways. For example, one can measure the amount of the protein using immunoassays or one can measure the amount of Mx RNA using amplification-based assays such as quantitative RT-PCR. Since the nucleic acid

sequence of Mx gene is known, one of ordinary skill in the art can design primers and/or probes to amplify and detect Mx mRNA using techniques known in the art.

**[00113]** In a seventh aspect, the invention provides a method of determining which nucleic acid sequence in a group of sequences encoding the same Type I interferon confers a more efficient expression. This method utilizes the techniques described in the sixth aspect of the invention above, including the interferons suitable for being tested, the nucleic acid sequences encoding said interferons, suitable vectors, the source cells, the target cells, the transfection techniques, and the methods for measuring the levels of Mx gene expression.

**[00114]** Briefly, if there is a group of N (N> 1) nucleic acid sequences encoding the same Type I interferon amino acid sequence, one prepares N vectors, differing only in the nucleic acid sequences that are being tested. Then the same amounts of these N vectors are transfected using the same techniques into the N batches of the same source cells cultured under identical conditions.

**[00115]** The same volume of the media from the cultured source cells (N samples) is then contacted with the N batches of the target cells, wherein each batch of these target cells is cultured under identical conditions. The first sample would be contacted with the first batch of the target cells, the second sample would be contacted with the second batch of the target cells, and so on; the Nth sample would be contacted with the Nth batch of the target cells. Then the respective levels of expression of the Mx gene in the N batches would be determined.

## EXAMPLES

### EXAMPLE 1. Preparation of surface-targeting expression system

**[00116]** All successful DNA vaccines for Atlantic salmon have been derived from enveloped viruses and are based on membrane-bound antigens. Exposure of the antigen on the surface of cells could therefore be important for efficacy.

**[00117]** PMCV is a naked virus, containing a dsRNA genome of 6.7 kb with three predicted open reading frames (Figure 1). The capsid protein (CP) is translated from ORF1, while ORF2 encodes the RNA dependent RNA polymerase (RdRp). The function of the ORF3 protein is still unknown,

but it encodes a protein with cytotoxic effect when over-expressed in cell culture and is not necessary for assembly of viral particles.

**[00118]** DNA vaccines expressing the PMCV ORF1 (capsid protein) and PMCV ORF3 (protein of unknown function) had already been tested without providing protection (data not shown). Since PMCV is a naked virus with no transmembrane regions predicted for the PMCV ORF1 protein, and it was demonstrated that the PMCV ORF1 protein would only be intracellularly expressed (data not shown), it was hypothesized that response may be elicited if the expressed ORF1 capsid protein is targeted to the cell membrane.

**[00119]** The aim of this experiment was to construct a DNA vaccine expressing the PMCV capsid protein (or parts of it) as a cell surface expressed antigen. The strategy was to fuse the N-terminal secretion signal peptide of the viral hemorrhagic septicemia virus G-protein (VHSV-G) according to SEQ ID NO: 7 upstream of the PMCV ORF1 antigen N-terminus. Additionally, the C-terminal transmembrane domain of VHSV-G according to SEQ ID NO: 8 was fused downstream of the antigen C-terminus, to incorporate the antigen at the cell surface (von Gersdorff Jørgensen et al., 2012), as illustrated in Fig. 2A. A secreted version of the vaccine antigen was also prepared by only using N-terminal secretion signal peptide and omitting the use of the C-terminal transmembrane domain (Fig. 2B).

**[00120]** Additional constructs were prepared similarly to the construct illustrated in Fig 2A, except where instead the signal peptide sequence and transmembrane domain sequence of the Atlantic salmon paramyxovirus fusion protein (SEQ ID NOs: 38 and 39, respectively) or Hemagglutinin (HE) protein of Infectious salmon anemia virus (ISAV) (SEQ ID NOs: 40 and 41, respectively) were used.

**[00121]** Routing of antigen expression was studied by immunofluorescence (IF) staining 6 days after transfection of CHH-1 cells with various DNA vaccine constructs. Transfection was done using the LIPOFECTAMIN® 3000 kit (Thermo Fisher) in 12-well plates. The pVAX1 expression vector (Invitrogen) was utilized to construct the DNA vaccines in this experiment, and 1 µg of plasmid DNA was used for transfection. Cells were fixed either with 3.7% formaldehyde only for surface antigen-expression or fixed with 3.7% formaldehyde followed by permeabilization of cells using 0.1% Triton X-100 for staining of intracellularly expressed proteins. Vaccine antigen was detected both using a monoclonal antibody and polyclonal antibodies against PMCV ORF1.

Appropriate fluorescent-labeled secondary antibodies (Rabbit anti-mouse FITC (DAKO F0261) or Polyclonal goat anti-rabbit Alexa Fluor Plus 488 (Invitrogen A32731) were used for detection. Staining permeabilized cells detecting intracellularly expressed antigen was routinely included as a positive control of the protocol.

**[00122]**As shown in Fig. 3, the use of VHSV G-protein could be used for routing expression of PMCV ORF-1 antigen to the cell surface. Using signal protein and transmembrane domain from Hemagglutinin (HE) protein of Infectious salmon anemia virus also resulted in membrane staining of PMCV ORF1 protein, while using regions from the fusion protein of Atlantic Salmon Paramyxovirus was not successful.

**[00123]**In the next experiment, *in vivo* testing in Atlantic salmon (*Salmo salar*) was conducted. Two variants of the antigen were tested, the full-length capsid protein (G-ORF1) and a truncated capsid protein (G-ORF1-DeltaD) expressing the first half of the capsid protein (PMCV ORF1 position 1-1293 of SEQ ID NO: 16). Both antigens were expressed both in a standard expression vector (pcDNA3.1) and as an alphavirus replicon vaccine based on salmonid alphavirus 3 (SAV-3). PBS and pcDNA-eGFP were included as negative vaccine control groups.

**[00124]**For all vaccines in the present study the antigen encoding genes were inserted downstream of a CMV promoter in the eukaryotic expression vector pcDNA3.1(+) (Invitrogen). All plasmids were diluted in sterile phosphate-buffered saline (PBS) to 10 µg per 50 µl injection volume. All fish received one intramuscular injection on each side of the fish and a total dose of 20 µg. Two non-immunized control groups were included, one group received 20 µg of a control vaccine (eGFP in pcDNA3.1) and one group received 2 x 50 µl PBS.

**Table 2.**

| Vaccine group | Description | Detailed description plasmid encoding antigen | Plasmid backbone | Final conc construct (µg/ml) | Dose µg (2 x 0.05 ml) |
|---|---|---|---|---|---|
| 1 | G-ORF1-Delta D | G-PMCV_ORF1_1-1293 (Delta D) | pcDNA3.1(+) | 200 | 20 |
| 2 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | pcDNA3.1(+) | 200 | 20 |
| 3 | repSAV-G-ORF1-Delta D | G-PMCV_ORF1_1-1293 (Delta D) in SAV replicon | pcDNA3.1(+) | 200 | 20 |
| 4 | repSAV-G-ORF1 | G-PMCV_ORF1_full_1-2583_in SAV replicon | pcDNA3.1(+) | 200 | 20 |
| 5 | eGFP | enhanced Green Fluorescent Protein (control) | pcDNA3.1(+) | 200 | 20 |
| 6 | PBS | | | | NA |

**[00125]**The fish (n=45 per group) were kept in freshwater and had an average weight of 48 grams. Prior to vaccination, the fish were anaesthetized using Tricain (PHARMAQ), tagged by shortening of the adipose fin and/or maxillae, allocated to a group, and intramuscularly injected twice under the same anaesthetic period (one injection on each side of the fish) with 0.05 ml of the test vaccines. Immunity was allowed to develop for 49 days at 12°C (light:dark 12:12) before the fish were anaesthetized again using Tricain (PHARMAQ) and challenged with infectious PMCV by intraperitoneal injection of 0.1 ml of homogenized spleen (isolate ID AL V1223 Spleen) originating from a Norwegian field outbreak of CMS. The fish were then sampled at three timepoints; 10 days, 20 days and 50 days post challenge (n=15 per group, per sampling-point). Heart ventricle and kidney were collected on RNALATER® from all sampled fish for subsequent RNA extraction. Hearts (ventricle and atrium) were also fixed in formalin from all fish on the last sampling point (day 50) for histopathological analysis. RNA was extracted from all samples stored on RNALATER® and analyzed by Real-Time RT-PCR for presence of PMCV RNA using a commercially available service from PHARMAQ Analytiq (Bergen, Norway). Hearts stored on formalin were sectioned and histopathology was scored from 0 to 3 according to severity, where 0 indicates no pathologic

finding and 3 indicate presence of severe pathology (service provided by PHARMAQ Analytiq, Bergen, Norway).

**[00126]**Vaccines expressing the full-length ORF1 of PMCV routed to the cell surface demonstrated significant protection against challenge compared to control groups (One-way ANOVA, with Dunnett's post test for histology in atrium, $p<0.0001$). This was shown both as increased Ct values in kidney and heart early after infection (results not shown), and reduced histopathology score 50 days after infection (Table 3). Antigen based on the first half of the capsid protein (G-ORF1-Delta D; PMCV ORF1 position 1-1293) failed to provide protection, and no additional increase in protection was provided using the salmon alphavirus replicon in contrast to the standard expression vector.

**Table 3**

| Vaccine-group | Description | Mean histoscore, Atrium | Standard deviation, histoscore | N | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 6 | PBS | 2,29 | 0,78 | 15 | NA |
| 5 | eGFP | 2,21 | 0,67 | 15 | 0,99 |
| 4 | repSAV-G-ORF1 | 1,49 | 0,88 | 15 | 0,028 |
| 3 | repSAV-G-ORF1-Delta D | 2,31 | 0,69 | 15 | 0,99 |
| 2 | G-ORF1 | 1,11 | 0,83 | 15 | 0,0005 |
| 1 | G-ORF1-Delta D | 2,35 | 0,83 | 15 | 0,99 |

Histopathological score in the heart atrium at 50 days post challenge with PMCV. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

**[00127]**The DNA vaccine candidate targeting the full-length antigen to the cell-surface gave an approximately 50% reduction of histopathological score and was accompanied by a reduced viral load (qPCR) in kidney and heart.

**[00128]**A follow up *in vivo* trial compared the efficacy of DNA vaccines expressing the PMCV ORF1 full-length protein as a cell surface expressed antigen, secreted antigen or an intracellularly expressed antigen. In addition, a codon-optimized version of the full-length capsid protein

encoded by SEQ ID NO: 45 was included. The study contained two parallel immunization tanks with five groups each of Atlantic salmon (n=30 per group, per tank).

**[00129]** For all vaccines in the present study the antigen encoding genes were inserted downstream of a CMV promoter in the eukaryotic expression vector NTC9385R (Nature Technology Corporation). All plasmids were diluted in sterile phosphate-buffered saline (PBS) to 10 µg per 50 µl injection volume. All fish received one intramuscular injection on each side of the fish and a total dose of 20 µg. A non-immunized control group was included and received 2 x 50µl PBS, as summarized in Table 4.

**Table 4.**

| Vaccine group | Description | Description plasmid encoding antigen | Plasmid backbone | Antigen expressed at | Final conc construct (µg/ml) | Dose µg (2 x 0.05 ml) |
|---|---|---|---|---|---|---|
| 1 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | NTC9385R | Cell-surface | 200 | 20 |
| 2 | ORF1 | PMCV_ORF1_full_1-2583 | NTC9385R | Intracellular | 200 | 20 |
| 3 | G-ORF1 Codon opt | G-PMCV_ORF1_full_1-2583-Codon optimized | NTC9385R | Cell-surface | 200 | 20 |
| 4 | G-ORF1 w/o TM | G-PMCV_ORF1_full_1-2583 without TM domain | NTC9385R | Secreted | 200 | 20 |
| 5 | PBS | | NA | NA | | |

The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

**[00130]** The fish were kept in freshwater and had an average weight of 26 grams at the time of vaccination. During administration of the test vaccines, the fish were anaesthetized using Tricain (PHARMAQ), tagged by shortening of the adipose fin and/or maxillae, allocated to a group, and intramuscularly injected twice under the same anaesthetic period (one injection on each side of the fish) with 0.05 ml of the test vaccines. Immunity was allowed to develop for 39 days at 12C (light:dark 12:12) before the fish were anaesthetized again using Tricain (PHARMAQ) and challenged with infectious PMCV by intraperitoneal injection of 0.1 ml of homogenized heart

(isolate ID AL V1273 Heart) originating from a Norwegian field outbreak of CMS. The fish (n=15 per group, per time-point) were then sampled 21 days and 56 days post challenge. Heart ventricle and kidney were collected on RNALATER® from all sampled fish for subsequent RNA extraction. Hearts (ventricle and atrium) were also fixed in formalin from all fish on the last sampling point (day 56) for histopathological analysis. RNA was extracted from all samples stored on RNALATER® and analyzed by Real-Time RT-PCR for presence of PMCV RNA using a commercially available service from PHARMAQ Analytiq (Norway). Hearts stored on formalin were sectioned and histopathology was scored from 0 to 3 according to severity, where 0 indicates no pathologic finding and 3 indicate presence of severe pathology (service provided by PHARMAQ Analytiq, Norway).

**[00131]**The results showed that significant protection against PMCV infection was achieved with vaccines expressing the full-length ORF1 of PMCV routed to the cell surface (G-ORF1 and G-ORF1 codon optimized). Expression of PMCV ORF1 in its native form (intracellular protein) did not provide detectable protection. Expression by secretion provided some protection (no difference detected in kidney at day 20; See Tables 5, 6).

**Table 5.** Tank 1. Viral RNA in kidney 3 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine group | Description | Mean Ct-value, PMCV | Standard deviation, Ct | n | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 5 | PBS | 20,95 | 0,96 | 16 | NA |
| 1 | G-ORF1 | 23,02 | 2,17 | 15 | 0,0042 |
| 3 | G-ORF1 Codon opt | 23,09 | 1,67 | 15 | 0,0031 |
| 4 | G-ORF1 w/o TM | 20,74 | 2,02 | 15 | 0,99 |
| 2 | ORF1 | 19,92 | 1,41 | 15 | 0,30 |

**Table 6.** Tank 2. Viral RNA in kidney 3 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine group | Description | Mean Ct-value, PMCV | Standard deviation, Ct | n | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 5 | PBS | 20,05 | 0,73 | 15 | NA |
| 1 | G-ORF1 | 22,12 | 1,95 | 15 | 0,0005 |
| 3 | G-ORF1 Codon opt | 23,06 | 1,93 | 15 | <0,0001 |
| 4 | G-ORF1 w/o TM | 20,06 | 1,16 | 15 | >0,99 |
| 2 | ORF1 | 19,90 | 0,67 | 15 | >0,99 |

**EXAMPLE 2: Preparation and determination of antigenic activity of CMS antigen**

**[00132]** Expressing the PMCV ORF1 full-length protein on the cell membrane provided partial protection in the challenge model. Despite this finding, it was observed *in vitro* that expression levels of the antigen were markedly lower than expression levels of a control GFP-construct. The project has investigated whether expressing truncated versions of the capsid protein by deleting regions could increase expression levels and immunogenicity of the vaccine antigen.

**[00133]** Expression system described in Example 1 (using VHSV-G signal peptide and transmembrane domain sequences) was prepared with different constructs based on PMCV ORF-1 antigen.

**[00134]** The first studies expressing membrane-bound PMCV capsid antigen from the VHSV-G expression cassette showed that including the first part of the PMCV ORF1 protein sequence was pivotal for success. When the capsid protein was divided into thirds, membrane bound protein was successfully achieved only for constructs comprising the first third (data not shown).

**[00135]** Following construction of PMCV ORF1 capsid deletion variants, these were tested by IF-staining and Western blot of transfected cells. For all deletion constructs, the deleted PMCV ORF1 sequence was replaced with a GGGGS linker (SEQ ID NO: 36). The strategy for making the various deletion variants was as follows:

(1) In the first round, relatively large deletions were tested (IntDel 1-7; 200 amino acids in length, overlapping by 100 amino acids, except IntDel7 which was 162 amino acids long). As inclusion of some N-terminal sequence was found to be required for successful routing of expressed protein to cell membranes, the first deletion (IntDel 1) was made at nucleotide positions 298-897 within PMCV ORF1 (SEQ ID NO: 16).

(2) In the second round, smaller deletion-variants were made focusing on the region covered by IntDel 4-7 (IntDel 11-18, adjacent, non-overlapping 50 amino acids in length, starting within IntDel 4). The first of these deletions (IntDel 11) was constructed at nucleotide positions 1384-1533 within PMCV ORF1 (SEQ ID NO: 16).

(3) In the third round, antigen variants with very small adjacent overlapping deletions were made (IntDel 21-42; 10 amino acids in length, overlapping by 2 amino acids). The first of these deletions (IntDel 21) was constructed at nucleotide positions 1936-1965 within PMCV ORF1 (SEQ ID NO: 16).

**[00136]** Expression of PMCV ORF1 capsid deletion variants in CHH-1 cells by IF-staining and Western blot. The procedure for immunofluorescence staining of cells was as follows: CHH-1 cells were trypsinated and seeded into 12- well plates (150 000 cells per well and 1 ml cell-medium). The cells were incubated at 15°C ON in a CO2 incubator (5% $CO_2$). The cells were transfected the day after seeding using 1 µg plasmid/well and LIPOFECTAMIN® 3000 (Thermo Fisher Scientific) according manufacturer's instructions. The cells were incubated at 15°C for 5 days in a CO2 incubator (5% $CO_2$) before immunostaining of cells. The cell-medium was carefully removed from all wells, and all wells were washed carefully with PBS. Cells were fixed by incubation with 3.7% formaldehyde (diluted in PBS) for 15 min at RT. Cells were then blocked with 5% non-fat dry milk in PBS for 1 h. Both mouse anti-HIS (6x-His Tag Monoclonal Antibody (4A12E4) Fisher scientific; cat # 37-2900 at 1:1500 dilution and mouse anti-PMCV ORF1 monoclonal antibody (mAb#10; 1:200 dilution) were used as primary antibodies and incubated at room temperature for 1 h. FITC-conjugated Polyclonal Rabbit Anti-mouse Immunoglobulin (DAKO F0261) was used as the

secondary antibody at 1:1000 dilution and incubated for another 1 h. Staining of cells were evaluated by fluorescence microscopy.

**[00137]** Evaluating expression levels of PMCV ORF1 deletion constructs by Western blotting was done as follows: CHH-1 cells were trypsinated and seeded into 6-well plates (1 000 000 cells per well and 3 ml cell-medium). The cells were incubated at 15°C ON in a $CO_2$ incubator (5% $CO_2$). The cells were transfected the day after seeding using 2.5 µg plasmid/well and LIPOFECTAMIN® 3000 (Thermo Fisher Scientific) according manufacturer's instructions. Cells were put back in sealed bags and incubated at 15°C in a CO2 incubator (5% $CO_2$) for six days before further analysis. Plates were then placed on ice and medium removed. Cells were first washed twice with ice-cold PBS. To prepare membrane lysates, cells were collected first adding 200 µl of 20 mM Tris-HCl, pH 7.5 with protease inhibitors, cells carefully scraped off and the cell suspensions transferred to pre-cooled tubes. The well was then flushed with another 100 µl to a total of 300 µl of cell suspension per transfection. As this is a detergent-free buffer, cells were lysed by passing cells through a syringe tip (25G, ~5 times) and centrifuged at 12 000 rpm for 10 min at 4°C. The supernatants were discarded, and the membrane pellets dissolved in 300 µl RIPA lysis-buffer (Abcam, #156034) with 1 x Protease inhibitor cocktail to release membrane-bound proteins. Samples were incubated for 30 min at 4°C while re-suspending the pellet occasionally by flicking the tube. Tubes were centrifuged at 12 000 rpm for 10 min at 4°C and the membrane-fraction lysates transferred to fresh tubes and kept on ice until immunoprecipitation. On ice, 1 µg of mouse IgG1anti 6x-His monoclonal antibody 4E3D10H2/E3 (Fisher Scientific #15442890, 1 mg/ml) was added to each sample and incubated for 4°C overnight on a rotator. Protein G-coupled beads were prepared (25 µl/sample) by washing the immobilized beads 3-5 x with ~1 ml cold PBST and re-suspended in 25µl of PBST with protease inhibitors per aliquote. On ice, 25 µl of pre-equilibrated beads were added to each sample and the sample-beads mixture incubated for 1 hour at RT with rotation. The beads were washed with 500 µl of ice cold PBST with proteinase inhibitors 3-5 times to remove non-specific binding with gentle mixing of the beads between each wash. After the last wash, as much buffer as possible was removed from the beads and 40 µl of 1x Laemmli Sample Buffer (BioRad #161-0737) added (prepared by mixing 20 µl 2x Laemmli Sample Buffer with 2 µl β-mercaptoethanol and 18 µl dH2O). Samples were incubated

at 70°C for 10 minutes, quickly centrifuged and magnetized, and eluent moved to a new vial. Before loading on a 4-20% Criterion™ TGX Stain-Free™ Gel, all samples were denatured at 98°C for 5 min. The gel was run for approximately 45 min at 200V and blotted using the TRANS-BLOT® TURBO™ Transfer System (BioRad), and TRANS-BLOT® TURBO™ Midi PVDF Transfer Packs #170-4157. 7 minutes turbo program was used. The membrane was immediately put in blocking-buffer (5% skimmed milk in PBST). Blocking was performed for 1 hour at room-temperature. The blot was incubated with primary antibody rabbit anti-delta PMCV ORF1 diluted 1: 500 in 1% skimmed milk/PBST over-night at 4°C in rotator. The following day the blots were washed 2 x 15 minutes with PBST and incubated with secondary antibody polyclonal swine anti-rabbit (DAKO #P0217) immunoglobulin HRP diluted 1:1000 in 2% skimmed milk and PRECISION PROTEIN™ StrepTactin-HRP Conjugate 1:10000 (to visualize ladder) for 1 hour at RT. The blot was washed 2 x 15 minutes with PBST before detection of signal by Clarity western ECL substrate (BioRad #170-5060).

**[00138]** The results can be summarized as follows: After the first round (200-amino-acid-long deletions) expression at the cell membrane was achieved only with antigen from constructs IntDel 4-7. Accordingly, the N-terminal portion is required for routing the antigen to the cell membranes. Constructs made in the second round (50-amino-acid-long deletions), focused therefore on the region covered by IntDel 4-7. All these constructs successfully expressed antigen at the cell membrane, except that cell surface expression was lower for IntDel 18 (the construct lacking the amino acid sequence set forth in SEQ ID NO: 15). The deletion constructs (IntDel 15-17) resulted in increased membrane expression levels. IntDel 6 (the construct lacking amino acids 500-699 of SEQ ID NO: 1) from the first round was also found to have higher expression levels. Except for IntDel 42 (the construct lacking amino acids set forth in SEQ ID NO: 14) doing slightly better than the others, no further candidates were revealed in the third round. The results are illustrated in Fig. 4.

**[00139]** Based on combined results from evaluation of immunofluorescence staining and Western blotting, several deletion candidates have been selected based on results from several vaccine trials in the project. For all vaccines in the first study the antigen encoding genes were inserted downstream of a CMV promoter in the eukaryotic expression vector NTC9385R (Nature Technology Corporation). All plasmids were diluted in sterile phosphate-buffered saline (PBS) to

10 µg per 50 µl injection volume. All fish received one intramuscular injection on each side of the fish and a total dose of 20 µg. A non-immunized control group was included and received 2 x 50µl PBS, as summarized in Table 7.

**Table 7**

| Vaccine group | Description | Description plasmid encoding antigen (positions relates to nucleotide position PMCV ORF1) | Plasmid backbone | Final conc construct (µg/ml) | Dose µg (2 x 0.05 ml) |
|---|---|---|---|---|---|
| 1 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | NTC9385R | 200 | 20 |
| 2 | G-IntDel-6 | G-PMCV ORF1_IntDel6 (pos 1798-2397) | NTC9385R | 200 | 20 |
| 3 | G-IntDel-15 | G-PMCV ORF1_IntDel15 (pos 1984-2133) | NTC9385R | 200 | 20 |
| 4 | G-IntDel-16 | G-PMCV ORF1_IntDel16 (pos 2134-2283) | NTC9385R | 200 | 20 |
| 5 | G-IntDel-17 | G-PMCV ORF1_IntDel17 (pos 2284-2433) | NTC9385R | 200 | 20 |
| 6 | PBS | | NA | | |

**[00140]** Atlantic salmon (*Salmo salar*) (n=30 per group) kept in freshwater and with average weight of 20 grams were anaesthetized using Tricain (PHARMAQ), tagged by shortening of the adipose fin and/or maxillae, allocated to a group, and intramuscularly injected twice under the same anaesthetic period (one injection on each side of the fish) with 0.05 ml of the test vaccines. Immunity was allowed to develop for 48 days at 12C (light:dark 12:12) before the fish were anaesthetized again using Tricain (PHARMAQ) and challenged with infectious PMCV by intraperitoneal injection of 0.1 ml of homogenized heart (isolate ID AL V1273 Heart) originating from a Norwegian field outbreak of CMS. The fish (n=15 per group, per sampling-point) were then sampled at two timepoints; 19 days and 49 days post challenge. Heart ventricle and kidney were collected on RNALATER® from all sampled fish for subsequent RNA extraction. Hearts (ventricle and atrium) were also fixed in formalin from all fish on the last sampling point (day 49) for histopathological analysis. RNA was extracted from all samples stored on RNALATER® and analyzed by Real-Time RT-PCR for presence of PMCV RNA using a commercially available service from PHARMAQ Analytiq (Norway). Hearts stored on formalin were sectioned and histopathology was scored from 0 to 3 according to severity, where 0 indicates no pathologic

finding and 3 indicate presence of severe pathology (service provided by PHARMAQ Analytiq, Norway).

**[00141]**The results for viral load at 3 weeks post challenge and histoscore at 7 weeks post challenge are shown in Tables 8 and 9, respectively. The combined data of all results showed that the IntDel variants 15, 16 and 17 (wherein the antigen lacked the amino acid sequences set forth in SEQ ID NOs 3, 4, or 5, respectively) performed better than full-length capsid protein (G-ORF1).

**Table 8**. Viral RNA in kidney 3 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine group | Description | Mean Ct-value, PMCV | Standard deviation, Ct | n | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 6 | PBS | 20,61 | 1,49 | 15 | NA |
| 1 | G-ORF1 | 23,77 | 2,57 | 15 | 0,0002 |
| 2 | G-IntDel-6 | 21,23 | 1,41 | 15 | 0,86 |
| 3 | G-IntDel-15 | 23,13 | 2,81 | 15 | 0,0033 |
| 4 | G-IntDel-16 | 22,92 | 1,20 | 15 | 0,0082 |
| 5 | G-IntDel-17 | 23,13 | 1,71 | 15 | 0,0032 |

**Table 9.** Histopathological lesions (histoscore) in atrium 7 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine-group | Description | Mean histoscore, Atrium | Standard deviation, histoscore | N | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 6 | PBS | 1,75 | 0,84 | 15 | NA |
| 1 | G-ORF1 | 1,60 | 0,83 | 15 | 0,99 |
| 2 | G-IntDel-6 | 1,54 | 0,99 | 15 | 0,95 |
| 3 | G-IntDel-15 | 0,75 | 0,75 | 15 | 0,012 |
| 4 | G-IntDel-16 | 1,10 | 0,63 | 15 | 0,16 |
| 5 | G-IntDel-17 | 1,32 | 0,87 | 15 | 0,53 |

**[00142]**An additional clinical trial was performed to evaluate three new antigen deletion candidates. For all vaccines in the first study the antigen encoding genes were inserted downstream of a CMV promoter in the eukaryotic expression vector NTC9385R (Nature Technology Corporation). All plasmids were diluted in sterile phosphate-buffered saline (PBS) to 10 µg per 50 µl injection volume. All fish received one intramuscular injection on each side of the fish and a total dose of 20 µg. A non-immunized control group was included and received 2 x 50µl PBS, as summarized in Table 10.

**Table 10**

| Vaccine group | Description | Description plasmid encoding antigen (positions relates to nucleotide position PMCV ORF1) | Plasmid backbone | Final conc construct (µg/ml) | Dose µg (2 x 0.05 ml) |
|---|---|---|---|---|---|
| 1 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | NTC9385R | 200 | 20 |
| 2 | G-IntDel-5 | G-PMCV ORF1_IntDel5 (pos 1498-2097) | NTC9385R | 200 | 20 |
| 3 | G-IntDel-7 | G-PMCV ORF1_IntDel7 (pos 2098-2583) | NTC9385R | 200 | 20 |
| 4 | G-IntDel-42 | G-PMCV ORF1_IntDel42 (pos 2440-2469) | NTC9385R | 200 | 20 |
| 5 | PBS | | NA | | |

**[00143]**Atlantic salmon (*Salmo salar*) (n=30 per group) kept in one tank of freshwater and with average weight of 28 grams were anaesthetized using Tricain (PHARMAQ), tagged by shortening of the adipose fin and/or maxillae, allocated to a group, and intramuscularly injected twice under the same anaesthetic period (one injection on each side of the fish) with 0.05 ml of the test vaccines. Immunity was allowed to develop for 58 days at 12C (light:dark 12:12) before the fish were anaesthetized again using Tricain (PHARMAQ) and challenged with infectious PMCV by intraperitoneal injection of 0.1 ml of homogenized heart (isolate ID AL V1289) originating from a Norwegian field outbreak of CMS. The fish (n=15 per group) were then sampled at two timepoints; 3 weeks and 7 weeks post challenge. Heart ventricle and kidney were collected on RNALATER® from all sampled fish for subsequent RNA extraction. Hearts (ventricle and atrium) were also fixed in formalin from all fish on the last sampling point (week 7) for histopathological

analysis. RNA was extracted from all samples stored on RNALATER® and analyzed by Real-Time RT-PCR for presence of PMCV RNA using a commercially available service from PHARMAQ Analytiq (Norway). Hearts stored on formalin were sectioned and histopathology was scored from 0 to 4 according to severity, where 0 indicates no pathologic finding and 4 indicate presence of severe pathology (service provided by PHARMAQ Analytiq, Norway).

**[00144]**The results for week 3 and week 7 are summarized in Tables 11 and 12, respectively.

**Table 11.** Viral RNA in kidney 3 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine group | Description | Mean Ct-value, PMCV | Standard deviation, Ct | N | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 5 | PBS | 19,44 | 1,13 | 15 | NA |
| 1 | G-ORF1 | 20,48 | 1,91 | 15 | 0,38 |
| 2 | G-Del5 | 22,58 | 2,69 | 15 | <0,0001 |
| 3 | G-Del7 | 21,12 | 1,99 | 15 | 0,06 |
| 4 | G-Del42 | 21,12 | 1,40 | 15 | 0,06 |

**Table 12.** Histopathological lesions (histoscore) in atrium 7 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine-group | Description | Mean histoscore, Atrium | Standard deviation, histoscore | N | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 5 | PBS | 2,14 | 0,36 | 14 | NA |
| 1 | G-ORF1 | 2,07 | 0,70 | 15 | 0,99 |
| 2 | G-Del5 | 1,64 | 1,15 | 14 | 0,28 |
| 3 | G-Del7 | 1,80 | 0,56 | 15 | 0,59 |
| 4 | G-Del42 | 1,20 | 0,94 | 15 | 0,0075 |

**EXAMPLE 3: Vaccine backbone selection**

**[00145]** To optimize vaccine efficacy, properties of the plasmid backbone such as vector size and functional elements are also important. Three vector candidates were compared with regards to *in vitro* antigen expression. The candidates included pcDNA3.1, pVAX1 (INVITROGEN™), NTC9385R (Nature Technology Corporation), and two variants of NTC9385R that co-express innate immunostimulatory elements: a RIG-I agonist that function as a type I interferon inducing adjuvant (NTC9385R-eRNA41H) and the RIG-I agonist as well as a Toll-like receptor 9 stimulating CpG motif (NTC9385R-eRNA41H-CpG). The NTC9385R NANOPLASMID™ is smaller in size than the other traditional vectors, allowing for improved uptake and persistence in transfected cells and harbors a modified promoter claimed to enhance antigen expression. It contains an RNA-based sucrose selection antibiotic free marker (RNA-OUT) that replaces the use of antibiotics in the production process and a modified replication origin that secures that the plasmid can only replicate in a specific *E. coli* production strain which is beneficial from a safety perspective. The smaller size can affect transfection efficiency and level and duration of expression. Additionally, some bacterial region protein marker genes like resistance marker genes have been shown to dramatically reduce vector expression. Bacterial regions larger than 1 kilobase have been shown to silence transgene expression in quiescent tissue such as the liver, likely due to untranscribed bacterial region mediated heterochromatin formation that spreads to the eukaryotic region and inactivates the promote (Suschak et al., 2017).

**[00146]** Evaluating *in vitro* expression levels of the vaccine antigen G-ORF1 in the five different plasmid backbones by Western blotting was done as follows: CHH-1 cells were trypsinated and seeded into 6-well plates (1 000 000 cells per well and 3 ml cell-medium). The cells were incubated at 15°C ON in a $CO_2$ incubator (5% $CO_2$). The cells were transfected the day after seeding using 2.5 µg plasmid/well and LIPOFECTAMIN® 3000 (Thermo Fisher Scientific) according manufacturer's instructions. Cells were put back in sealed bags and incubated at 15°C in a $CO_2$ incubator (5% $CO_2$) for five days before further analysis. Plates were then placed on ice and medium removed. Cells were first washed twice with ice-cold PBS. Total cell lysates were prepared by adding 150 µl NP40 lysis buffer (Invitrogen, #FNN021) with 1 x Protease inhibitor cocktail (Roche, #1697498) directly to the wells. Lysates were transferred to pre-cooled

Eppendorf tubes, incubated for 30 min at 4°C while re-suspending cells by flicking the tube occasionally, centrifuged at 12 000 rpm for 10 min at 4°C and the supernatant transferred to fresh tubes and kept on ice until immunoprecipitation. On ice, 1 µg of mouse IgG1anti 6x-His monoclonal antibody 4E3D10H2/E3 (Fisher Scientific #15442890, 1 mg/ml) was added to each sample and incubated for 4°C overnight on a rotator. Protein G-coupled beads were prepared (25 µl/sample) by washing the immobilized beads 3-5 x with ~1 ml cold PBST and re-suspended in 25µl of PBST with protease inhibitors per aliquot. On ice, 25 µl of pre-equilibrated beads were added to each sample and the sample-beads mixture incubated for 1 hour at RT with rotation. The beads were washed with 500 µl of ice cold PBST with proteinase inhibitors 3-5 times to remove non-specific binding with gentle mixing of the beads between each wash. After the last wash, as much buffer as possible was removed from the beads and 40 µl of 1x Laemmli Sample Buffer (BioRad #161-0737) added (prepared by mixing 20 µl 2x Laemmli Sample Buffer with 2 µl β-mercaptoethanol and 18 µl dH2O). Samples were incubated at 70°C for 10 minutes, quickly centrifuged and magnetized, and eluent moved to a new vial. Before loading on a 4-20% CRITERION™ TGX STAIN-FREE™ Gel, all samples were denatured at 98°C for 5 min. The gel was run for approximately 45 min at 200V and blotted using the TRANS-BLOT® TURBO™ Transfer System (BioRad), and TRANS-BLOT® TURBO™ Midi PVDF Transfer Packs #170-4157. 7 minutes turbo program was used. The membrane was immediately put in blocking-buffer (5% skimmed milk in PBST). Blocking was performed for 1 hour at room-temperature. The blot was incubated with primary antibody rabbit anti-delta PMCV ORF1 diluted 1: 500 in 1% skimmed milk/PBST over-night at 4°C in rotator. The following day the blots were washed 2 x 15 minutes with PBST and incubated with secondary antibody polyclonal swine anti-rabbit (DAKO #P0217) immunoglobulin HRP diluted 1:1000 in 2% skimmed milk and PRECISION PROTEIN™ StrepTactin-HRP Conjugate 1:10000 (to visualize ladder) for 1 hour at RT. The blot was washed 2 x 15 minutes with PBST before detection of signal by Clarity western ECL substrate (BioRad #170-5060).

**[00147]** For all vaccines in the present study the full-length PMCV ORF1 capsid gene routed for expression at the cell membrane (G-ORF1), was inserted downstream of a CMV promoter in different eukaryotic expression vectors according to the table below. All plasmids were diluted in sterile phosphate-buffered saline (PBS) to 10 µg per 50 µl injection volume. All fish received

one intramuscular injection on each side of the fish and a total dose of 20 µg. A non-immunized control group was included and received 2 x 50 µl PBS, as summarized in table 13.

**Table 13**

| Vaccine group | Description | Description plasmid encoding antigen | Plasmid backbone | Final conc construct (µg/ml) | Dose µg (2 x 0.05 ml) |
|---|---|---|---|---|---|
| 1 | G-ORF1-pcDNA3.1 | G-PMCV_ORF1_full_1-2583 | pcDNA3.1(+) | 200 | 20 |
| 2 | G-ORF1-pVAX1 | G-PMCV_ORF1_full_1-2583 | pVAX1 | 200 | 20 |
| 3 | G-ORF1-NTC9385R | G-PMCV_ORF1_full_1-2583 | NTC9385R | 200 | 20 |
| 4 | G-ORF1-NTC9385R-eRNA41H-CpG | G-PMCV_ORF1_full_1-2583 | NTC9385R-eRNA41H-CpG | 200 | 20 |
| 5 | G-ORF1-NTC9385R-eRNA41H | G-PMCV_ORF1_full_1-2583 | NTC9385R-eRNA41H | 200 | 20 |
| 6 | PBS | | NA | | |

**[00148]** Atlantic salmon (*Salmo salar*) (n=30 per group) kept in freshwater and with average weight of 29 grams were anaesthetized using Tricain (PHARMAQ), tagged by shortening of the adipose fin and/or maxillae, allocated to a group, and intramuscularly injected twice under the same anaesthetic period (one injection on each side of the fish) with 0.05 ml of the test vaccines. Immunity was allowed to develop for 59 days at 12C (light:dark 12:12) before the fish were anaesthetized again using Tricain (PHARMAQ) and challenged with infectious PMCV by intraperitoneal injection of 0.1 ml of homogenized heart (isolate ID AL V1273 Heart) originating from a Norwegian field outbreak of CMS. The fish (n=15 per group) were then sampled at two timepoints; 20 days and 48 days post challenge. Heart ventricle and kidney were collected on RNALATER® from all sampled fish for subsequent RNA extraction. Hearts (ventricle and atrium) were also fixed in formalin from all fish on the last sampling point (day 48) for histopathological analysis. RNA was extracted from all samples stored on RNALATER® and analyzed by Real-Time

RT-PCR for presence of PMCV RNA using a commercially available service from PHARMAQ Analytiq (Norway). Hearts stored on formalin were sectioned and histopathology was scored from 0 to 3 according to severity, where 0 indicates no pathologic finding and 3 indicate presence of severe pathology (service provided by PHARMAQ Analytiq, Norway).

**[00149]** Western blot analysis of transfected CHH-1 cells demonstrated that all three NTC9385R plasmid variants mediated superior antigen expression compared to the pcDNA3.1 and pVAX1 plasmid backbone (Figure 5). Clinical trials showed at least as good protection for NTC9385R-based vaccines as those based on pcDNA3.1/pVAX1 (See Table 14 and Table 15 for three and seven weeks, respectively). The combined dataset showed a trend towards superior protection by NTC9385R-based vaccine backbones. The NTC9385R plasmids with immunostimulatory elements did not provide additional effect.

**Table 14**. Viral RNA in kidney 3 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine group | Description | Mean Ct-value, PMCV | Standard deviation, Ct | n | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 6 | PBS | 21,73 | 1,25 | 15 | NA |
| 1 | G-ORF1-pcDNA3.1 | 22,66 | 1,20 | 15 | 0,24 |
| 2 | G-ORF1-pVAX1 | 22,57 | 1,51 | 15 | 0,35 |
| 3 | G-ORF1-NTC9385R | 23,78 | 1,71 | 15 | 0,0008 |
| 4 | G-ORF1-NTC9385R-eRNA41H-CpG | 23,52 | 1,42 | 15 | 0,0034 |
| 5 | G-ORF1-NTC9385R-eRNA41H | 23,03 | 1,28 | 15 | 0,053 |

**Table 15**. Viral RNA in kidney 7 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine group | Description | Mean Ct-value, PMCV | Standard deviation, Ct | n | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 6 | PBS | 22,45 | 1,53 | 15 | NA |
| 1 | G-ORF1-pcDNA3.1 | 23,75 | 1,73 | 15 | 0,26 |
| 2 | G-ORF1-pVAX1 | 23,77 | 2,01 | 15 | 0,23 |
| 3 | G-ORF1-NTC9385R | 23,94 | 2,42 | 15 | 0,14 |
| 4 | G-ORF1-NTC9385R-eRNA41H-CpG | 25,63 | 1,77 | 15 | 0,0001 |
| 5 | G-ORF1-NTC9385R-eRNA41H | 24,44 | 2,02 | 15 | 0,031 |

**EXAMPLE 4: Adjuvant selection**

**[00150]** Molecular adjuvants show great promise for both increasing immunogenicity and extending the longevity of the immune response, and these molecular adjuvants expressing cytokines, chemokines, or co-stimulatory molecules may be co-administered with the DNA vaccine plasmid encoding the antigen. Cells transfected by molecular adjuvant plasmids secrete the adjuvant into the surrounding region, stimulating local antigen presenting cells (Suschak et al., 2017). Adjuvant activity of fish type I interferons have already been shown in a virus DNA vaccination model for infectious salmon anemia virus (ISAV) in Atlantic salmon (Chang et al., 2015). In the paper, it was demonstrated that Type I IFNs enhanced the antibody response against ISAV-hemagglutinin protein and provided increased protection against viral challenge.

**[00151]** The following molecular adjuvants: IFNa, IFNb, IFNc, IFNd, IFNγ, IL-2, IL-4, IL-12 and VHSV-G were tested *in vivo* together with the full-length PMCV ORF1 vaccine antigen (G-ORF1) for adjuvant activity using the high-throughput model for rapid efficacy screening of candidates.

**[00152]** This model is based on a surprising discovery that that differences in histopathological lesions 50 days post challenge could be predicted by viral RNA levels in heart and kidney in the same groups 20 days post challenge. Therefore, for rapid screening of many groups, only one sampling point 3 weeks post challenge needs to be used.

**[00153]** For all groups in the *in vivo* screening study of adjuvants, the vaccine antigen and plasmid encoded adjuvants were administered on separate plasmids. The gene encoding the antigen (G-ORF1) and various plasmid encoded adjuvant genes were all inserted downstream of a CMV promoter in the eukaryotic expression vector pcDNA3.1(+) (Invitrogen). Plasmids were diluted in sterile phosphate-buffered saline (PBS) to 10 µg per 50 µl injection volume according to the table below. All fish received one intramuscular injection on each side of the fish and a total dose of 20 µg plasmid (20 µg of each plasmid if given antigen plus adjuvant). Two non-immunized control groups were included, one group received 20 µg of a control vaccine (eGFP in pcDNA3.1) and one group received 2 x 50 µl PBS, as summarized in Table 16.

**[00154]** Atlantic salmon (*Salmo salar*) (n=15 per group) kept in freshwater and with average weight of 31 grams were anaesthetized using Tricain (PHARMAQ), tagged by shortening of the adipose fin and/or maxillae, allocated to a group, and intramuscularly injected twice under the same anaesthetic period (one injection on each side of the fish) with 0.05 ml of the test vaccines. Immunity was allowed to develop for 50 days at 12C (light:dark 12:12) before the fish were anaesthetized again using Tricain (PHARMAQ) and challenged with infectious PMCV by intraperitoneal injection of 0.1 ml of homogenized heart (isolate ID ALV1223 Heart) originating from a Norwegian field outbreak of CMS. The fish (n=15 per group) were then sampled 21 days post challenge. Heart ventricle and kidney was collected on RNALATER® from all sampled fish for subsequent RNA extraction. RNA was extracted from all samples stored on RNALATER® and analyzed by Real-Time RT-PCR for presence of PMCV RNA using a commercially available service from PHARMAQ Analytiq (Norway).

**[00155]** Following 7 weeks of immunization and 3 weeks of challenge, the model found that while most of the adjuvant strategies failed in improving efficacy, one adjuvant – IFNb significantly increased the level of effect. The results are listed in Table 17.

**Table 16**

| group | Description (Antigen) | Detailed description construct A (Antigen) | Description (Adjuvant) | Detailed description construct B (Adjuvant) | Final conc construct A (µg/ml) | Final conc construct B (µg/ml) | Dose µg (2 x 0.05 ml) |
|---|---|---|---|---|---|---|---|
| 1 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | None | | 200 | | 20 |
| 2 | eGFP | enhanced Green Fluorescent Protein (control) | None | | 200 | | 20 |
| 3 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | IFNa1 | IFNa1 adjuvant construct | 200 | 200 | 20 each |
| 4 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | IFNb | IFNb adjuvant construct | 200 | 200 | 20 each |
| 5 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | IFNc | IFNc adjuvant construct | 200 | 200 | 20 each |
| 6 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | IFNd | IFNd adjuvant construct | 200 | 200 | 20 each |
| 7 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | Interleukin 2 | IL-2 adjuvant construct | 200 | 200 | 20 each |
| 8 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | Interleukin 4 | IL-4 adjuvant construct | 200 | 200 | 20 each |
| 9 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | Interleukin 12 | IL-12 adjuvant construct | 200 | 200 | 20 each |
| 10 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | IFNg | IFN gamma adjuvant construct | 200 | 200 | 20 each |
| 11 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | VHSV-G | VHSV-g protein DNA vaccine | 200 | 200 | 20 each |
| 12 | G-ORF1 | G-PMCV_ORF1_full_1-2583 | rORF1 (PMCV) | Rec.protein PMCV ORF1 (E.coli)* | 200 | NA | 20 |
| 13 | PBS | PBS control | | | | | |

**Table 17**. Viral RNA in kidney 3 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the G-ORF1 control group (no adjuvant) using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine group | Antigen construct | Adjuvant | Mean Ct-value, PMCV | Standard deviation, Ct | n | p-value (compared to G-ORF1 alone – group 1) |
|---|---|---|---|---|---|---|
| 13 | PBS | None | 19,49 | 0,96 | 14 | 0,0002 |
| 1 | G-ORF1 | None | 23,12 | 2,60 | 31 | NA |
| 2 | eGFP | None | 22,04 | 1,51 | 16 | 0,73 |
| 3 | G-ORF1 | IFNa1 | 23,17 | 3,29 | 15 | 0,99 |
| 4 | G-ORF1 | IFNb | 26,06 | 3,05 | 15 | 0,0033 |
| 5 | G-ORF1 | IFNc | 23,53 | 3,27 | 14 | 0,99 |
| 6 | G-ORF1 | IFNd | 21,82 | 2,50 | 16 | 0,52 |
| 10 | G-ORF1 | IFNg | 21,09 | 1,69 | 14 | 0,11 |
| 7 | G-ORF1 | Interleukin 2 | 20,70 | 3,20 | 13 | 0,04 |
| 8 | G-ORF1 | Interleukin 4 | 21,72 | 2,01 | 15 | 0,45 |
| 9 | G-ORF1 | Interleukin 12 | 23,03 | 3,24 | 16 | 0,99 |
| 11 | G-ORF1 | VHSV-G | 20,25 | 2,52 | 15 | 0,004 |
| 12 | G-ORF1 | rORF1 (PMCV) | 19,42 | 0,74 | 10 | 0,001 |

**[00156]** IFNb and IFNc were selected for a follow up fish trial. For all groups in this trial, the vaccine antigen and plasmid encoded adjuvants were administered on separate plasmids. The gene encoding the antigen (G-ORF1) and the plasmid encoded adjuvant genes (IFNb or IFNc) were all inserted downstream of a CMV promoter in the eukaryotic expression vector pcDNA3.1(+) (Invitrogen). Plasmids were diluted in sterile phosphate-buffered saline (PBS) to 10 µg per 50 µl injection volume according to the table below. All fish received one intramuscular injection on each side of the fish and a total dose of 20 µg plasmid (20 µg of each plasmid if given antigen plus adjuvant). A non-immunized control group was included and received 2 x 50µl PBS, as summarized in Table 18.

**Table 18**

| Group | Description (Antigen) | Description (Adjuvant) | Detailed description construct A (Antigen) | Detailed description construct B (Adjuvant) | Final conc construct A (µg/ml) | Final conc construct B (µg/ml) | Dose µg (2 x 0.05 ml) |
|---|---|---|---|---|---|---|---|
| 1 | G-ORF1 | None | G-PMCV_ORF1_full_1-2583 | | 200 | | 20 |
| 2 | G-ORF1 | IFNb | G-PMCV_ORF1_full_1-2583 | IFNb adjuvant construct | 200 | 200 | 20 each |
| 3 | None | IFNb | | IFNb adjuvant construct | | 200 | 20 |
| 4 | G-ORF1 | IFNc | G-PMCV_ORF1_full_1-2583 | IFNc adjuvant construct | 200 | 200 | 20 each |
| 5 | None | IFNc | | IFNc adjuvant construct | | 200 | 20 |
| 6 | PBS | | PBS control | | | | |

**[00157]**Atlantic salmon (Salmo salar) (n=30 per group) kept in freshwater and with average weight of 29 grams were anaesthetized using Tricain (PHARMAQ), tagged by shortening of the adipose fin and/or maxillae, allocated to a group, and intramuscularly injected twice under the same anaesthetic period (one injection on each side of the fish) with 0.05 ml of the test vaccines. Immunity was allowed to develop for 59 days at 12C (light:dark 12:12) before the fish were anaesthetized again using Tricain (PHARMAQ) and challenged with infectious PMCV by intraperitoneal injection of 0.1 ml of homogenized heart (isolate ID ALV1273 Heart) originating from a Norwegian field outbreak of CMS. The fish (n=15 per group) were then sampled at two timepoints; 20 days and 48 days post challenge. Heart ventricle and kidney were collected on RNALATER® from all sampled fish for subsequent RNA extraction. Hearts (ventricle and atrium) were also fixed in formalin from all fish on the last sampling point (day 48) for histopathological analysis. RNA was extracted from all samples stored on RNALATER® and analyzed by Real-Time RT-PCR for presence of PMCV RNA using a commercially available service from PHARMAQ Analytiq (Norway). Hearts stored on formalin were sectioned and histopathology was scored

from 0 to 3 according to severity, where 0 indicates no pathologic finding and 3 indicate presence of severe pathology (service provided by PHARMAQ Analytiq, Norway).

**[00158]** The results showed that IFNb was superior to IFNc (Table 19). The difference between the two adjuvants was most profound at the 7 weeks post challenge sampling point (data from earlier time point not shown). The group receiving adjuvant only was also partly protected reflecting that IFNs are critical effectors of both innate and adaptive immune responses.

**Table 19.** Viral RNA in heart 7 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine group | Antigen | Adjuvant | Mean Ct-value, PMCV | Standard deviation, Ct | n | p-value (compared to PBS) |
|---|---|---|---|---|---|---|
| 6 | PBS | PBS | 23,17 | 2,40 | 15 | NA |
| 1 | G-ORF1 | PBS | 25,64 | 2,61 | 15 | 0,40 |
| 2 | G-ORF1 | IFNb | 30,77 | 5,61 | 15 | 0,0001 |
| 3 | None | IFNb | 30,38 | 4,40 | 15 | 0,0002 |
| 4 | G-ORF1 | IFNc | 25,16 | 4,79 | 15 | 0,64 |
| 5 | None | IFNc | 22,48 | 2,15 | 15 | 0,99 |

**EXAMPLE 5 – Adjuvant delivery**

**[00159]** The aim of this experiment was to determine the best way to deliver the antigen and the molecular adjuvant.

**[00160]** Plasmid-encoded adjuvants can be delivered either on a separate plasmid or included on the same plasmid as the one encoding the antigen. If expressed from the same plasmid as the antigen, this can e.g. be done in the following ways:

1. From a separate but identical promoter as the vaccine antigen.
2. From a separate similar (but not identical) promoter as the vaccine antigen.

3. As a fusion protein with the antigen – separated by a P2A peptide sequence to facilitate self-cleavage.

**[00161]** In this example, different delivery options for the adjuvant were tested. The cell surface expressed PMCV ORF1 antigen (G-ORF1) was given to all groups. The adjuvant was provided in the following ways:

1. On a separate plasmid (G-ORF1+IFNb)
2. Expressed together with the antigen, downstream of the same CMV promoter and separated from the antigen by a self-cleavage 2A peptide (G-ORF1-P2A-IFNb)
3. Expressed on the same plasmid as the antigen but downstream of a second CMV promoter (G-ORF1-CMV-IFNb),
4. One group did not receive the adjuvant only the antigen (G-ORF1).
5. A non-vaccinated control group receiving PBS was also included.

**[00162]** For all vaccines in the present study the genes were inserted downstream of a CMV promoter in the eukaryotic expression vector pVAX1 (Invitrogen). The antigen used was G-ORF1 (G-PMCV_ORF1_full_1-2583). All vaccines were diluted in sterile phosphate-buffered saline (PBS) to 10 µg per 50 µl injection volume. All fish received one intramuscular injection on each side of the fish and a total dose of 20 µg. For fish receiving the antigen and the plasmid encoded adjuvant on separate plasmids, the dose for each plasmid was 20 µg. A non-immunized control group was included and received 2 x 50 µl PBS. In addition, one group received a codon-changed version of the plasmid encoded adjuvant, as summarized in Table 20.

**Table 20**.

| Group | Construct 1 | Construct 2 | Adjuvant administration | Dose µg (2 x 0.05 ml) |
|---|---|---|---|---|
| 1 | G-ORF1 | IFNb codon changed | IFNb adjuvant codon changed provided on separate plasmid | 20 each |
| 2 | G-ORF1 | IFNb | IFNb adjuvant provided on separate plasmid | 20 each |
| 3 | G-ORF1-P2A-IFNb | | IFNb provided on same plasmid and under same promoter as antigen | 20 |
| 4 | G-ORF1-CMV-IFNb | | IFNb provided on same plasmid but downstream of a second CMV promoter | 20 |
| 5 | G-ORF1 | | No adjuvant control group | 20 |
| 6 | PBS | | | |

**[00163]**Atlantic salmon (*Salmo salar*) (n=30 per group) kept in freshwater and with average weight of 25 grams were anaesthetized using Tricain (PHARMAQ), tagged by shortening of the adipose fin and/or maxillae, allocated to a group, and intramuscularly injected twice under the same anaesthetic period (one injection on each side of the fish) with 0.05 ml of the test vaccines. Immunity was allowed to develop for 49 days at 12C (light:dark 12:12) before the fish were anaesthetized again using Tricain (PHARMAQ) and challenged with infectious PMCV by intraperitoneal injection of 0.1 ml of homogenized heart (isolate ID ALV1273 Heart) originating from a Norwegian field outbreak of CMS. The fish (n=15 per group) were then sampled at two timepoints; 19 days and 47 days post challenge. Heart ventricle and kidney were collected on RNALATER® from all sampled fish for subsequent RNA extraction. Hearts (ventricle and atrium) were also fixed in formalin from all fish on the last sampling point (day 47) for histopathological analysis. RNA was extracted from all samples stored on RNALATER® and analyzed by Real-Time RT-PCR for presence of PMCV RNA using a commercially available service from PHARMAQ Analytiq (Norway). Hearts stored on formalin were sectioned and histopathology was scored from 0 to 3 according to severity, where 0 indicates no pathologic finding and 3 indicate presence of severe pathology (service provided by PHARMAQ Analytiq, Norway).

**[00164]**The results from the clinical trial are exemplified by histoscore data from heart at 7 weeks post challenge (Table 21).

**Table 21.** Histopathological lesions (histoscore) in atrium 7 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine-group | Description | Mean histoscore, Atrium | Standard deviation, histoscore | N | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 6 | PBS | 2,46 | 0,54 | 15 | NA |
| 5 | G-ORF1 | 1,73 | 1,00 | 15 | 0,20 |
| 2 | G-ORF1+IFNb | 1,00 | 0,90 | 15 | 0,0014 |
| 4 | G-ORF1-CMV-IFNb | 1,13 | 1,03 | 15 | 0,0035 |
| 3 | G-ORF1-P2A-IFNb | 1,73 | 1,22 | 15 | 0,20 |
| 1 | G-ORF1-CMV-IFNb_codon changed | 1,04 | 0,96 | 15 | 0,003 |

**[00165]** Antigen expression levels and adjuvant activity for the different constructs have also been assessed *in vitro* (results not shown). The combined results suggest that the preferred approach is to provide the adjuvant on the same plasmid as the antigen under an identical but separate promoter as the antigen but delivery on different plasmids can also be effective.

**EXAMPLE 6 – Duration of vaccine efficiency**

**[00166]** The initial studies showed that fish receiving adjuvant only were partly protected, reflecting that IFNs are critical effectors of both innate and adaptive immune responses. To study the duration of protection obtained by including a molecular adjuvant in a DNA construct, a long-term follow-up vaccine trial was carried out.

**[00167]** For all vaccines in the present study the genes were inserted downstream of a CMV promoter in eukaryotic expression vectors from Nature Technology Corporation (NTC). The backbones were either NTC9385R or NTC9385R-eRNA41H-CpG. For the group receiving both an antigen and the plasmid encoded adjuvant, these were either provided on separate plasmids (20 µg each plasmid) or on the same plasmid (20 µg). All fish received one intramuscular injection on

each side of the fish (0.05 ml). A non-immunized control group was included and received 2 x 50 µl PBS. For details about vaccine groups, see Table 22 below.

**Table 22**

| Group | Construct 1 | Backbone construct 1 | Construct 2 | Backbone construct 2 | Adjuvant administration | Dose µg (2 x 0.05 ml) |
|---|---|---|---|---|---|---|
| 1 | G-ORF1 | NTC9385R | IFNb | NTC9385R | Antigen + adjuvant - on two separate plasmids | 20 each |
| 2 | G-ORF1 | NTC9385R | | | Antigen only | 20 |
| 3 | IFNb | NTC9385R | | | Adjuvant only | 20 |
| 4 | G-ORF1-CMV-IFNb | NTC9385R | | | Antigen + adjuvant on same plasmid | 20 |
| 5 | G-ORF1-CMV-IFNb | NTC9385R-eRNA41H-CpG | | | Antigen + adjuvant on same plasmid | 20 |
| 6 | PBS | | | | | |

**[00168]** Atlantic salmon (*Salmo salar*) (n=60 per group) kept in freshwater and with average weight of 17 grams were anaesthetized using Tricain (PHARMAQ), tagged by shortening of the adipose fin and/or maxillae, allocated to a group, and intramuscularly injected twice under the same anaesthetic period (one injection on each side of the fish) with 0.05 ml of the test vaccines. The fish were then kept in a reservoir tank where immunity was allowed to develop for 78 days, 120 days or 183 days at 12C (light:dark 12:12). At each of these time-points cohorts of fish (n=15 per group on time-points 78 dpv and 183 dpv, and n=30 per group on time-point 120 dpv) were moved to a challenge tank. They were anaesthetized again using Tricain (PHARMAQ) and challenged with infectious PMCV by intraperitoneal injection of 0.1 ml of homogenized heart (isolate ID ALV1273 Heart) originating from a Norwegian field outbreak of CMS. The fish (n=15 per group) were then sampled 3 weeks post challenge. Heart ventricle and kidney were collected on RNALATER® from all sampled fish for subsequent RNA extraction. For the cohort that was challenged 120 days post vaccination, an additional sampling was done 7 weeks post challenge. In addition to heart and kidney for RNALATER®, hearts (ventricle and atrium) were also fixed in formalin from all fish on this last sampling point (week 7) for histopathological analysis. RNA was extracted from all samples stored on RNALATER® and analyzed by Real-Time RT-PCR for presence

of PMCV RNA using a commercially available service from PHARMAQ Analytiq (Norway). Hearts stored on formalin were sectioned and histopathology was scored from 0 to 3 according to severity, where 0 indicates no pathologic finding and 3 indicate presence of severe pathology (service provided by PHARMAQ Analytiq, Norway).

**[00169]**The efficacy of the vaccines was evaluated by challenge after up to 26 weeks of immunization (challenge at 12, 18- and 26-weeks post immunization). The challenge at week 18 was followed for 50 days with two sampling points, at 20- and 50-days post challenge, where heart and kidney tissues were sampled for analysis by qPCR and evaluation of histopathology in heart as described above.

**[00170]**The results showed that significant protection against PMCV infection was achieved with all vaccines until the last challenge, 183 days / 2196 degree days post vaccination. The combination of G-ORF1 and IFNb provided better protection than the two components separately (Tables 23, 24). The overall results show that the vaccine provide long and durable protection.

**Table 23**. Tank 1. Viral RNA in heart 3 weeks post challenge with three different durations of immunization before challenge. Mean Ct-value for PMCV with standard deviation is indicated for each group. N=15 per group, per sampling-point.

| Vaccine-group | Description | 12 weeks immunization | 18 weeks immunization | 26 weeks immunization |
|---|---|---|---|---|
| 6 | PBS | 27,42 ± 2,07 | 29,33 ± 1,62 | 29,33 ± 1,74 |
| 2 | G-ORF1 | 28,70 ± 2,19 | 30,50 ± 2,38 | 29,62 ± 3,59 |
| 3 | IFNb | 33,82 ± 3,48 | 32,65 ± 3,68 | 28,35 ± 6,22 |
| 1 | G-ORF1+IFNb | 35,88 ± 3,25 | 33,25 ± 3,36 | 32,21 ± 1,95 |

**Table 24**. Tank 2. Viral RNA in heart 3 weeks post challenge with three different durations of immunization before challenge. Mean Ct-value for PMCV with standard deviation is indicated for each group. N=15 per group, per sampling-point.

| Vaccine-group | Description | 12 weeks immunization | 18 weeks immunization | 26 weeks immunization |
|---|---|---|---|---|
| 6 | PBS | 27,68 ± 2,32 | 27,58 ± 2,04 | 28,80 ± 1,90 |
| 4 | G-ORF1-CMV-IFNb | 36,73 ± 4,69 | 33,75 ± 2,80 | 32,93 ± 3,31 |
| 5 | G-ORF1-CMV-IFNb | 33,58 ± 3,10 | 30,70 ± 2,14 | 32,63 ± 4,13 |

**Table 25**. Tank 1. Histopathological lesions (histoscore) in atrium 7 weeks post challenge after an immunization period of 18 weeks. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine-group | Description | Mean histoscore, Atrium | Standard deviation, histoscore | N | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 6 | PBS | 1,54 | 1,08 | 12 | NA |
| 2 | G-ORF1 | 0,60 | 0,74 | 15 | 0,01 |
| 3 | IFNb | 0,43 | 0,78 | 15 | 0,0025 |
| 1 | G-ORF1+IFNb | 0,43 | 0,65 | 14 | 0,0028 |

**Table 26**. Tank 2. Histopathological lesions (histoscore) in atrium 7 weeks post challenge after an immunization period of 18 weeks. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnet's post test. NA=Not applicable.

| Vaccine-group | Description | Mean histoscore, Atrium | Standard deviation, histoscore | N | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 6 | PBS | 1,68 | 0,75 | 11 | NA |
| 4 | G-ORF1-CMV-IFNb | 0,07 | 0,18 | 15 | <0,0001 |
| 5 | G-ORF1-CMV-IFNb | 0,50 | 0,76 | 8 | 0,0002 |

**EXAMPLE 7 – Optimization of adjuvant efficiency**

**[00171]**In an attempt to further optimize the adjuvant potential of IFNb, the inventors identified homologous genes in Atlantic salmon and closely related species. As salmonids have experienced an evolutionary recent whole genome duplication, many salmon genes are found in duplicates (diverged homologues). Three new genes, similar but not identical to the initially tested IFNb-adjuvant were selected (Table 27). These were:

-Atlantic salmon (Salmo salar) - IFNb1 (diverged paralog gene)

-Brown trout (Salmo trutta)-IFN a3-like gene

-Sockeye salmon (Onchorhynchus nerka) – IFNa3-like gene.

**Table 27**

| Gene | GenBank accession # | | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|
| IFNb Salmo salar (SEQ ID NO: 6) | | 1 | | *6* | *5* | *11* |
| IFNb1 *Salmo salar* | EU768890.1 | 2 | 96.74 | | *9* | *13* |
| IFNa3 like *Salmo trutta* | XM_029727364.1 | 3 | 97.28 | 95.11 | | *11* |
| IFNa3 like *Onchorhynchus nerka* | XM_029635101.1 | 4 | 94.02 | 92.93 | 94.02 | |

Pairwise comparison of alterative IFNb adjuvants showed as amino-acid identity (%) and number of different amino acids (italicized)

**[00172]**As vaccine integration into the salmon genome through homologous recombination might be a concern, risk mitigation can be obtained by reducing the sequence similarity between the adjuvant and the salmon genome. This was done by first translating the plasmid encoded adjuvants to protein sequences. Thereafter these sequences were reverse translated from proteins back into nucleotide sequences (back-translation) based on codon frequency distribution (codons assigned with a probability given by the frequency of use in Atlantic salmon). Reverse translation was done using CLC Main Workbench (Qiagen) with software settings to use codons based on frequency distribution.

**[00173]**The new adjuvant sequences returned after this procedure were between 74-80% identical to the wild type IFNb sequences at the nucleotide. The activities of the four original native adjuvant candidates, including the four codon-changed versions (referred to as RT) were then compared using an *in vitro* assay.

**[00174]** Thus, the wild type nucleic acid sequence for IFNb (SEQ ID NO: 18) was about 76% identical to the RT nucleic acid sequence for IFNb (SEQ ID NO: 17), and the wild type nucleic acid sequence for IFNb1 (SEQ ID NO: 19) was about 78% identical to the RT nucleic acid sequence for IFNb1 (SEQ ID NO: 20)

**[00175]** To facilitate testing of the different IFNb candidates and assess their adjuvant potential, an *in vitro* screening assay was established. The principle of this test is to quantify Mx-expression (IFN-induced gene) by RT-qPCR. Mx proteins belong to the superfamily of large GTPases with antiviral activity against a wide range of RNA viruses. *In vivo*, the expression of Mx genes is tightly regulated by the presence of type I interferons (IFNs), and their induction has been described during several viral infections. As CHH-1 cells do not express Mx in response to IFNb stimulation (the potential result of lacking receptors) and TO cells not easily can be transfected, the assay has been set up in the following way:

**[00176]** Functionality/activity of IFNb genes was studied by transfecting CHH-1 cells with the various adjuvant constructs with non-adjuvanted constructs included as negative controls. Seven days post transfection, cell culture medium containing IFN molecules secreted from the CHH-1 cell transfectants was collected and transferred to another fish cell-line (TO cells) in different dilutions. The final ratio of conditioned medium used for incubation of TO cells was typically 1:6; 1:60, 1: 600 and 1:6000, allowing the *in vitro* model also to provide quantitative measures of adjuvant activity. Following three days of incubation with conditioned medium, TO cells were sampled by carefully removing the cell culture medium and extracting RNA from the cells. The Mx response induced by IFNb was analyzed by qPCR using the following primers/probe:

SEQ ID NO: 42 Mx Fwd: GATGCTGCACCTCAAGTCCTATTA

SEQ ID NO: 43 Mx Rev: CGGATCACCATGGGAATCTGA

SEQ ID NO: 44 Mx Probe: 6-FAM-CAGGATATCCAGTCAACGTT-MGB

**[00177]** The results are provided in Table 28 below:

**Table 28**

| Construct | Ratio CHH-1 conditioned medium transferred to TO-cells | | |
|---|---|---|---|
| | 1:6 | 1:60 | 1:600 |
| *Salmo Salar* IFNb wild type (SEQ ID NO: 18 | 21.61 | 24.29 | 34.44 |
| *Salmo Salar* IFNb RT (SEQ ID NO: 17) | 20,86 | 22,33 | 26,39 |
| *Salmo Salar* IFNb1 wild type (SEQ ID NO: 19) | 20.94 | 21.77 | 23.66 |
| *Salmo Salar* IFNb1 RT (SEQ ID NO: 20) | 20.57 | 21.05 | 21.84 |
| *Salmo trutta* IFNa3 like wild type | 20.66 | 22.17 | 27.04 |
| *Salmo trutta* IFNa3 like RT | 21.49 | 21.96 | 26.82 |
| *Onchorhynchus nerka* INFa3 wild type | 20.99 | 24.04 | 31.26 |
| *Onchorhynchus nerka* INFa3 RT | 21.28 | 22.87 | 28.13 |
| eGFP | No Ct | No Ct | No Ct |
| Non template control | No Ct | | |

**Real-time Ct values for Mx in TO cells incubated with different concentrations of conditioned medium from transfected CHH-1 cells. Lower Ct values indicate higher levels of IFN induction.**

**[00178]** These results demonstrate that RT constructs have slightly higher immunomodulatory activity compared to the wild type sequences. The results also clearly show that IFNb1 possesses higher immunomodulatory activity than IFNb.

**[00179]** Based on the *in vitro* studies, the four RT INFb adjuvants were selected for testing as vaccine adjuvants in fish (all groups receiving the PMCV capsid antigen (G-ORF1) and adjuvant on two separate plasmids). In addition, one group received G-ORF1 vaccine antigen plus an eGFP encoding plasmid as negative adjuvant control group. All fish received one intramuscular injection on each side of the fish. All fish received the antigen and the plasmid encoded adjuvant on separate plasmids, the dose for each plasmid was 20 µg. A non-immunized control group was included and received 2 x 50 µl PBS, as summarized in Table 29.

**Table 29**

| Group | Description (Antigen) | Detailed description Antigen | Antigen backbone | Description (Adjuvant) | Adjvuant backbone | Final conc A µg/ml | Final conc B (µg/ml) | Dose µg (2 x 0.05 ml) |
|---|---|---|---|---|---|---|---|---|
| 1 | G-ORF1 | G-PMCV_ORF 1_full_1-2583 | NTC9385R | IFNb *S.salar* | pVAX1 | 200 | 200 | 20 each |
| 2 | G-ORF1 | G-PMCV_ORF 1_full_1-2583 | NTC9385R | IFNb1_*S.salar* | pVAX1 | 200 | 200 | 20 each |
| 3 | G-ORF1 | G-PMCV_ORF 1_full_1-2583 | NTC9385R | IFNb_*S. trutta* IFNa3-like | pVAX1 | 200 | 200 | 20 each |
| 4 | G-ORF1 | G-PMCV_ORF 1_full_1-2583 | NTC9385R | IFNb_*O. nerka* INFa3-like | pVAX1 | 200 | 200 | 20 each |
| 5 | G-ORF1 | G-PMCV_ORF 1_full_1-2583 | NTC9385R | eGFP | pcDNA3.1(+) | 200 | 200 | 20 each |
| 6 | PBS | PBS control | NA | | | | | |

**[00180]** Atlantic salmon (*Salmo salar*) (n=30 per group) kept in freshwater and with average weight of 20 grams were anaesthetized using Tricain (PHARMAQ), tagged by shortening of the adipose fin and/or maxillae, allocated to a group, and intramuscularly injected twice under the same anaesthetic period (one injection on each side of the fish) with 0.05 ml of the test vaccines. Immunity was allowed to develop for 48 days at 12C (light:dark 12:12) before the fish were anaesthetized again using Tricain (PHARMAQ) and challenged with infectious PMCV by intraperitoneal injection of 0.1 ml of homogenized heart (isolate ID ALV1273 Heart) originating from a Norwegian field outbreak of CMS. The fish (n=15 per group) were then sampled at two timepoints; 19 days and 49 days post challenge. Heart ventricle and kidney were collected on RNALATER® from all sampled fish for subsequent RNA extraction. Hearts (ventricle and atrium) were also fixed in formalin from all fish on the last sampling point (day 49) for histopathological analysis. RNA was extracted from all samples stored on RNALATER® and analyzed by Real-Time RT-PCR for presence of PMCV RNA using a commercially available service from PHARMAQ

Analytiq (Norway). Hearts stored on formalin were sectioned and histopathology was scored from 0 to 3 according to severity, where 0 indicates no pathologic finding and 3 indicate presence of severe pathology (service provided by PHARMAQ Analytiq, Norway).

**[00181]** Results are shown in tables 30 and 31 below.

**Table 30.** Tank 1. Viral RNA in kidney 3 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine group | Description | Mean Ct-value, PMCV | Standard deviation, Ct | n | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 6 | PBS | 19,89 | 0,79 | 15 | NA |
| 1 | G-ORF1 + IFNb *S.salar* | 25,42 | 1,51 | 15 | <0,0001 |
| 2 | G-ORF1 + IFNb1 *S.salar* | 27,66 | 1,31 | 15 | <0,0001 |
| 3 | G-ORF1 + IFNb a3 *S. trutta* | 27,13 | 1,09 | 15 | <0,0001 |
| 4 | G-ORF1 + IFNb a3 *O. nerka* | 26,74 | 1,25 | 15 | <0,0001 |
| 5 | G-ORF1 + EGFP | 23,23 | 2,10 | 15 | 0,028 |

**Table 31.** Tank 2. Viral RNA in kidney 3 weeks post challenge. The indicated p-value was calculated by comparing individual groups with the PBS control group using a One-way ANOVA, with Dunnett's post test. NA=Not applicable.

| Vaccine group | Description | Mean Ct-value, PMCV | Standard deviation, Ct | n | p-value (compared to PBS) |
|---|---|---|---|---|---|
| 6 | PBS | 20,73 | 1,44 | 15 | NA |
| 1 | G-ORF1 + IFNb *S. salar* | 26,55 | 0,94 | 15 | <0,0001 |
| 2 | G-ORF1 + IFNb1 *S. salar* | 26,27 | 0,99 | 15 | <0,0001 |
| 3 | G-ORF1 + IFNb a3 *S. trutta* | 26,72 | 1,15 | 15 | <0,0001 |
| 4 | G-ORF1 + IFNb a3 *O. nerka* | 26,43 | 1,09 | 11 | <0,0001 |
| 5 | G-ORF1 + EGFP | 21,63 | 1,76 | 15 | 0,20 |

**[00182]**The overall conclusion is that all tested RT adjuvants tested provide similar protection, although the protection elicited using IFNb1 RT construct was slightly higher than then protection elicited by IFNb RT construct. Codon-changed versions of these adjuvants are preferred to reduce risk of homologous recombination.

**[00183]**The best way to deliver the adjuvant is on the same plasmid as the antigen while using a separate but identical promoter as the antigen but delivery of the antigen and the adjuvant using different plasmids is also effective.

**[00184]**All publications cited in the specification, both patent publications and non-patent publications, are indicative of the level of skill of those skilled in the art to which this invention pertains. All these publications are herein fully incorporated by reference to the same extent as if each individual publication were specifically and individually indicated as being incorporated by reference.

**[00185]**Although the invention herein has been described with reference to particular embodiments, it is to be understood that these embodiments are merely illustrative of the principles and applications of the present invention. It is therefore to be understood that numerous modifications may be made to the illustrative embodiments and that other arrangements may be devised without departing from the spirit and scope of the present invention as defined by the following claims.

**CLAIMS**

1. A nucleic acid sequence that encodes SEQ ID NO: 6 or 50 or an amino acid sequence that is at least 95% identical to SEQ ID NO: 6 or SEQ ID NO: 50 wherein said nucleic acid sequence is 65-90% identical to SEQ ID NO: 18 or 65-90% identical to SEQ ID NO: 19, and wherein codon frequency is preserved in said nucleic acid sequence.

2. The nucleic acid sequence according to claim 1, wherein said nucleic acid sequence is 70-79% identical to SEQ ID NO: 18 or SEQ ID NO: 19.

3. The nucleic acid sequence according to claim 1 or claim 2, wherein said amino acid sequence is at least 98% identical to SEQ ID NO: 6 or SEQ ID NO: 50.

4. The nucleic acid sequence according to any one of claims 1-3, wherein at least 50% of amino acids differing from SEQ ID NO: 6 or SEQ ID NO: 50 are conservative substitutions.

5. The nucleic acid sequence according to claim 1 that is at least 90% identical to SEQ ID NO: 20 or SEQ ID NO: 17.

6. The nucleic acid sequence according to claim 5, that is at least 95% identical to SEQ ID NO: 20 or SEQ ID NO: 17.

7. An expression cassette comprising the nucleic acid sequence according to any one of claims 1-6 under operative control of a second promoter.

8. The expression cassette of claim 7, wherein said second promoter is cytomegalovirus (CMV) immediate-early enhancer and promoter.

9. The expression cassette of claim 7 or 8 further comprising a nucleic acid sequence encoding an antigen.

10. The expression cassette of claim 9, wherein the antigen comprises a structural protein of a non-enveloped virus or a portion of said structural protein, said portion capable of eliciting protective immune response.

11. The expression cassette of claim 10, wherein said antigen is expressed as a fusion protein, comprising:

a)N-terminal secretion signal sequence of a secreted or a first membrane-bound protein upstream of the amino acid sequence of said antigen;

b) a transmembrane domain of a second membrane-bound protein downstream of the amino acid sequence of said antigen;

12. The expression cassette of claim 11, wherein said first and second proteins are independently selected from the group consisting of Atlantic salmon paramyxovirus fusion protein, Hemagglutinin (HE) protein of Infectious salmon anemia virus (ISAV) and viral hemorrhagic septicemia virus G-protein (VHSV-G).

13. The expression cassette of claim 11 or 12 wherein the first membrane-bound protein is identical to the second membrane-bound protein.

14. The expression cassette of claim 13, wherein said membrane bound protein is viral hemorrhagic septicemia virus G-protein (VHSV-G).

15. The expression cassette of claim 14, wherein said N-terminal secretion signal sequence is at least 90% identical to SEQ ID NO: 7 or 48, or wherein said secretion signal sequence comprises a portion of SEQ ID NO: 48 or a sequence at least 90% identical to SEQ ID NO: 48, wherein said portion comprises SEQ ID NO: 7 or a sequence that is at least 90% identical to SEQ ID NO: 7.

16. The expression cassette of claim 15 wherein at least half of differing amino acids in said N-terminal secretion signal are conservative substitutions.

17. The expression cassette of any one of claims 11-16, wherein said transmembrane domain is at least 90% identical to SEQ ID NO: 8 or 49, or wherein said transmembrane fragment comprises a portion of SEQ ID NO: 49 or a sequence at least 90% identical to SEQ ID NO: 49, wherein said portion comprises SEQ ID NO: 8 or a sequence that is at least 90% identical to SEQ ID NO: 8.

18. The expression cassette of claim 17 wherein at least half of differing amino acids in said Transmembrane domain are conservative substitutions.

19. The expression cassette of any one of claims 10-18, wherein said structural protein is a capsid protein.

20. The expression cassette of claim 10-18, wherein said non-enveloped virus is Piscine Myocarditis Virus (PMCV).

21. The expression cassette of claim 20, wherein said antigen comprises SEQ ID NO: 1 or an amino acid sequence that is 95% identical thereto.

22. The expression cassette of claim 20, wherein Piscine Myocarditis Virus (PMCV) ORF-1 antigen comprises, from N- to C- terminus:

a. a sequence that is at least 90% identical, or at least 91% identical, or at least 92% identical, or at least 93% identical, or at least 94% identical, or at least 95% identical, or at least 96% identical, or at least 97% identical, or at least 98% identical, or at least 99% identical, or 100% identical to SEQ ID NO: or SEQ ID NO: 35, and
b. an amino acid sequence that is at least 95%, 96%, 97%, 98%, 99%, or 100% identical to any one of SEQ ID NOs: 25-33,

wherein,

i) said Piscine Myocarditis Virus (PMCV) ORF-1 antigen lacks SEQ ID NO: 15 or a sequence at least 90% identical to SEQ ID NO: 15; and/or

ii) compared to SEQ ID NO: 1, said Piscine Myocarditis Virus (PMCV) ORF-1 antigen comprises an internal deletion that is at least four consecutive amino acids long.

23. The expression cassette of claim 21, wherein the antigen comprises SEQ ID NO: 15, or a sequence at least 90% identical thereto, and any one of SEQ ID NOs 4, 5, 25, 26 27, 29, 30, 32, or an amino acid sequence that is at least 90% identical to SEQ ID NO: 4, 5, 25, 26 27, 29, 30, or 32 wherein, compared to SEQ ID NO: 1, said protein comprises an internal deletion that is at least four consecutive amino acids long, and, optionally, wherein said protein comprises a linker in place of said internal deletion

24. The Piscine Myocarditis Virus (PMCV) ORF-1 antigen of claim 22 which includes the sequence that is at least 95% identical to SEQ ID NO: 25 and lacks SEQ ID NO: 14 or a sequence that is at least 90% identical to SEQ ID NO: 14.

25. The expression cassette of claim 22 wherein the antigen is at least 95% identical to SEQ ID NO: 11 or SEQ ID NO: 12 or SEQ ID NO: 46.

26. The expression cassette of claim 25 wherein at least 50% of amino acids differing between the antigen and SEQ ID NO: 11 or SEQ ID NO: 12 or SEQ ID NO: 46 are conservative substitutions.

27. The expression cassette according to claim 9 wherein said antigen is an outer surface protein of an enveloped virus, or a fragment of said outer surface protein, wherein said fragment is capable of eliciting protective immune response.

28. The expression cassette according to claim 27 wherein said enveloped virus is a coronavirus and said outer surface protein is Spike protein.

29. The expression cassette according to claim 9 wherein said antigen comprises a protein from midgut of a sea louse or a fragment thereof, wherein said fragment is at least 90% identical to SEQ ID NO: 21.

30. The expression cassette of any one of claims 9-29, wherein said nucleic acid sequence encoding the antigen is under operative control of a first promoter.

31. The expression cassette of claim 30, wherein said first promoter is cytomegalovirus (CMV) immediate-early enhancer and promoter.

32. A vaccine comprising the expression cassette according to any one of claims 9-31.

33. A vaccine comprising the expression cassette according to claim 7 or 8, and further comprising an antigen.

34. A method of vaccinating a salmonid against an infection, the method comprising administering the vaccine according to claim 32 or 33 to said salmonid.

35. The method of claim 34, wherein said vector comprises the expression cassette that comprises the nucleic acid sequence encoding the antigen, wherein the antigen is a non-enveloped virus.

36. The method of claim 35, wherein the non-enveloped virus is PMCV.

37. The method of any one of claims 34-36, wherein said salmonid weighs between 15 and 200 grams.

38. The method of any one of claims 34-36, wherein said salmonid weighs between 40 and 110 grams.

39. The method of any one of claims 34-38, wherein said salmonid is Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), or Coho Salmon (*Oncorhynchus kisutch*).

40. The method of claim 39, wherein said salmonid is *Salmo salar.*

41. A method of evaluating the efficiency of a Type I interferon as an adjuvant, the method comprising:

a) transfecting a nucleic acid sequence encoding an interferon into a source cells;
b) culturing said source cells, wherein said interferon is secreted into a source cell media;
c) contacting cultured target cells with at least a portion of said source cell media, wherein said target cells naturally express Mx gene in response to interferon; and
d) measuring Mx gene expression.

42. The method of claim 41, wherein said Type 1 interferon is Interferon B.

43. The method of claim 42 wherein the nucleic acid encoding an interferon is the nucleic acid sequence according to any one of claims 1-5.

44. The method of any one of claims 41-43 wherein the source cells are CHH-1 cells.

45. The method of any one of claims 41-44 wherein the target cells are TO-cells.

46. The method of any one of claims 41-45, wherein the step of measuring the Mx gene expression comprises RT-PCR.

47. A method of determining which nucleic acid sequence in a group of N sequences (N>1) encoding the same Type I interferon confers a more efficient expression the method comprises: for each member of the group of N sequences

a) transfecting an Nth nucleic acid sequence encoding an interferon into an Nth batch of source cells;
b) culturing said Nth batch source cells, wherein said interferon is secreted into a source cell media;
c) contacting an Nth batch of cultured target cells with at least a portion of said source cell media from said Nth batch of the source cells, wherein said target cells naturally express Mx gene in response to interferon;
d) measuring Mx gene expression in said Nth batch of the target cells;
e) comparing the Mx gene expression in each batch of the target cells from 1 to N.

48. The method of claim 47, wherein said Type 1 interferon is Interferon B.

49. The method of claim 48 wherein the nucleic acid encoding an interferon is the nucleic acid sequence according to any one of claims 1-5.

50. The method of any one of claims 47-49 wherein the source cells are CHH-1 cells.

51. The method of any one of claims 47-50 wherein the target cells are TO-cells.

52. The method of any one of claims 47-51, wherein the step of measuring the Mx gene expression comprises RT-PCR.

53. A vaccine according to claim 32 or claim 33 for use in a method of protecting a salmonid against infection.

54. The vaccine of claim 53, wherein said vector comprises the expression cassette that comprises the nucleic acid sequence encoding the antigen, wherein the antigen is a non-enveloped virus.

55. The vaccine of claim 54, wherein the non-enveloped virus is PMCV.

56. The vaccine of any one of claims 53-55, wherein said salmonid weighs between 15 and 200 grams.

57. The vaccine of any one of claims 53-55, wherein said salmonid weighs between 40 and 110 grams.

58. The method of any one of claims 53-57, wherein said salmonid is Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), or Coho Salmon (*Oncorhynchus kisutch*).

59. The method of claim 58, wherein said salmonid is *Salmo salar.*

FIG 1.

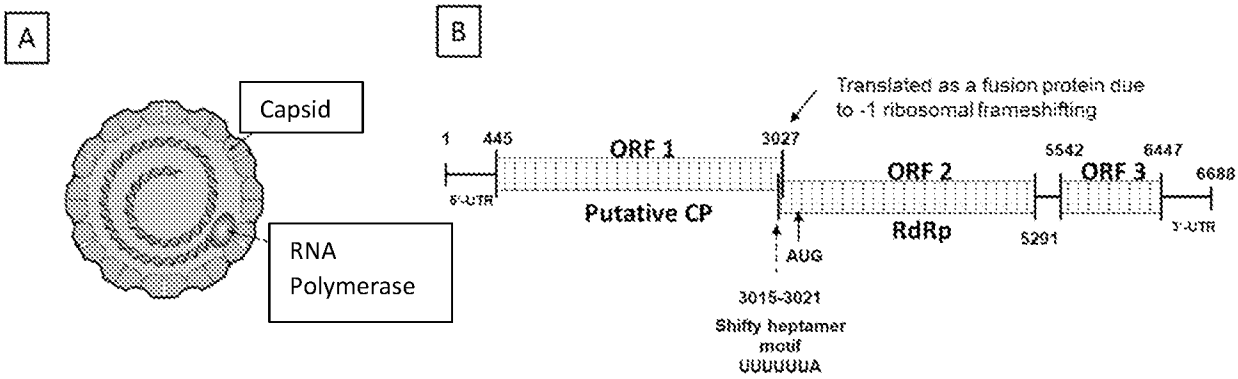

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Polymerase
B
Translated as a fusion protein due to -1 ribosomal frameshifting
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445
ORF 1
3027
5542
6447
ORF 2
ORF 3
6688
5'-UTR
Putative CP
RdRp
5291
3'-UTR
AUG
3015-3021
Shifty heptamer motif
UUUUUUA

FIG. 2.

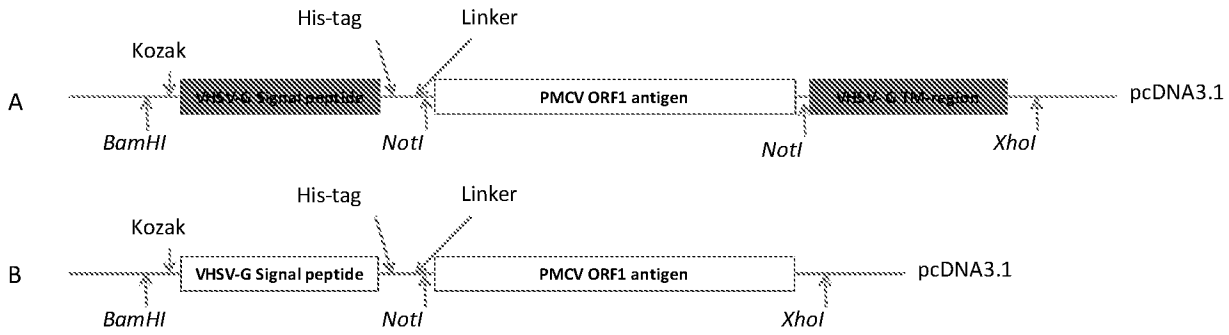

A
Kozak
His-tag
Linker
VHSV-G Signal peptide
PMCV ORF1 antigen
VHSV-G TM-region
pcDNA3.1
BamHI
NotI
NotI
XhoI
B
Kozak
His-tag
Linker
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NotI
XhoI

FIG 3.

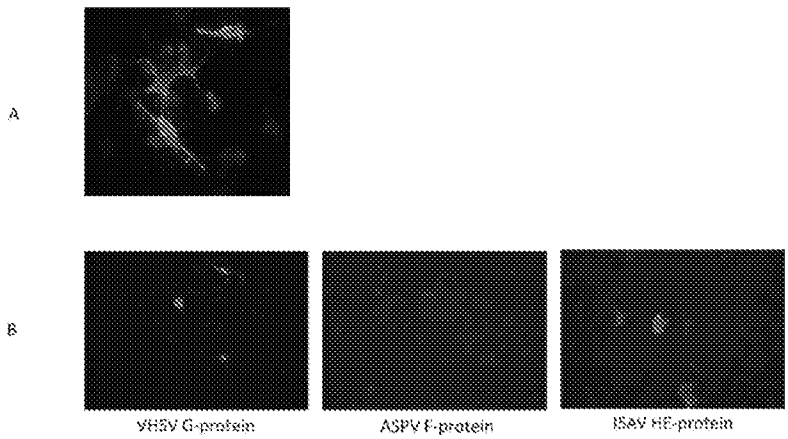



FIG. 4

A

| Description | Fluoresence intensity | |
|---|---|---|
| | Exp. 1 | Exp. 2 |
| Mock | - | - |
| ORF1 full | ++ | ++ |
| Del5 | ++ | ++ |
| Del6 | ++ | ++(+) |
| Del7 | ++ | ++ |
| Del11 | ++(+) | ++ |
| Del12 | ++ | ++ |
| Del13 | ++ | ++ |
| Del14 | ++ | ++ |
| Del15 | ++ | ++(+) |
| Del16 | +++ | +++ |
| Del17 | +++ | +++(+) |
| Del18 | (+) | (+) |

B

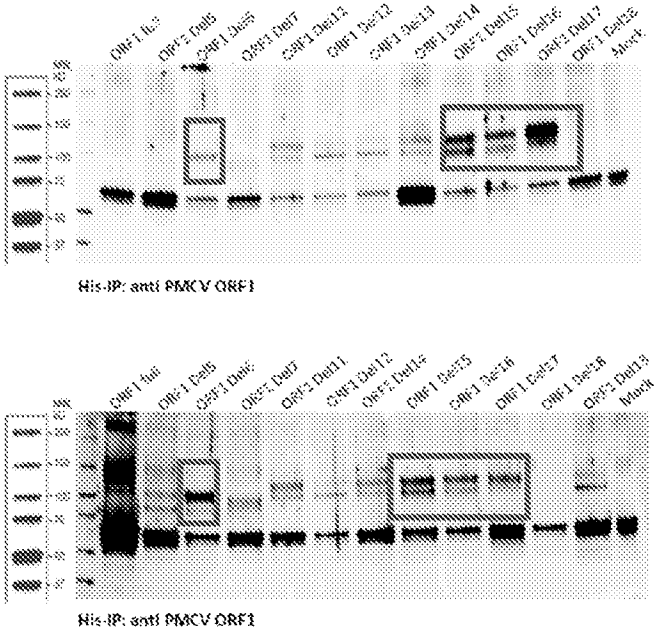
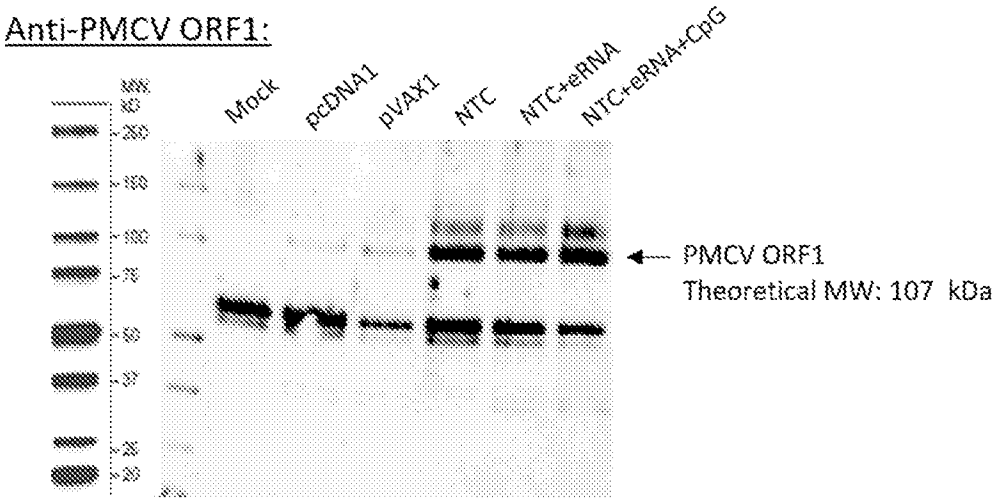


FIG 5


Anti-PMCV ORF1:
Mock
pcDNA1
pVAX1
NTC
NTC+eRNA
NTC+eRNA+CpG
PMCV ORF1
Theoretical MW: 107 kDa

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2022/081916

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/12 A61K39/39 A61P31/14
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P C12N

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | WO 2014/118385 A1 (UNIV TROMSØ [NO]) 7 August 2014 (2014-08-07) *** claims 1, 7, 10, 11, SEQ ID NO: 3, 8 *** | 1-8 |
| Y | US 2006/147458 A1 (HAMBLIN PAUL A [GB] ET AL) 6 July 2006 (2006-07-06) *** para. 34-36, claim 1 *** | 1-8 |

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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

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

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

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 29 March 2023 | 30/05/2023 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Heder, Andreas |



Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2022/081916

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed.

   b. [ ] furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

      [ ] accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. [ ] With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/081916

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

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

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

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

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

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

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

see additional sheet

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

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

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

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

1-8

**Remark on Protest**

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

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

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-8

Nucleic acid and expression cassette comprising it, as such, encoding SEQ ID NO: 6 or 50, or 95% identical polypeptide, having 65-90% identity to SEQ ID NO: 18 or 19, and preserved codon frequency

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2. claims: 20-26, 36, 55(completely); 9-19, 30-35, 37-40, 53, 54, 56-59(partially)

Group of inventions referring to (i) expression cassette encoding both the polypeptide of invention 1, and an antigen of PMCV (Piscine Myocarditis Virus), or (ii) vaccine comprising expression cassette encoding the polypeptide of invention 1, and an antigen of PMCV, or (iii) PMCV ORF-1 antigen as such

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3-5. claims: 27-29(completely); 9-19, 30-35, 37-40, 53, 54, 56-59(partially)

Like invention 2, except that the antigen is (3) spike protein of coronavirus, (4) protein from midgut of sea louse, or (5) antigen not belonging to any of inventions 2-4

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6. claims: 41-46

Method of evaluating IFN Type I as adjuvant

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7. claims: 47-52

Method of comparing different IFN Type I for more efficient expression

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2022/081916

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| | | ZA 200506548 B | 27-12-2007 |