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POISSONS ET SON UTILISATION
(54) Title: NUCLEIC ACID SEQUENCES OF FISH CARDIOMYOPATHY SYNDROME VIRUS AND THE USE THEREOF

(57) **Abrégé/Abstract:**

The present invention relates to nucleic acid sequences originating from a virus causing cardiomyopathy syndrome (CMS) and the use thereof within veterinary immunology and diagnosis.

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(54) Title: NUCLEIC ACID SEQUENCES OF A FISH VIRUS AND THE USE THEREOF

(57) Abstract: The present invention relates to nucleic acid sequences originating from a virus causing cardiomyopathy syndrome (CMS) and the use thereof within veterinary immunology and diagnosis.

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Nucleic acid sequences of fish cardiomyopathy syndrome virus and the use thereof

The present invention relates to nucleic acid sequences originating from a virus causing cardiomyopathy syndrome (CMS) in fish and the use thereof within veterinary immunology and diagnosis. More specifically, the invention provides
5 nucleic acid sequences coding proteins and the use thereof in inter alia recombinant vaccines, DNA vaccines and in the providing of a live recombinant vaccine. The protein coding sequences is also useful in respect of developing methods providing for immunological determination of CMS virus infection. The invention thus also relates to antibodies, and the use thereof in immunoassays.

10 The present invention also relates to primers useful in methods for detection of CMS virus in a test sample.

Finally, the present invention provides diagnostic kits comprising recombinant proteins, antibodies and primers, respectively.

15 Background of the invention.

Cardiomyopathy syndrome (CMS) is a disease affecting primarily large Atlantic salmon in the second year in seawater close to harvest, and therefore the economic impact is significant. Affected fish may suddenly die without showing signs of disease, or may show symptoms such as abnormal swimming behaviour and
20 anorexia. CMS is diagnosed on the basis of histopathology, showing severe inflammation and degeneration of spongy myocardium in the atrium and ventricle. A possible secondary effect of circulatory disturbance is multifocal liver necrosis which is commonly observed.

The cause of CMS was for a long time unknown. Although intracellular inclusions
25 and virus-like particles were reported, the reports were not consistent as apparent from a review provided in Kongtorp *et al.* Cardiomyopathy syndrome (CMS): a literature review, National Veterinary Institute, Norway, November 2005. In 1997 Grotmol *et al.* reported the presence of nodavirus-like particles in endothelial cells in the heart of Atlantic salmon diagnosed as suffering from CMS. The particles had
30 a diameter of 25 nm (Grotmol *et al.*, 1997, *Dis Aquat Org*, vol 29, pp 79-84).

Experimental transmission of cardiomyopathy syndrome in Atlantic salmon, *Salmo salar* have been reported, cf. Bruno and Noguera, *Disease of Aquatic Organism*, vol. 87: 235-242, 2009 and Fritsvold *et al.*, *Disease of Aquatic Organism*, vol 87: 225-234, 2009.

35 The infectious nature of CMS was finally demonstrated by the isolation and cultivation of a virus causing CMS in fish as reported in the Norwegian patent application NO 2008 2869. In particular, NO 2008 2869 discloses an isolated virus being deposited with the European Collection of Cell Culture (ECACC), Health

Protection Agency, Porton Down, Salisbury, Wiltshire (UK), SP4 0JG UK on the 29 March 2007 under accession number 07032909.

5 The virus disclosed in the above mentioned Norwegian patent application may have the ability of introducing one or more symptoms selected from the group consisting of: skin haemorrhages, raised scales, exophthalmos, ascites, fibrinous casts over the liver capsule, blood or blood clots filling the pericardial cavity, ruptures in the cardiac atrial wall, dilation of the cardiac atrium, compression of the cardiac ventricle, inflammation of the spongy myocardium and the epi- and endocardium, liver lesions including multifocal to anastomosing necrosis of
10 hepatocytes and fibrinous coating of the capsule, congestion of the spleen and/or the gills.

A further understanding of the CMS causing agent has been provided by the isolation and cultivation of the CMS virus disclosed in NO 2008 2869. The host cells for cultivation of the CMS virus disclosed in NO 2008 2869 have shown to
15 results in low yield of the CMS virus. There is still a need for further knowledge to be able to develop efficient means for controlling the disease and to be able to develop efficient vaccines, such as recombinant vaccines. Although the virus was successfully isolated and host cells for the cultivation thereof is known from NO2008 2869, the further identification and providing of knowledge of the genome
20 and the sequence thereof have proven difficult due to unexpected characteristics of the genome and the organisation thereof.

It is therefore an object of the present invention to provide nucleic acid sequences originating from CMS virus. Through the providing of the nucleic acid sequences of the present invention, various useful applications thereof are provided as outline
25 below.

Summary of the invention

The present invention thus provides an isolated nucleic acid sequence originating from CMS virus having a sequence selected from the group consisting of SEQ ID
30 No. 1, SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 6, and variants or fragments thereof being at least 70 % homologous with any of the sequences SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 6.

According to one aspect the nucleic acid sequences of the invention is at least 80%, preferably 90 %, more preferably 95% homologous with the nucleic acid selected
35 from the group consisting of SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 6.

Another aspect of the invention relates to a vector comprising nucleic acid sequences according to the present invention, and host cells comprising said vectors.

According to another aspect of the invention, vectors are provided comprising nucleic acids selected from the group consisting of SEQ ID No. 2 and SEQ ID No. 6, and variants or fragments thereof being at least 70 % homologous with any of the sequences SEQ ID No. 2, and SEQ ID No. 6 is operable linked to control sequences directing the expression of said sequences.

According to yet another aspect of the invention, DNA vaccines are provided comprising a nucleic acid sequence selected from the group consisting of SEQ ID No. 2 and SEQ ID No. 6, and variants or fragments thereof being at least 70 % homologous with any of the sequences SEQ ID No. 2, and SEQ ID No. 6.

Another aspect of the invention relates to recombinant proteins encoded by a nucleic acid sequence selected from the group consisting of SEQ ID No. 2 and SEQ ID No. 6, and variants or fragments thereof being at least 70 % homologous with any of the sequences SEQ ID No. 2, and SEQ ID No. 6. Said recombinant proteins according to the present invention has according to one aspect of the invention an amino acid sequence selected from the group consisting of SEQ ID No. 3 and SEQ ID No. 7, or a variant or fragment thereof.

The present invention also provides a recombinant vaccine comprising at least one of the recombinant proteins according to the present invention.

Furthermore, according to another aspect of the invention, an antibody is provided that recognises and binds to a recombinant protein according to the present invention. The antibody is according to one aspect a monoclonal antibody. According to another aspect, the antibody of the invention is a polyclonal antibody.

According to yet another aspect of the invention, an immunoassay is provided for the detection of CMS virus specific antibody against CMS virus in a biological sample, characterized by comprising the following steps:

- a) contacting at least one of the recombinant proteins according to the present invention and a biological sample suspected to comprise antibodies against a CMS virus; and
- (b) determining whether the at least one recombinant protein binds to a CMS virus specific antibody present in the biological sample wherein said binding represent an indication that the animal has been in contact with CMS virus.

According to yet another aspect of the invention an immunoassay is provided for detection of CMS virus in a biological sample, characterized by comprising the following steps:

- (a) contacting one or more antibodies that specifically recognise and binds to a recombinant protein according to the invention and a biological sample suspected to comprise CMS virus; and
- (b) determining whether the antibody binds to CMS virus present in the

biological sample wherein said binding represent an indication that the sample contains a CMS virus.

The present invention also provides according to another aspect a primer comprising a sequence of at least about 10 nucleotides, wherein said primer
5 hybridize to a nucleic acid sequence originating from CMS virus having a sequence selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 6, and variants or fragments thereof being at least 70 % homologous with any of the sequences SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 6. The primer according to the present invention may according to one aspect
10 consist of at least 15 nucleotides, preferably at least 20 nucleotides.

According to yet another aspect of the invention, a method for the detection of CMS virus in a biological sample is provided, wherein the method comprises the following steps:

a) preparing a biological sample comprising nucleic acid sequences isolated from a
15 biological sample suspected to comprise CMS virus for a reverse transcription reaction with a specific or random primer;

b) subjecting the mixture of a) for a polymerase chain reaction with a primer pair specific to the reverse transcribed nucleic acid sequence corresponding to the genome sequence of the virus, wherein each primer of said primer pair hybridize to
20 a nucleic acid sequence originating from CMS virus having a sequence selected from the group consisting of SEQ ID No. 1, SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 6, and variants or fragments thereof being at least 70 % homologous with any of the sequences SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 6; and

c) determining whether the binding of the primers to nucleic acid sequences in the sample and amplification of the sequence between them have occurred indicating
25 the presence of CMS virus in the sample tested.

According to one aspect of the above method, each primer of the primer pair is selected from a group consisting of SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15,
30 SEQ ID No. 16, SEQ ID No. 17 SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20 and SEQ ID No. 21.

Finally, the present invention relates to the use of the nucleic acid sequences according to any of the claim 1-2 for the preparation of DNA vaccine, recombinant vaccine or a live recombinant microorganism.

35

Brief description of the figures

Figure 1 discloses the genomic sequence of the CMS virus corresponding to SEQ ID No. 1.

Figure 2 discloses the nucleic acid sequence of the open reading frame 1 corresponding to SEQ ID No. 2. The open reading frame 1 comprise the sequence starting at the nucleotide in position 445 and ending at the nucleotide in position 3028 of SEQ ID No. 1.

5 Figure 3 discloses the amino acid sequence coded by the sequence depicted in figure 2, i.e. the amino acid sequence corresponding to SEQ ID No. 3.

Figure 4 discloses the nucleic acid sequence of the open reading frame 2 corresponding to SEQ ID No. 4. The open reading frame 2 comprise the sequence starting at the nucleotide in position 3114 and ending at the nucleotide in position 5292 of SEQ ID No. 1.

10 Figure 5 discloses the amino acid sequence coded by the sequence depicted in figure 4, i.e. the amino acid sequence corresponding to SEQ ID No. 5.

Figure 6 discloses the nucleic acid sequence of the open reading frame 3 corresponding to SEQ ID No. 6. The open reading frame 3 comprise the sequence starting at the nucleotide in position 5542 and ending at the nucleotide in position 6448 of SEQ ID No. 1.

Figure 7 disclose the amino acid sequence coded by the sequence depicted in figure 6, i.e. the amino acid sequence corresponding to SEQ ID No. 7.

20 Figure 8 shows graphically the genome organization of piscine myocarditis virus (PMCV). The genome includes three large open reading frames (ORFs). The predicted ORF1 and ORF2 resemble two overlapping open reading frames. ORF2 is translated as a fusion protein with ORF1 due to ribosomal -1 frameshifting or as an individual protein (start codon indicated). ORF3 is a non-overlapping frame .

Figure 9 shows recombinant proteins coded by ORF1 and ORF3 and expressed in E.coli separated by gel electrophoresis. Figure 9A shows the expression of ORF-1 and a partial ORF 1 (nucleotide 1-852) in E.coli. The ORF1 (1-852) is expressed as a protein of approximately 30 kD (lanes 3 and 4), while the full length protein is approximately 90 kD (lanes 6 and 7). Lanes 2 and 5 are the soluble protein fractions from the fermentate. A protein size ladder is run in lane 1. Figure 9B shows the expression of ORF-3. The ORF3 is expressed as a protein of approximately 30 kD (lanes 3 and 4). Lane 2 is the soluble protein fraction. A protein size ladder is run in lane 1.

Figure 10 is a Western blot showing that an antiserum raised against recombinant ORF 1 protein produced in E.coli recognizes a band corresponding to the ORF 1 protein in heart homogenate from Atlantic salmon diagnosed with CMS (lane 2). No band of this size is recognized in heart homogenate from a healthy Atlantic salmon (lane 3). A protein size ladder is run in lane 1.

Figure 11 constitutes Western blots showing binding of ORF3 by Atlantic salmon serum from fish diagnosed with CMS (A, left panel). Control serum from healthy Atlantic salmon (B, right panel) shows no binding to ORF3. Some unspecific binding to a protein of approximately 10-15 kD is visible.

- 5 Figure 12 represents a bivariate plot showing the relationship between histoscore of spongy part of the heart ventricle and virus load (expressed as Cp values) in heart tissue for Sample 1 (n=40).

The present invention and the various embodiments thereof will now be described in detail in the following.

10

Detailed description of the invention

Definitions

- The present invention relates to isolated nucleic acid sequences and variants or fragments thereof being at least 70% homologous with the isolated nucleic acid sequences. The term “% homology” is to be understood to refer to the percentage of nucleotides that two or more sequences or fragments thereof contains that are the same. A specified percentage of nucleotides can be referred to as e.g. 70% homologous, 80% homologous, 85% homologous, 90% homologous, 95% homologous, 99% homologous or more over a specified region when compared and aligned for maximum correspondence. The skilled person will acknowledge that various means for comparing sequences are available. For example, one non-limiting example of a useful computer homology program useful for determining the percent homology between sequences includes the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990, J. of Molec. Biol., 215:403-410, "The BLAST Algorithm; Altschul et al., 1997, Nuc. Acids Res. 25:3389-3402, , Karlin and Altschul 1990, Proc. Nat'l Acad. Sci. USA, 87:2264-68; 1993, Proc. Nat'l Acad. Sci. USA 90:5873-77).

- The term "fragments or variants thereof" used in respect of the nucleic acid sequences and recombinant proteins according to the present invention is to be understood to encompass nucleic acid sequences and recombinant proteins that only differs from the isolated sequences SEQ ID No. 1-7 by way of some amino acid or nucleotide additions, deletions or alteration that have little effect, if any, on the functional activity of the claimed sequences. The skilled person will acknowledge that modifications of a protein coding nucleotide sequence may be introduced which does not alter the amino acid sequence, e.g. the substitution of a nucleotide resulting in that the triplett affected by the substitution still codes for the same amino acid. Such alterations may be introduced to adapt the nucleic acid sequence to the codons preferably used by a host cell and thus to enhance the expression of a desired recombinant protein. Furthermore, the addition of nucleic acid sequences coding

polypeptides which facilitates purification may be added without affecting the activity of the resulting recombinant protein.

The skilled person will further acknowledge that also alterations of the nucleic acid sequence resulting in modifications of the amino acid sequence of the recombinant protein it codes may have little, if any, effect on e.g. the proteins ability to induce protection against CMS virus if the alteration does not have any impact on the resulting three dimensional structure of the recombinant 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 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 recombinant protein with substantially the same functional activity as the protein having the amino acid sequences as depicted in SEQ ID No. 3, 5 and 7 and thus to be expected to constitute a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal or C-terminal portions of the protein molecule would also not be expected to alter the functional activity of the protein. Each of the proposed modifications is well within the routine skills in the art, as is determination of retention of biological activity of the encoded products. Therefore, where the terms "nucleic acid sequences of the invention" or "recombinant protein of the invention" are used in either the specification or the claims each will be understood to encompass all such modifications and variations which result in the production of a biologically equivalent protein.

25

Thus, the present invention therefore encompass recombinant proteins and fragments thereof which differs in respect of amino acid substitutions, addition or deletions compared with the recombinant proteins having the sequence of SEQ ID No. 3, SEQ ID No. 5, and SEQ ID No. 7, respectively, but which exert substantially the same functional activity as the proteins having the sequence of SEQ ID No. 3, SEQ ID No. 5, and SEQ ID No. 7.

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The terms "antigen", immunogenic protein or peptide", "antigenic determinant" or "epitope" when used in connection with the present invention is to be understood to refer to a recombinant protein or fragment thereof according to the invention being able to induce protection against CMS in fish or to be able to bind to an antibody which recognise and bind to the CMS virus. A fragment of a recombinant protein having a sequence as depicted in SEQ ID No. 3, SEQ ID No. 5, and SEQ ID No. 7, respectively may constitute such an antigen or epitope. A non-limiting example of a fragment of the present invention is e.g. a fragment constituting for example 8-11 amino acids of the sequence SEQ ID No. 3, SEQ ID No. 5, or SEQ ID No. 7.

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The term "vaccine" as used herein refers to a material that can produce an immune response that blocks the infectivity, either partially or fully, of an infectious agent, which in respect of the present invention is the cardiomyopathy syndrome virus affecting fish such as e.g. salmonids. Thus, when administering to a fish, the vaccines of the invention is immunised against the disease caused by CMS virus. The immunising component of the vaccine may be e.g. DNA as in a DNA vaccine, a recombinant protein or fragment thereof according to the present invention, or a live recombinant microorganism.

As outlined below, the present invention also provides primers (i.e. oligonucleotide sequences) useful in polymerase chain reaction techniques to be used as diagnostic tools. The term "primer" as used herein refers to an oligonucleotide either naturally occurring (e.g. as a restriction fragment) or produced synthetically which is significantly complementary to a CMS virus target sequence and thus capable of hybridizing to nucleic acid sequences of the present invention. The primer may be labelled to facilitate detection, e.g. using fluorescent labels or other label means well known to the skilled person.

Detailed description of the embodiments of the invention

20

Nucleic acid sequences

The present inventors have provided the genomic sequence of the CMS virus. GF-1 cells were infected with homogenate obtained from salmon suspected of being infected with CMS virus. Culture supernatant was harvested, pelleted by centrifugation and subjected to DNase and RNase treatment. RNA was then isolated and cDNA synthesised using random primers. Thereafter, double stranded DNA was provided and amplified by PCR using random primers, and the obtained products were sequenced and analysed to obtain part of the genomic sequence according to the present invention depicted in figure 1 and SEQ ID No. 1. The sequence of the rest of the genome was obtained by PCR with specific primers and 3' and 5' RACE (Rapid Amplification of cDNA Ends).

The further analysis of SEQ ID No. 1 by the present inventors revealed three open reading frames which are believed to include the coding region for three viral proteins. Sequence comparisons with other known viral sequences has revealed that the open reading frame 1 (SEQ ID No. 2) according to the present invention shows very weak homology to other viral sequences. Furthermore, the analysis of the open reading frame 1 (SEQ ID No. 2) indicates that it comprise a nucleic acid sequence coding a coat protein. The amino acid sequence of the open reading frame 1 is

shown in figure 3 and SEQ ID No. 3. Said sequence show only very weak homology with other known proteins. (Poilos et al., 2006, Jour. General Virology, 87, 987-996, Tang et al. 2008, PNAS, 105:45, 17526- 17531).

5 A further isolated nucleic acid sequence of the invention (open reading frame 2, SEQ ID No. 4) comprises a sequence coding an RNA dependent RNA polymerase and the amino acid sequence thereof is depicted in figure 5 and SEQ ID No. 5. Comparison with other known viral polymerase coding sequences shows that this sequence is weakly homologous with a totivirus polymerase, in particular a *Giardia lamblia virus* which belongs to Totiviridae when a homology search is conducted
10 with the BLAST program (Accession no. AAB01579).

The third open reading frame (SEQ ID No. 6) of the invention comprises a sequence which is also believed to include the coding sequence of a coat protein, the amino acid sequence thereof being depicted in figure 6/SEQ ID No. 6. Surprisingly, said sequence show only very weak homology with other viral coat proteins, and no
15 other members of the Totiviridae family comprise this particular open reading frame. (Poilos et al., 2006, Jour. General Virology, 87, 987-996).

With the providing of the above sequences, further progress in respect of controlling and preventing cardiomyopathy syndrome in fish is achieved. The sequences of the open reading frames 1 and/or 3 (SEQ ID No. 2 and 6) may form
20 the basis for the development of recombinant proteins to be used in a vaccine, or the development of DNA vaccines or a live recombinant microorganism.

Recombinant proteins being coded for by sequences based on the open reading frames 1 and 3 may also form the basis for the development of diagnostic tools, i.e. through the production of antibodies and the development of immunoassays for the
25 detection of the presence of CMS virus in biological samples.

Finally, the sequences provided may form the basis for development of diagnostic tools wherein the nucleic acid sequences or fragments thereof is used to detect the presence of CMS virus in fish.

The various embodiments of the invention being the result of the providing of the
30 nucleic acid sequences of the invention are outlined further below. The skilled person will acknowledge that said embodiments should not be understood to involve a limitation of the scope of the claimed nucleic acid sequences. Other uses of the claimed nucleic acid sequences are also understood to be covered by the scope of the present invention.

35

Recombinant proteins

According to one aspect of the invention, the invention provides recombinant cardiomyopathy virus proteins.

The skilled person is well familiar with the various available biotechnological techniques providing for the expression of isolated nucleic acid sequences for the preparation of recombinant proteins by heterologous expression in various host cell systems using commonly available genetic engineering techniques and recombinant DNA expression systems. Various protocols for the expression of recombinant protein are known, cf. e.g. "Recombinant Gene Expression Protocols, in Methods in Molecular Biology, 1997, Ed. Rocky S Tuan, Human Press (ISSN 1064-3745) or Sambrook et al., Molecular Cloning: A laboratory Manual (third edition), 2001, CSHL Press, (ISBN 978-087969577-4). For example, the nucleic acid sequences of the open reading frames 1 and 3 or fragments or variants thereof may be inserted in suitable expression vectors comprising all the necessary transcriptional and translational regulatory sequences specifically adapted for directing the expression of the desired protein coding nucleic acid sequence in a suitable host cell. Suitable expression vectors are e.g. plasmids, cosmids, viruses or artificial yeast chromosomes (YAC's). A non-limiting list of examples of suitable expression vector systems is e.g. pET-type of vectors (available from Invitrogen), pcDNA3.1 vectors (available from Invitrogen), or the pBR-type, pUC-type or pGEM-type of expression vectors. The obtained expression vector including the open reading frames (SEQ ID No. 2, or SEQ ID No. 6) or a fragment thereof is then introduced in suitable host cells for the production of the desired recombinant protein.

According to one embodiment of the invention, a vector is provided comprising a sequence selected from the group consisting of SEQ ID No. 2 and SEQ ID No. 6, and variants or fragments thereof being at least 70 % homologous with any of the sequences SEQ ID No. 2, and SEQ ID No. 6 operatively linked to control sequences directing the expression of said sequences. The term "operatively linked", means that the desired sequence coding for a recombinant protein is linked to all the necessary transcriptional and translational regulatory sequences specifically adapted for directing the expression of the desired protein coding nucleic acid sequence in a suitable host cell.

It should be understood that various modifications may be introduced in the nucleic acid sequences of the present invention utilising techniques well known to the skilled person for example to facilitate expression. By the use of site directed mutagenesis or synthesis, modification may be introduced to adapt the coding sequence to the desired host used to express the sequence and thus produce the recombinant protein. The skilled person is well aware of the fact of the presence of host specific codons, and that the adaption of a heterologous nucleic acid sequence to the host specific codons increase the expression efficiency. Other modification may also be introduced, e.g. to facilitate isolation and purification, i.e. by adding a sequence coding for a peptide or protein useful for such purposes. Also, nucleic acid sequences coding signal peptide providing for secretion of the desired recombinant protein from the host cell may also be linked to the nucleic acid

sequences of the present invention. In case the recombinant protein is expressed in a live recombinant microorganism (see below), the nucleic acid sequence may be modified to enable the anchorage of the recombinant protein in the membrane or cell wall of the microorganism resulting in the presentation of the recombinant protein on the surface of the live recombinant microorganism. According to such an embodiment of the invention, the nucleic acid sequence of the present invention is modified by adding a nucleic acid sequence coding a protein or peptide resulting in the anchorage of the resulting recombinant protein in the membrane or cell wall of the live recombinant microorganism.

- 5
- 10 The present invention thus provides vectors comprising the nucleic sequence as depicted in figure 2 and/or 6 (SEQ ID No. 2 and SEQ ID No. 6) or variants or fragments thereof. The present invention also provides host cells comprising the vectors of the invention.

- 15 Various commercially available host cells specifically adapted for the production of recombinant proteins may be used, both prokaryotic host cells and eukaryotic host cells. Non-limiting examples of suitable prokaryote host cells is e.g. *Escherichia Coli*, *Bacillus subtilis*, *Lactobacillus sp*, *Caulobacter crescentus*, *Yersinia ruckeri*, or *Vibrio anguillarum*, Non-limiting examples of suitable eukaryote host cells is e.g. yeast such as *Saccharomyces cerevisiae* or *Chinese hamster ovary cells*. Also
- 20 viruses may be used for the production recombinant expression of a desired protein, cf. e.g. alphavirus, adenovirus or baculovirus vector systems (Bac-to-Bac® Baculovirus Expression Systems, Invitrogen, PichiaPink™ Yeast Expression System, Invitrogen).

- 25 According to one embodiment of the invention, a pET vector available from Invitrogen and specifically adapted for the expression of recombinant proteins in *Escherichia Coli* is used.

- Recombinant protein may also be provided by using cell free expression systems. Recombinant proteins being the results of expression of the open reading frames 1 or 3 (SEQ ID No. 2 or 6) or fragments or combinations thereof may be used in a
- 30 vaccine to obtain protection against further CMV virus infections.

Recombinant vaccine composition

- Recombinant proteins prepared by heterologous expression of the nucleic acid sequences according to the present invention may be used as immunogenic
- 35 component(s) of a recombinant vaccine composition.

The present invention therefore provides a vaccine composition comprising at least one recombinant protein having an amino acid sequence as depicted in figure 3 or 7 (SEQ ID No. 3 or SEQ ID No. 7), or fragments or variants thereof.

According to one aspect of the invention, the at least one recombinant protein used in a vaccine consist of a fragment of the coating protein coded for by the sequences of the open reading frames 1 or 3 that induce an immune response in the individual being administered the recombinant vaccine of the invention. Such fragments may
5 constitute an epitope of the coating protein coded for by the open reading frames 1 or 3, also known as an *antigenic determinant*. The skilled person is well familiar with the fact that the coat proteins of a virus may comprise one or more epitopes that constitutes the part of the coat protein that is recognized by the immune system and thus result in the development of protection against further infection. The
10 epitopes may be found as linear, continuous sequence of amino acids, such as e.g. 8-11 amino acids, within a coat protein or another surface structure of a pathogen organism. Such an epitope is commonly named a linear epitope. An epitope of a coat protein may also be the result of a three dimensional structure of the coat protein, e.g. formed by the assembly of amino acids of the sequence that are not
15 sequential. Such epitopes are commonly named discontinuous epitopes, and are often larger than a linear epitope.

Linear and discontinuous epitopes comprised in the open reading frame 1 and 3 of the invention may be determined by the use of epitope mapping technology available to the skilled person, c.f. Methods in Molecular Biology: Epitope Mapping
20 Protocols, 1996, vol. 66, Ed. Glenn E Morris, Human Press (ISBN 978-0-89603-375-7). It is to be understood that vaccines comprising recombinant proteins according to the present invention which constitute protective epitopes of the coat proteins of CMS virus prepared by expression of fragments of the open reading frames 1 or 3, or variants or fragments thereof, is covered by scope of the present
25 invention. More specifically, epitopes which are recognized by neutralizing antibodies will be of particular interest.

Epitopes that are found in those parts of the recombinant proteins according to the present invention that are particular specific for the CMS virus are preferred.

The recombinant vaccine composition according to the invention comprising
30 recombinant proteins or fragments thereof as outlined above may further comprise an adjuvant. Examples of adjuvants frequently used in fish and shellfish farming are muramyl dipeptides, lipopolysaccharides, several glucans and glycans, mineral oil, Montanide™ and Carbopol®. An extensive overview of adjuvants suitable for fish and shellfish vaccines is given in the review paper by Jan Raa, 1996, Reviews in
35 Fisheries Science 4(3): 229-228.

The vaccine of the invention may further comprise a suitable pharmaceutical carrier. In a currently preferred embodiment the vaccine is formulated as an emulsion of water in oil. The vaccine may also comprise a so-called "vehicle". A vehicle is a device to which the antigen adheres, without being covalently bound to
40 it. Such vehicles are i.a. biodegradable nano/micro-particles or -capsules of PLGA

(poly-lactide-co-glycolic acid), alginate or chitosan, liposomes, niosomes, micelles, multiple emulsions and macrosols, all known in the art. A special form of such a vehicle, in which the antigen is partially embedded in the vehicle, is the so-called ISCOM (European patents EP 109.942, EP 180.564 and EP 242.380)

- 5 In addition, the vaccine may comprise one or more suitable surface-active compounds or emulsifiers, e.g. Cremophore®, Tween® and Span®. Also adjuvants such as interleukin, CpG and glycoproteins may be used.

It is to be understood that the vaccine may further be in a formulation comprising an antigen from a bacterial source, an antigenic material obtained from a viral source
 10 other than the fish virus as defined above, an antigenic material obtained from a parasitical source, and/or an antigenic material obtained from a fungal source. Polyvalent vaccines containing antigens from typical fish pathogens other than CMS virus are well known in the art and are already commercially available. In addition, representative isolates of relevant fish pathogens are available from
 15 various sources.

In particular embodiments of the invention said antigen from a bacterial source is selected from the group consisting of: live, attenuated or killed bacteria of the species *Piscirickettsias* sp., *Aeromonas* sp., *Vibrio* sp., *Listonella* sp., *Moritella* *viscosa*, *Photobacterium damsela*, *Flavobacterium* sp., *Yersinia* sp., *Renibacterium* *sp.*, *Streptococcus* sp., *Lactococcus* sp., *Leuconostoc* sp., *Bifidobacterium* sp.,
 20 *Pediococcus* sp., *Brevibacterium* sp., *Edwardsiella* sp., *Francisella* sp., *Pseudomonas* sp., *Cytophaga* sp., *Nocardia* sp., *Mycobacterium* sp., parts or subunits of these bacteria, and any combination hereof.

Isolates of such bacteria are available, e.g. from LGC Promochem/American Type
 25 Culture Collection ATCC repository and distribution center (ATCC) including strains of *A. salmonicida* (ATCC 33658), *V. salmonicida* (ATCC 43839), *V. anguillarum* serotype O1(ATCC 43305) and O2(ATCC 19264), and *Moritella viscosa* (ATCC BAA-105). In addition, cultures of *Piscirickettsias salmonis* have been deposited in the European Collection of Cell Culture (ECACC), Health
 30 Protection Agency, Porton Down, Salisbury, Wiltshire (UK), SP4 0JG UK on the 9 June 2006 under the following accession numbers: 06050901, 06050902, 06050903 and 07032110.

Other specific embodiments pertain to a vaccine, wherein said antigenic material obtained from a viral source other than the fish virus as defined above is from a
 35 virus selected from the group consisting of: Viral Hemorrhagic Septicemia Virus (VHSV), Viral Hemorrhagic Septicemia Virus (VHSV), Infectious Hematopoietic Necrosis virus (IHNV), Infectious Pancreatic Necrosis Virus (IPNV), Spring Viremia of Carp (SVC), Channel Catfish Virus (CCV), Infectious Salmon Anaemia virus (ISAV), pancreatic disease virus (SPDV), Iridovirus, and heart and skeletal
 40 muscle inflammation virus (HSMIV), parts or subunits of any one of these viruses,

and combinations hereof. Representative species of such viruses are available to the skilled artisan, for instance from the following deposits: infectious pancreatic necrosis virus (IPNV, ATCC VR-1318, country of origin: unknown), Viral Hemorrhagic Septicemia Virus (VHSV, ATCC VR_1389, country of origin: Denmark); Infectious Hematopoietic Necrosis virus (IHNV, ATCC VR-1392, country of origin: USA)); Pancreatic Necrosis Virus; Spring Viremia of Carp (SVC, ATCC VR-1390, country of origin: Denmark); Channel Catfish Virus (CCV) (ATCC VR-665, country of origin: USA); Infectious Salmon Anaemia (ISA) virus (ATCC VR-1554, country of origin: Canada).

Patent deposits have previously been made by the present applicant of the following viral species: Heart and Skeletal Muscle Infection Virus (HSMIV, patent deposit nr ECACC 04050401, country of origin: Norway).

In more specific embodiments, said antigenic material obtained from a viral source other than the fish virus as defined above is from the group consisting of: Glycoprotein of Viral Hemorrhagic Septicemia Virus (VHSV), nucleoprotein of Viral Hemorrhagic Septicemia Virus (VHSV), glycoprotein of Infectious Hematopoietic Necrosis virus (IHNV), structural proteins of Infectious Pancreatic Necrosis Virus (IPNV), G protein of Spring Viremia of Carp (SVC), and a membrane-associated protein, tegumin or capsid protein or glycoprotein, of Channel Catfish Virus (CCV), antigenic fragments of any of one of these proteins and combinations hereof.

In other embodiments said antigenic material from a parasitic source is from a source selected from the group consisting of *Lepeophtheirus Sp.*, *Caligus Sp.*, and *Ichthyophthirius Sp.*, parts of any one of these parasites, and combinations hereof.

In yet other embodiments said antigenic material is from a fungal source selected from the group consisting of *Saprolegnia Sp.*, *Branchiomyces sanguinis*, *Branchiomyces demigrans* and *Ichthyophonus hoferi*.

The vaccine according to the invention may in particular be formulated for administration to a fin fish. More specifically the vaccine may be (formulated) for administration to a teleostei. The teleostei include, but are not limited to salmonids, basses, breams, cods, snappers, flatfish, catfish, yellowtails and tilapias.

In a presently preferred embodiment the vaccine is formulated for administration to Atlantic salmon (*Salmo Salar* L.), Rainbow trout (*Oncorhynchus mykiss*) and/or Coho salmon (*Oncorhynchus kisutch*).

In further embodiments of the invention the vaccine is formulated for administration by a route selected from the group consisting of: bath, immersion, intraperitoneal injection, intramuscular injection and oral administration. Optionally, the vaccine would be administered to young fish in the fresh-water stage.

Live recombinant microorganism

According to another embodiment of the invention, the sequences of the open reading frames 1 and/or 3 may also be used to develop a live recombinant microorganism to be used in a vaccine against CMS virus infection. A live recombinant organism function as a carrier for genetic information according to the present invention by the insertion of the open reading frames 1 or 3 or variants or fragments thereof. Said open reading frames or variants or fragments thereof may be inserted in the microorganism in a way making the microorganism capable of expressing said nucleic acid sequences. The nucleic acid sequences or variants or fragments thereof according to the invention may be inserted in the genome of the microorganism or by the introduction of a vector comprising the desired nucleic acid sequences. The live recombinant microorganism may thus be a carrier of nucleic acid sequences coding for the recombinant proteins or variants or fragments thereof according to the present invention. As mentioned above, the live recombinant organism may e.g. carry one or more recombinant proteins according to the present invention on the surface, e.g. wherein said recombinant proteins are anchored in the membrane or the cell wall of the live recombinant organism.

The live recombinant microorganism may be e.g. a virus, a bacteria or a parasite. The live recombinant microorganism may function in the same way as a live fish vaccine, i.e. the microorganism will replicate in the target fish and result in an immunological response against the recombinant proteins coded by the nucleic acids of the invention.

The live recombinant microorganism may have the advantage of being efficiently cultured and thus being optimised for desired fermentation processes and high yield production of vaccine.

The skilled person will be able, by the use of common biotechnological techniques for cloning and constructions of recombinant organisms, to construct a live recombinant microorganism according to the present invention.

The live recombinant microorganism may also be the carrier of nucleic acid coding for other fish pathogens thus providing for protection against a variety of pathogens. The live recombinant organism may thus include nucleic acids coding e.g. coating proteins or fragments thereof of other fish viruses, such as e.g. SAV, IPNV, IHNV or HSMIV.

According to one embodiment of the invention, the live recombinant microorganism is a virus able to replicate in fish, preferably salmonid fish. For example, IHNV may be used as live recombinant microorganism, c.f. WO 03/097090 reporting the use of a modified novirhabdovirus to obtain vaccines. The use of live recombinant microorganism as carrier for nucleic acids coding antigens is also suggested for other fish pathogens, e.g. in WO 2007/031572.

A vaccine composition comprising a live recombinant microorganism may further as for the recombinant vaccine described above comprise adjuvants, carrier, surface-active compounds or emulsifiers as those described above. Furthermore, the advantage of combining different vaccine components is equally applicable to the live recombinant vaccine according to the present invention as to recombinant vaccine composition as disclosed above.

DNA vaccine

According to one aspect of the invention, a DNA vaccine is provided comprising nucleic acids according to the present invention.

DNA vaccines is based on the administration of nucleic acids sequence(s) coding an antigen to a patient, and wherein the expression of said nucleic acid(s) results in an immune response in said patient. For the purpose of the present invention, the patient is a fish. A DNA vaccine may consist of a naked plasmid DNA comprising a nucleic acid sequence coding the desired antigen of a pathogen operably linked to suitable promoter sequences and other suitable control sequences enabling the expression of said nucleic acid sequence by the cells of the patient after administration thereof. A DNA vaccine may also be carried in a live host microorganism being involved in the delivering the genetic material to the patient.

Various DNA vaccines to combat fish pathogens are known. Heppell et al. reports of the protection against live virus after injection with a DNA vaccine comprising viral haemorrhagic septicaemia virus G and N genes cloned into an expression plasmid and injected into rainbow trout (Fish and Shellfish Immunology, 1998, vol 8, issue 4, pp 271-286). Lorenzen and LaPatra describes a DNA vaccine against a fish rabdovirus in "DNA vaccines for aquacultured fish", Rev. sci.tech. Off int. Epiz., 2005 (1), 201-213. A DNA vaccine resulting in protective effects is further more reported by Mikalsen et al., 2005, "Protective effects of a DNA vaccine expressing the infectious salmon anaemia virus hemagglutinin esterase in Atlantic salmon", Vaccine, 23:30, pp 4895-4905.

In EP 1 818 406 A1, a DNA expression system that may be used to produce a DNA vaccine that prevents infectious disease in aquatic animals is described, wherein the expression is directed by a cytomegalovirus promoter.

WO 2007/31572 discloses inter alia a DNA vaccine useful to combat SAV infections in salmonid fish.

WO 2009/002376 discloses a DNA vaccine useful to combat viral infection in carp (Spring viremia of carp virus).

EP 2 011 876 regards the development of a DNA fish vaccine useful to combat KOI Herpes disease that affects carps.

Based on the prior art and the common general knowledge referred to above, the skilled person is able to construct a DNA vaccine comprising the open reading frame 1 or 3 of the present invention or fragments or variants thereof utilizing methods commonly used in the field of genetic engineering.

5 DNA vaccines consisting of naked plasmid comprising the open reading frames 1 or 3 or fragments or variant thereof may be administered to fish by intramuscular injection or particle mediated deliver by gene gun. The latter involves the coating of small gold particles with the DNA vaccine, and the administration is then effected by air-pressured mediated intradermal delivery. Intramuscular injection of a DNA
10 vaccine according to the present invention is preferred as it is more cost effective than the gene gun method, cf. Lorenz and LaPatra, *supra*. Methods for the administration of DNA vaccines to fish is furthermore disclosed in "Intramuscular Injection of DNA vaccines in Fish" by Heppel and Davis in "DNA Vaccines: Methods and Protocols", Methods of Molecular Medicine, 2000, vol. 29 (ISBN 978-
15 0-89603-508-5).

Antibodies and immunoassays

The recombinant proteins according to the present invention may be used to obtain antibodies capable of binding a CMS virus and thus being useful in immunological
20 methods designed for the determination of CMS virus in biological samples.

Another aspect of the invention thus relates to an antiserum or an isolated antibody or antibodies (monoclonal or polyclonal) which selectively binds to a CMS virus or to a component or part of said virus. Antiserum is conventionally obtained for instance by immunising a laboratory animal with the appropriate antigenic
25 determinant. Once the concentration of antibodies in serum from the animal reaches a desired level, the animal is bled. The serum thus obtained should contain antibodies produced in response to the immunogenic stimulus.

Likewise, techniques for the preparation of antibodies are known to the skilled person. The techniques include the traditional hybridoma technology and alternative
30 techniques such as mRNA display, ribosome display, phage display and covalent display.

In some embodiments of the invention the isolated antibody comprises a marker, e.g. a radiolabel, a fluorescent tag, a chemiluminescent label or an enzyme. The antibody may be a polyclonal or monoclonal antibody. Protocols for the labelling of
35 antibodies by coupling of the various markers mentioned above are well known to the skilled person.

For sufficient protection and control of the disease caused by CMS virus, means for efficient diagnosis of CMS virus is needed. The present invention therefore provides immunoassays, useful for the diagnosis of CMS virus in fish. An

immunoassay within the meaning of the present invention is a biochemical test that measures the presence and/or concentration of the CMS virus in a biological sample, such as e.g. serum. An immunoassay thus utilise the specific binding of an antibody to its antigen. Both the presence of antigens (e.g. present on the CMS virus) and the presence of antibodies against the CMS virus may be measured in the biological sample.

The present invention further provides an immunoassay for detection of CMS virus in a biological sample, comprising (a) contacting the recombinant proteins according to the present invention and a biological sample suspected to comprise antibodies against a CMS virus; and (b) determining whether the one or more antibodies binds to a CMS virus present in the biological sample wherein said binding represent an indication that the sample contains a CMS virus.

According to another aspect of the invention, an immunoassay for detection of CMS virus in a biological sample is provided, comprising (a) contacting one or more antibodies that specifically recognise and binds to a recombinant protein according to the invention and a biological sample suspected to comprise CMS virus; and (b) determining whether the antibody binds to CMS virus present in the biological sample wherein said binding represent an indication that the sample contains a CMS virus.

The complex formed in the immunoassays according to the present invention, e.g. the binding of an antibody to a CMV virus present in a biological sample or the binding of an antibody present in a biological sample to a recombinant protein or fragment thereof according to the present invention, may be determined by means of e.g. densitometry, fluorimetry or colorimetry or the like.

The immunoassays of the invention as described above may be a standard Enzyme-linked immunosorbent assay (ELISA). The skilled person will be able to provide ELISA protocols using antibodies developed against the recombinant proteins according to the present invention, or the recombinant proteins. Reference is in this respect made to the ELISA Guidebook, Methods in Molecular biology, vol. 149, ed. By John R Crowther, Humana Press, 2001.

The present invention also provides a diagnostic kit for carrying out the immunoassays according to the present invention. According to one embodiment, the kit according to the invention comprise antibodies recognizing a recombinant protein according to the invention and usual means for carrying out an immunoassay, such as e.g. wells of an ELISA plate, reagent to produce a colour reaction upon the binding of the antibodies to a CMS virus in a biological sample to be tested. According to another embodiment, a kit is provided comprising one or more recombinant proteins according to the invention and usual means for carrying out an immunoassay, such as e.g. wells of an ELISA plate, reagent to produce a

colour reaction upon the binding of said recombinant proteins to antibodies in a biological sample to be tested.

Primer sequences and method of detection of CMS virus infection

5 The nucleic acid sequences of the present invention may furthermore be used to detect the presence of CMS virus in fish. The present invention therefore also relates to a diagnostic method wherein primers specifically hybridize to a nucleic acid in a test sample that is similar to the nucleic acid sequences of the invention. Such tests may take the form of a PCR test or a hybridization test without
10 amplification.

The detection of nucleic acids present in a biological sample is widely applied in both human and veterinary diagnosis, wherein nucleic acids from e.g. pathogens present in biological samples are isolated and hybridized to one or more primers. The one or more primers to be used in hybridization based detection methods is
15 constructed so that they are able to specifically bind to a nucleic acid sequence if present in the sample to be tested. The primers may be linked to a signalling unit, such as e.g. a radiolabel, a luminescent molecule or a fluorescent molecule enabling the visualisation of the binding of the primers to a target sequence.

A primer as used herein refers to a nucleic acid sequence, which can be used as a
20 probe in a method of detection of the presence of CMS virus in fish. The primers according to the present invention consist of at least 10, preferably at least 15, and more preferably at least 20 nucleotides that are able to hybridize to nucleic acid sequences isolated from a CMS virus.

The primer according to the present invention is able to hybridized to another
25 nucleic acid molecule, such as genomic RNA originating from CMS virus or cDNA prepared from said genomic RNA, under appropriate conditions of temperature and solution ionic strength, cf. e.g. Sambrook et al., Molecular Cloning: A laboratory Manual (third edition), 2001, CSHL Press, (ISBN 978-087969577-4). The condition of temperature and ionic strength determine what the skilled person will
30 recognise as the "stringency" of the hybridization. The suitable stringency for hybridisation of a primer to target nucleic acids depends on inter alia the length of the primer and the degree of complementation, variables well known to the skilled person. A minimum length of a hybridisable primer is usually at least about 10 nucleotides, preferably about 15 nucleotides and more preferably about 20
35 nucleotides.

Various protocols for detection of nucleic acid sequences by PCR are available to the skilled person; cf. e.g. Methods in Molecular Biology, vol. 226, PCR protocols, second ed., Ed. Barlett and Stirling, Humana Press, 2003. The skilled person will furthermore, with the knowledge of the genomic CMS virus sequence depicted in

figure 1 (SEQ ID No. 1) be able to provide suitable primers for the purpose of using said primers in a method for detection of the presence of CMS virus according to the present invention. Reference is in this respect made to the textbook PCR Primer Design, vol. 402 of the series Methods in Molecular Biology, Ed. A. Yuryev, 2007, Humana Press.

According to one embodiment of the invention, the following primers are provided:

5'-TCCAGTGCCTTGATGTCTG-3 (SEQ ID NO. 8)
5'-CATCTCCATCCGCTAAGTACG-3 (SEQ ID NO. 9)
5'-TGCCTGTCGTTGAGTTTAGC-3 (SEQ ID NO. 10)
5'-CCCGAATGAAGCAAGATGG-3 (SEQ ID NO.11)
5'-GAAAGCCCAGACTCAGGATG-3 (SEQ ID NO. 12)
5'-ACACCAGGTGACCGAAAAG-3 (SEQ ID NO. 13)
5'-ACATGGTGCGAGGTAACGAC-3 (SEQ ID NO. 14)
5'-AGTTCCTGCCCGTAGATGG-3 (SEQ ID NO. 15)
5'-GGCGAGAATGGTGTGTTGTG-3 (SEQ ID NO. 16)
5'-CCTGGTCTCACTCCCAAGAG-3 (SEQ ID NO. 17)
5'- AGGGAACAGGAGGAAGCAGAA -3 (SEQ ID.NO 18)
5'- CGTAATCCGACATCATTTTGTG -3 (SEQ ID NO. 19)
5'- GGAAGCAGAAGTGGTGGAGCGT-3 (SEQ ID NO. 20)
5'-CCGGTTTTGCGCCCTTCGTC-3 (SEQ ID NO. 21)

10 Experiments

Example 1. Identification of virus sequences.

Although isolation of the virus causing CMS was reported in patent application NO2008 2869, identification of the genome of this virus has proven difficult. The virus is cultivatable in GF-1 cells, but the output of virus is very low in this system. Many different methods for cloning sequences from unknown viral genomes have been tested, but none with success.

Previous studies indicate that CMS is a chronic disease developing over several months prior to the terminal clinical phase. Consistent histopathological changes are observed only in the heart, and sometimes histopathological changes are observed in the liver as well. Nothing is known about the distribution of virus at the different

stages of the disease. It is often difficult to give salmon a clear CMS diagnosis, as many other diseases give the same clinical signs i.e. pancreas disease and heart and skeletal muscle inflammation (Kongtorp RT, Halse M, Taksdal T, Falk K, J Fish Dis. 2006 Apr;29(4):233-44.

- 5 Gf-1 cells were infected with homogenate made from Atlantic salmon originating from an aquaculture site experiencing disease problems compatible with CMS. After 14 days, cell cultures started to show mild cpe (cytopathogene effect), and this developed into a stronger cpe after 21 days. The cpe was not possible to reproduce in more than three passages, and became weaker at each passage.
- 10 50 ml of supernatant from infected cultures were collected, passed through a 0.22 μ m filter and centrifuged at 100 000 x g for three hours to collect the virus as a pellet. After removal of almost all supernatant from the resulting virus pellet, the pellet was resuspended, and the volume adjusted with fresh cell culture medium resulting in 500 μ l virus suspension. This was digested with 250U DNase I and 5 μ g
- 15 RNase A in RDD-buffer (included with Qiagen DNase I) for 1h at 37°C.

- RNA was prepared from the sample using QIAamp viral kit (Qiagen) according to the manufacturers instructions. Single stranded cDNA was synthesized using SuperScript®III reverse transcriptase kit (Invitrogen) and random primers with a fixed 5' overhang (FR26RVN primer 5' GCC GGA GCT CTG CAG ATA TCN
- 20 NNN NN 3'; Djikeng et al., BMC Genomics 2008, 9:5). The RNA was incubated at 95°C prior to cDNA synthesis to avoid problems caused by a possible secondary structure of the RNA. After synthesis the RNA strand was digested using RNase H before double stranded DNA was made using a combination of the 2nd strand buffer components of the Universal RiboClone® cDNA Synthesis System (Promega) and
 - 25 Exo-Klenow polymerase and including additional supply of the random primers. A single primer PCR reaction using primer against the random primer fixed overhang (FR20RV 5' GCC GGA GCT CTG CAG ATA TC 3'; Djikeng et al., BMC
 - 30 Genomics 2008, 9:5) and standard *Taq* polymerase (Invitrogen) was performed (initial denature of cDNA 95°C for 5 minutes, 40 cycles of 94°C for 30 seconds, primer annealing 60°C for 15 seconds and synthesis 72°C for 1 minute and then
 - 35 final synthesis 72°C for 10 minutes), and the resulting products were separated by 1% agarose gel electrophoresis. PCR products in the size range 500 – 1000 bp were excised from the gel in one piece, purified and cloned using the TOPO-TA cloning system (Invitrogen). Approximately 300 clones were sequenced and subjected to analysis.

Sequences with no significant virus host cell/tissue related hits after NCBI Blastn or BlastX analysis were subjected to analysis in the ContigExpress program of Vector NTI software, resulting in identification of two different sequences without overlap. One sequence included two contigs (contig 1 = 2,4 kB, contig 2 = 4,0 kB with an

initial BlastX hit of RNA polymerase of several totivirus (best hit $E=1e-04$ over appr. 430bp).

5 The smallest sequence contig 1 (2,4kb) showed no homology to other known sequences, while the largest sequence showed weak homology with the RNA polymerase from a totivirus in a 870bp region (Expect = $7e-15$, amino acid identities = 81/312 (25%), Positives = 138/312 (44%), Gaps = 32/312 (10%). The assembly of both sequences showed various numbers of individual clone sequences in different regions resulting in low quality overlap in some regions. When
10 analyzing the sequences by PCR, it became evident that some parts of the sequences are difficult to amplify by PCR, indicating strong secondary structures in these regions. Especially PCR in the region between the end part of ORF 1 and the first part of ORF2 is predicted to contain strong secondary structures since PCR in this region is very inefficient and difficult to perform (approximately nucleotide 2800 – 3200). [Without being bound by any specific theory, it is believed that this may
15 partly explain the difficulties experienced with the isolation and characterization of the CMS virus genome].

A sequence joining the two sequence parts was identified by PCR using primers. Two primers, PMCV-bridge F (5'-AGCAGGAGCAACAGCACAC-3') and PMCV-
20 bridge R (5'-TACCCGCCAAATGGTACTTC-3'), were designed to span the region between the two Contigs, resulting in a product of 96 bp. By manually going through the unassigned sequences obtained, a sequence 3' of contig 2 was identified. The 5' and 3' ends of the genome were identified using 5' and 3' RACE.

25 Finally, a genome of 6688 bp was assembled, containing three open reading frames. This is the first time a putative member of the *Totiviridae* is identified in a vertebrate. The finding of reading frame 3 (SEQ ID No. 6) was also very surprising. No other known *Totiviridae* members have this open reading frame.

30 Example 2

Expression of recombinant protein in a prokaryot expression system.

ORF1

35 Gene sequences from open reading frame 1 was made synthetically as an E.coli-optimised sequence and cloned into a pET vector system using standard methods. A suitable E.coli host cell was transformed, and recombinant protein expressed and purified as disclosed in details below.

ORF1 and a partial ORF1 consisting of the first 852 nucleotides (ORF1 (1-852)) in the open reading frame was cloned into the vector pET14b (Novagen) using standard techniques. The plasmid was transformed into OneShot®

- 5 BL21(DE3)pLysS Chemically competent *E.coli* from Invitrogen (cat no C606003). For preparation of a pre-culture, 2 colonies of each transformants were picked and grown in 3ml LB + antibiotics ((Ampenicillin (50mg/ml), Chloramphenicol (34mg/ml)).

- 10 After growth for about 7,5hours, 1ml of each pre-cultures were transferred to 50ml pre-warmed medium containing antibiotics.

The cultures were induced by the addition of IPTG (0,5mM) after about growth for 2 hours at an OD between 0.7 and 1 and the temperature was lowered to 16 °C. The cultures were grown over night at this temperature.

Construct	OD induction	OD harvest
ORF1(1-852)	0,8	3,09
ORF1(1-852)	0,78	3,18
ORF1	0,7	4,2
ORF1	0,76	4,2

15

The cultures were harvested 15h after induction and the bacteria were pelleted at 3300xg for 20min. The supernatants were discarded and the pellets stored at -20 °C until purification of inclusion bodies. The isolation of inclusion bodies was done using protein extraction reagents B-PER® (Pierce) according to the manufacturer's instructions.

20

The isolated proteins separated by gel electrophoresis using the Phast gel system according to the manufacturer's instructions (GE Healthcare Life Sciences) are shown in figure 9A.

25 B. ORF3

An expression vector for ORF3 was constructed using a vector system with a Pm/xyIS induction system (Blatny, J. M., Brautaset, T., Winther-Larsen, H. C., Haugan, K., og Valla, S. 1997a. Construction and use of a versatile set broad-host-range cloning and expression vectors based on the RK2 replicon. Appl. Environ. Microbiol. 63:370-379, Blatny, J. M., Brautaset, T., Winther-Larsen, H. C.,

30

Karunakaran, P., og Valla, S. 1997b. Improved broad-host-range RK2 vectors for high and low regulated gene expression levels in gram-negative bacteria. *Plasmid* **38**:35-51).

5 The vector had the cop 271 version of the replication protein TrfA, giving a copy number of 15-30 in *E. coli*, and a c-myc and 6xHis tag fused to the 3' end of ORF3.

The plasmid was transformed into *E. coli* RV308 and fermented according to Sletta et al 2007 (**Sletta, H., Tøndervik, A., Hakvåg, S., Aune, T. E. V., Nedal, A., Aune, R., Evensen, G., Valla, S. og Brautaset, T.** 2007. The presence of N-terminal secretion signal sequences leads to strong stimulation of the total expression levels of three tested medically important proteins during high-cell-density cultivations of *Escherichia coli*. *Appl. Environ. Microbiol.* **73**:906-912.)

10 Inclusion bodies were isolated from the fermentate using protein extraction kit B-PER® (Pierce) according to the manufacturer's instructions. The isolated proteins separated by gel electrophoresis using the Phast gel system according to the
15 manufacturers instructions (GE Healthcare Life Sciences) are shown in figure 9B.

Example 3

Expression of recombinant protein in a eukaryotic expression system.

20

Gene sequences from open reading frame 1 and 3 was cloned into a vector system using standard methods. A suitable eukaryotic host cell was transformed, and recombinant protein expressed and then purified as described in details below.

25 A partial ORF1 consisting of the first 852 amino acids of the open reading frame and all of ORF3 was cloned into the vector pcDNA4 (Invitrogen) using standard techniques. HeLa cells grown on coverslips were transfected using FuGENE® (Promega) according to the manufacturer's instructions. To determine whether the open reading frames were expressed, transfected cells were fixed in 3%
30 paraformaldehyde for 15 minutes, washed in PBS and quenched with 50 mM NH₄Cl for 15 min. washed in PBS and incubated with primary anti-His antibody (Antibody commercially available from Qiagen, 1:100) for 2 hrs at room temperature. After washing with PBS, samples were incubated with secondary Ab commercially available from Jackson ImmunoResearch Laboratories, Inc. for 45
35 min (Goat a-mouse IgG-CY3 1:500 and Donkey a-mouse IgG-CY3 1:500, respectively). After washing with PBS, the coverslips were dipped in ddH₂O before mounting in a high quality polyvinyl alcohol anti-fade medium Mowiol® (Polysciences, Inc.) containing 2ug/ml Hoechst for staining of the nucleus.

No structures were seen one day after transfection. After two days of transfection ORF1(1-852)-transfected cells showed rather big structures containing the expressed His-tagged protein. Fewer cells transfected with ORF3 showed the same structures. The negative control cells showed no specific staining. This shows that the ORF1 and ORF3 proteins can be expressed in a eukaryotic expression system.

Example 4

Immunogenicity of recombinant ORF 1 proteins

Rabbits were immunized with recombinant ORF 1 produced as described above in example 2 using standard methods. Rabbits were injected with 60-100mg protein, and boosted three consecutive times. Serum was harvested after 10 weeks, and tested in Western blotting.

Heart homogenate from Atlantic salmon diagnosed with CMS and heart homogenate from healthy fish was subjected to PAGE and blotted to a PVDF (polyvinylidene fluoride) membrane using a PhastGel® apparatus (GE Healthcare). The membrane was incubated with antiserum (1:250) for 120 minutes, washed in PBS and incubated with a secondary antibody coupled to alkaline phosphatase (1:500, DAKO, Denmark) for 60 minutes. The blot was visualized with NBT-BCIP (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) (Promega) according to the manufacturer's instructions.

The Western blots show the presence of a specific band with molecular weight of approx 90kD in the lane with heart homogenate from Atlantic salmon diagnosed with CMS (Figure 10). This corresponds well with the estimated molecular weight of ORF1. These results show that recombinant ORF1 protein elicits an immune response producing an antiserum recognizing a protein from the CMS virus. Thus, ORF1 is a good candidate for antigen in a vaccine against CMS.

30

Example 5.

Immunogenicity of recombinant ORF 3 proteins.

Recombinant ORF 3 protein was produced as described in example 2 and subjected to PAGE and blotted to PVDF (polyvinylidene fluoride) membranes using a PhastGel® apparatus (GE Healthcare). The membranes were blocked for 2 hours at 37°C in TBS-0,05% tween (TTBS) containing 5% skimmed milk powder.

35

Fish serum from either Atlantic salmon diagnosed with CMS or a healthy control fish was diluted to 1:2 with TTBS+5% skimmed milk powder and the membrane was incubated O/N at 4°C. The membranes were washed 3x5min in TTBS+1% skimmed milk powder before 1 hr incubation with 4C10 mouse anti-trout IgM (1:1000) in 3x5min in TTBS+1% skimmed milk powder at RT. The membranes were washed 3x5 min in TBS-tween before 1 hr incubation with Goat-anti-mouse IgG-AP (1:500) in TTBS only. The membrane was then washed 3x5 min in TTBS before incubation at RT for 5 minutes in NBT/BCIP substrate buffer before NBT/BCIP (Promega) was added to visualize the proteins.

10

The serum from the Atlantic salmon diagnosed with CMS recognised the recombinant ORF3 proteins, while the serum from the healthy fish did not (Figure 11). This shows that the recombinant ORF 3 protein has common epitopes with the virus causing CMS. Thus, ORF3 is a good candidate for antigen in a vaccine against CMS.

15

Example 6

DNA vaccine protecting against CMS

Open reading frame 1(nucleotide 1-852) and 3 were cloned into a suitable DNA vaccine vector, pcDNA4 (Invitrogen) using standard techniques. The resulting vector was injected intramuscularly into Atlantic salmon proven to be free of CMS virus in a dose of 100 µg/fish. After an immunization period of 6 weeks at 12 degrees Celsius, the fish were i.p. challenged with salmon cardiomyopathy virus. A group injected with PBS instead of vaccine was included as a control. Tissue samples from challenged vaccinated and unvaccinated fish were collected for analysis by Real Time RT-PCR to assess the viral load in the kidney three weeks after challenge. The samples were kept in RNeasy kit® (Ambion) until analysis. RNA was extracted using the RNeasy kit® (Quiagen). All RNA samples were diluted to 50 ng/µl prior to cDNA synthesis. For cDNA synthesis and Real Time PCR, the SuperScript® III Platinum® Two-Step qRT-PCR Kit with SYBR® Green was used. For the Real Time PCR, the primers PMCV ORF2-3F/ORF2-3R (ORF2-3F= (5'-GGAAGCAGAAGTGGTGGAGCGT-3') and ORF2-3R= (5'-CCGGTTTTGCGCCCTTCGTC-3') were used, and the cycling conditions were: 50°C – 2 min, 95°C – 2 min, (95°C – 15 sck, 60°C – 1min) x 45 cycles.

35

Since the highest viral load is found in the kidney throughout the CMS virus infection, this was used as a measure for vaccine efficacy. The viral load in the kidney of the infected fish and control fish reported as cycle threshold (Schmitgen

TD, Liyak KJ, Nat. Protoc. 2008; 3(6): 1101-8 “Analyzing real-time PCR data by the comparative C(T) method”), Ct were as follows:

	Ct average
ORF1(1-852)	27,33
ORF3	26,27
PBS control	24,62

- 5 A Ct value difference of 3 represents a 100 fold difference in viral load. The average Ct value from analysis of kidney tissue for the control group was 24,62. Both the group vaccinated with ORF1 (1-852) and ORF3 had higher average Ct values than the control. A higher Ct value means that the viral load was lower in the vaccinated groups than in the control group. This result shows that the ORF1(1-852) and ORF3 DNA vaccine constructs were effectively lowering the viral load in the fish and thus has a potential as DNA vaccines against CMS.
- 10

Example 7

PCR diagnostics

15

The following primers have been tested and found suitable for PCR diagnostics for the CMS virus

PRIMER	FORWARD (5' – 3')	REVERSE (5' – 3')	PROD. LENGTH
PMCV	ACACCAGGTGACCGAAAAG	TCCAGTGCCTTGATGTCTG (SEQ ID NO. 8)	237 bp
A01	ACATGGTGCGAGGTAACGAC	CATCTCCATCCGCTAAGTACG (SEQ ID NO. 9)	124 bp
B04	AGTTCCTGCCCCGTAGATGG	TGCCTGTCGTTGAGTTTAGC (SEQ ID NO. 10)	531 bp
F02	GGCGAGAATGGTGTGTTGTG	CCCGAATGAAGCAAGATGG (SEQ ID NO. 11)	269 bp
A09	GAAAGCCCAGACTCAGGATG	CCTGGTCTCACTCCCAAGAG (SEQ ID NO. 17)	242 bp
ORF2-3	GGAAGCAGAAGTGGTGGAGC GT	CCGGTTTTGCGCCCTTCGTC (SEQ ID NO. 21)	107 bp

It is also possible to design Real Time PCR methods using primers and probes, for instance

PRIMER	FORWARD (5' – 3')	REVERSE (5' – 3')	PROBE
PMCV-F2	AGGGAACAGGAGGAAGCAGAA	CGTAATCCGACATCATTTTGT GA (SEQ ID NO. 19)	TGGTGGAGCG TTCAA

5 The 5' half of the virus genome is much richer in GC content than the 3' part. We predict that PCR in the 3' part of the genome is more efficient than in the 5' end. There is also a part in the middle of the genome in the region between and partially overlapping with ORF1 and ORF 2 forming a pseudoknot, thus making PCR in this region difficult.

10 Example 8

Clinical CMS correlates with presence of PMCV in heart tissue.

15 To examine whether clinical CMS in farmed fish coincides with the presence of PMCV in heart tissue, samples were collected from three fish farms. Two of these (1 and 2) had a CMS diagnosis, while site three had no prior history of CMS. Real-time RT-PCR on field samples was run on the MXpro 3000P (Stratagene) with all reactions performed in triplicate using the following primers:

ORF2-3F (5'-GGAAGCAGAAGTGGTGGAGCGT-3') and
20 ORF2-3R (5'-CCGGTTTTGCGCCCTTCGTC-3').

25 Fish collected from Sample 1 (at slaughter) were found with histomorphological changes typical of CMS with scores ranging from 0.6 to 4 (covering the entire scale; Fig. 12). The relationship between histoscores and Cp values as assessed by real-time RT-PCR shows that there is a clear tendency that higher virus amount being found in heart tissue with high histoscores ($r^2=0.76$). As it has been suggested that the biggest fish die most frequently from CMS the Cp values were also plotted against the condition factor ($\text{weight} \times 100 / \text{length}$) of the fish without any correlation being found ($r^2=0.08$).

30 For Sample 2 (clinical CMS), 10 out of 10 fish were positive by real-time RT-PCR and all showed typical histopathological changes of CMS.

For Sample 3 (previous history of HSMT), no fish were found positive for PMCV by real-time RT-PCR while all were found positive by real-time RT-PCR for PRV. None of the fish had changes typical of CMS.

- 5 These findings show that clinical cases of CMS correlate with presence of PMCV in heart tissue and further substantiate that PMCV is the causative agent of CMS.

CLAIMS:

1. An isolated nucleic acid molecule from the Fish Cardiomyopathy Syndrome (CMS) virus comprising a sequence selected from the group consisting of: SEQ ID No. 1; SEQ ID No. 2;
5 SEQ ID No. 4; SEQ ID No. 6; a sequence having at least 70% identity with the full length of SEQ ID No. 2; a sequence having at least 70% identity with the full length of SEQ ID No. 4; and a sequence having at least 70% identity with the full length of SEQ ID No. 6.
2. The isolated nucleic acid molecule according to claim 1, wherein the sequence has at
10 least 80% identity with the full length of the nucleic acid selected from the group consisting of SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 6.
3. A vector comprising the nucleic acid molecule according to claim 1 or 2.
- 15 4. A vector comprising 1) a sequence selected from the group consisting of: SEQ ID No. 2; SEQ ID No. 6; a sequence having at least 70% identity with the full length of SEQ ID No. 2; and a sequence having at least 70% identity with the full length of SEQ ID No. 6 operatively linked,
2) to control sequences directing the expression of said sequences.
- 20 5. The vector according to claim 3 or 4, wherein the vector is a pET vector.
6. An isolated host cell comprising the vector according to claim 4 or 5.
7. A DNA vaccine comprising a nucleic acid molecule comprising: a sequence selected
25 from the group consisting of SEQ ID No. 2; SEQ ID No. 6; a sequence having at least 70% identity with the full length of SEQ ID No. 2; and a sequence having at least 70% identity with the full length of SEQ ID No. 6.
8. A recombinant protein encoded by a nucleic acid molecule comprising a sequence
30 selected from the group consisting of: SEQ ID No. 2; SEQ ID No. 6; a sequence having at least

70% identity with the full length of SEQ ID No. 2; and a sequence having at least 70% identity with the full length of SEQ ID No. 6.

9. The recombinant protein according to claim 8 comprising an amino acid sequence
5 selected from the group consisting of SEQ ID No. 3 and SEQ ID No. 7.

10. A recombinant vaccine comprising at least one recombinant protein according to claim 8 or 9.

10 11. An antibody recognising and specifically binding to the recombinant protein according to claim 8 or 9.

12. The antibody according to claim 11, wherein said antibody is a monoclonal antibody.

15 13. The antibody according to claim 11, wherein said antibody is polyclonal.

14. An immunoassay for detection of specific antibodies against Fish Cardiomyopathy Syndrome (CMS) virus in a biological sample, comprising the following steps:

(a) contacting at least one of the recombinant proteins according to claim 9 and a
20 biological sample suspected to comprise antibodies against a CMS virus; and

(b) determining whether the at least one recombinant protein binds to a CMS virus specific antibody present in the biological sample wherein said binding represent an indication that the biological sample has been in contact with CMS virus.

25 15. An immunoassay for detection of Fish Cardiomyopathy Syndrome (CMS) virus in a biological sample, comprising the following steps:

(a) contacting one or more antibodies that specifically recognise and bind to the recombinant protein according to claim 9 with a biological sample suspected to comprise CMS virus; and

(b) determining whether the one or more antibodies binds to CMS virus present in the biological sample wherein said binding represent an indication that the sample contains a CMS virus.

5 16. The immunoassay of claim 15, wherein the antibody is a monoclonal antibody.

17. A primer comprising a sequence of at least 10 nucleotides, wherein said primer hybridizes to a nucleic acid molecule originating from CMS virus having a sequence selected from the group consisting of: SEQ ID No. 1; SEQ ID No. 2; SEQ ID No. 4; SEQ ID No. 6.

10

18. The primer according to claim 17, wherein said primer is at least 15 nucleotides.

19. The primer according to claim 18, wherein said primer is at least 20 nucleotides.

15 20. The primer according to claim 17 or 18, wherein said primer is selected from the group consisting of SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17 SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20 and SEQ ID No. 21.

20 21. A method for detection of the Fish Cardiomyopathy Syndrome (CMS) virus in a biological sample characterised by comprising the following steps:

a) preparing a mixture of a biological sample comprising nucleic acid molecules isolated from a biological sample suspected to comprise CMS virus for a reverse transcription reaction with a specific or random primer;

25 b) subjecting the mixture of a) to a polymerase chain reaction with a primer pair specific to the reverse transcribed nucleic acid sequence corresponding to the genome sequence of the virus, wherein each primer of said primer pair hybridize to a nucleic acid molecule from CMS virus having a sequence selected from the group consisting of: SEQ ID No. 1; SEQ ID No. 2; SEQ ID No. 4; SEQ ID No. 6; a sequence having at least 70% identity with the full length of
30 SEQ ID No. 2; a sequence having at least 70% identity with the full length of SEQ ID No. 4; and a sequence having at least 70% identity with the full length of SEQ ID No. 6; and

c) determining whether the binding of the primers to nucleic acid molecules in the sample and amplification of the sequence between them has occurred indicating the presence of CMS virus in the sample tested.

5 22. The method according to claim 21, wherein each primer of the primer pair is selected from the group consisting of SEQ ID No. 8, SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15, SEQ ID No. 16, SEQ ID No. 17 SEQ ID No. 18, SEQ ID No. 19, SEQ ID No. 20 and SEQ ID No. 21.

10 23. A diagnostic kit for the detection of a Fish Cardiomyopathy Syndrome (CMS) virus comprising one or more primers according to any one of claims 17-20 and instructions for use thereof.

15 24. A use of the isolated nucleic acid molecule according to claim 1 or 2 for the preparation of a DNA vaccine, recombinant vaccine or a live recombinant microorganism.

20 25. The isolated nucleic acid molecule according to claim 1, wherein the sequence has at least 90% identity with the full length of the nucleic acid selected from the group consisting of SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 6.

26. The isolated nucleic acid molecule according to claim 1, wherein the sequence has at least 95% identity with the full length of the nucleic acid selected from the group consisting of SEQ ID No. 2, SEQ ID No. 4 and SEQ ID No. 6.

Figure 1.

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1  AAAAAGACAA CAGAAAAACC GAGAAAATCT CTCTTTTTC TGAACATAAC TGGTGCATAG
61  TTCAGTAACA AACCATCCGC TTTGCAACAG GGAAGCGGGC CGTAACCTCA GTGGTGACAT
121 GGGGAACGGA CATTAGCTTA CTCCGCTAAG GGAGTGAGGA GACCTGACTG AGGAAGTAGG
181 ATGCACCTCA GGACTTAGAA TTCGGTTTAG TCATCTATGG TAGTGACCCC AAAACGACTA
241 GTTCGGGAAC GGGGGACCCC ACTGCCGCAG GGGTATCGAC GGGCTACTAC ACCCACTTCA
301 GCCTTCCCTC CGGGGGGGGT AGAAAGGTGG GTCTGAGCGGT GAGGAGAAAG GTTGTCAAAAT
361 TAAAACAACT CTCCGACTGC GATCTTATTT CGGTAGTCCC GGGCCGGCGT TGATACTCAG
421 GTAAGGTCCG GATGTCAACC AACTATGGAA CCAAACACAT CTGTCATTGC AACGGAGCAG
481 CAGCAGGCTG CCATGAGAGA GGTGGAGGCC GAGGCGGCGG CCAGAGACGA AGTGGTGGAG
541 AAGATCGCAT TCGCTGAAGG AGCGATGATG GTACAGACGA GGAGGTACC ATCAGGAAAG
601 TCGTCGGTAG GAGGTTTTCT CGGCGAAGTG GCACAGAACA TACGTGCCAT GAATCGGTCA
661 TTGCACACAG ATACCAACAT GCTGACCGAA GGGGCGATGG TGGACAGAGC GAGGGCAAAA
721 GTACACAAAA TCATTAGGGA AGGGAATTTG GACTCTAGGG TATTTTCAAA CACGGGGAGC
781 AACACTATGT TGTCACGTGT GGTACCAGCA GTACCGGGAC CACCGGCAGT ACCGGAGCAT
841 TGGGACGTTG CGCCGTCCTG GTTCGTATGC AGACCGGGGA AAAAGGGGGG GATAAAGATC
901 ACACAAAGCG CATCAATGGC AGCATTAAC CCACTATTTA GAGGCGCAGA CGTGGGGCCA
961 ATCGGGACAG CAGTCAGGGC GGATGTAAAC GCATTTTCAA TGAATGCAGT TCTGGGAGCA
1021 CTAAGAGCCG GGGGATTTAA CACCGAACAT TCCCTGGTGT CATTCGTTGA ACCACTAATT
1081 CGGATCTTGC TAATGGGGGT ACAAACACAA GACAGGGGGA CCAGCCATG GGATTGGGTT
1141 GGAGGGATGA GTTCGCGAAT AGTCAATCCC CTAGTATTCA CAACAAGCGG GAACTTCTTC
1201 CCAGGGGGAC CAAATTTGAG GGTGTGGGGA GCCAACGATA CAGTGGCCAG GATAGTAAAC
1261 GTTGAGGACT ACATGCGCGA GGC GGCCGGG GAGGGGAGGT TCGACGCTGG ATGGGGACCG
1321 GAATTCTGGG GTGGGACAGG GGACGACGCA GTGGCGGTGG TACCGATAAG GGCAGTAGAA
1381 GCAGGGCTAG GAGAAGTAAA CGCAGGGTGG ACATTGGCAC ACATGGAATA CCCAGTCAAG
1441 GTTAGACTAC TTGACGTCGA CGACCGAACA ATTGGACCAG GGGGGAGCCT GCCCTAAAC
1501 GCAAACAGAG AATACACGGC GGCAGGAGCT ACGCATGTAC CCGGGCCCTA TGCCAGGGTA
1561 CTGTACGTCG TCGTGGACCA AAACGCAGAG AGGTGTGTGG GGGTGAGAGT GCAGGGACAG
1621 GGTGCTGTAA TTGACGTGGA TCCGGCGTTG AATTACGTGA TAGGGGGAGC GGATTGGGG
1681 ATGTTGCCGT TGATACAGTG GAGTGTAGGG CTGGGGGGCCG AGGACATGGC GCAGGGATCG
1741 ATTGCACAGA CGCAGCGATG GGTGAGGATG TATGGAAACG AGGACGATTG GGAATCAGCG
1801 TGGCATCTAG TGTCTAGCGC GTACACAGTG TACAGCCCGG CATTCAGGAG ATCGGGTGTC
1861 GCAGTGAGAG GAGGATTCTG GCGCAACCA GCTGCAGGGG CAGCACCGTT TCCACTAGGA
1921 GGATTGGCAG GGTGGGTGAG GTACGACAAT CAGGCACGGG CGGCGCAGGT TGCACTTTGC
1981 AGAGAGAGGG CGGATATGGC GGAGTGTCTT TGGGGGGGGT ACAGGGAGAG AGGGGTGAGA
2041 CCGGGGAGTG TGGCAAACCT GCAGTACGTA AGGTTCGATC CCACAGTGGC TGTAGGAGTA
2101 GCTGCTCACT TCTGGTCGGT AGTGAAGGTG ATGGTGGCTC CCGTCCAGA CAGAGCGGCT
2161 GCTCTGGCGG ACATGGCGTG GGGGAAGGGG AAGGTGCAAG CCATGGGTGA GGATGTGATC
2221 AACGGGCAGA TGGGACAACC TGAGTCCATG ATGAGAGGGG TGGCGCTGAA CGAGAACCAG
2281 GGAAGTACGG CGGCTACAGT CAGGAGGGTG GTTGGGCTGG AGAACGAGTC GATGCAAAAC
2341 ACGCACTGGA GTACAACGGA GGTAGCAATG AACGGGTACT ACGGGAGAGC AGGAGCAACA
2401 GCACACCACG CTGCATTTCC GTTGTCGAG GGGGGGACAA TGCGAAAACG AATACCAGCT
2461 ATAGAGATGA GGGAGAACGG GGTGGAGGGG GACCTGATGA ACGATGATCT CTATTCAATT
2521 GGAACGGCAG CGGGGTACCT GGCGGTAGAG GGGATGGCAG GTGCGCAGGG GGGTATCTGG
2581 GACGTGGTCC AGTACCAGCT GCCTGGGCTG GACGATGAGG CGAGGGGGGT GATGAACACG
2641 GTGGGGGCGA TGGGGGGATG GACGAGGGCG GTGACACCAG TAGACAATGT GGCCACCATG
2701 AGGGACAACG GGGTTGAGGG GGAACCTTGT GGAATAGTGA TGTCTCTACC AACAGTGGG
2761 ACCGCTGTGG TGGATAGGTT AGCTAATTTT GGATTACCAC CAGCAGGGGC GGAATTAAGA
2821 GAAGTACCAT TTGGCGGGTA CCAAAGATCA GTCACAAACA CCAACCACAG AGTCAAGGTG
2881 AGTGTGAGTG GGGGGCGAGC AGTTGTTCAA AAAGGGAACA AAGCCGAGAT GAATCCAGTC
2941 TTTGTCAATA GGACACCAGG ACAAACGACC CTAGGCCAAC CAACAACAGA CACTACAGGG
3001 ATGACAACTG CAGATTTTTT AGATATATAG ACCCTGGGC ACCAAAAAGA AGTAGAAAGA
3061 GGAATGCAGT TGGAGCACTA CTACAATTAG ACCCAAATT AGGACTTAAG AGAATGATAA
3121 CCAAAGCATT GGCCAGGGG CGAGAAGAAG ATTGGATTAA GATAACCACC AATACTACAA
3181 AGATCAAATT GAAATCACTA AGGCCACTGG ATGGATGGGA GCATTCAGGA TGGGGACTGG
3241 GACTGAGGTT AGCCTTAGAA CTAGTTTCTG AGCCAACGAG AGAGACAATG TCAGGGGTAA
3301 TTAGACAAAT TAGGATAGGG AAGCTAGATC CAACCAAGGT ATTTAAATCA ATTAGTAACC
3361 AGGCGAAAGT TAGCGGGTGC GGGCTATGTA GGGAGTGGAA GGAGTACGTA AATTGGGAAT
3421 TACTAGGGGG GTACCAGATG ATAACAGCCC AACAGATGGG TGAAGAGGTA GCACACCAGG
3481 TGACCGAAAA GGCTAAAAGC GAGGAAGCGG GAAGCGATAG GATAAATAGG GCATTAGAAAT

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3541 CAATTTTAGG GCCACTAAGG GGTACATGCA CGGTACCGTG CTCACAAAGG GAATTTTAT
3601 TATGTAGGGA TAAATGGGCC AAAAACACGT CCGGGTACAT AGGGTTAGGG CAAAATTTAG
3661 GGCGACAGAA GATAGACGTA GCACTTAATT CAGACATCAA GGCCTGGAA AGGATGATGG
3721 AGGGAGAATT CGAGAACAGA CCATTTATTA AAAGTGAACC AGGGAAAAAGC AGGCCGGTAG
3781 TTAATAGTAA TATATCGTGT TACCTAAATT TAGAGTATGG GTGGGTAACC ATTA AAAAAGA
3841 CACTGAAAAA ATACTTAGGC AAGAGGACAA CAATTTTCGA CGACGCAAGC CAAAAGGTAC
3901 TGTTATGGCA AGAAATGATA ATTGGAGCAG ATAACCATAG GGAGATTAAG GTGCCACTAG
3961 ACTACTCGCG GTTCGATTCA ACAATTGGGA AAACGCAAAAT CATGAAGTGT GTTAGACTGC
4021 TATTAGACAT GGTGCGAGGT AACGACAATT GGAAAAGGTC AGTTACTGAA AGATTTCGAGA
4081 GCAAACGGT GTACATTGAC GGTACGGAA AACTCAGGTG GGAAAACAGC GTACTTAGCG
4141 GATGGAGATG GACAAGTATA ATCACATCTG TAATAAAATTT GGCCATTCTT AAGGCGACCG
4201 GAGCCGATGC GAGCGGAGTA GGGATTAAAG TCCAGGGAGA TGATGTTAAA ATCAGCTTCC
4261 AAACAATTCG AGAAGCGGAA AGGTGTGTCG AAGAAAATTAA CGCTTTAGGG TATGAAATTA
4321 ACCCGGGTAA AGTATTTGCG TCAAGGAAAA GGGACGAGTA TCTCAGGATG GTAGCGGAAG
4381 AGGGGAAAAAT GGCAGGTTAT GTAATTAGGT GTCTACCGAA AATTGTATTT ACATCACCAG
4441 CAGAAGAGGT TACCACATGG GAAGAACGAA TTAGGGGAAC AGTGTCAAAA TGGATGAGGG
4501 TGTTATCACG GGGGGGGGAT AGGGAAGTAG CTAAATATTG GATGAAACGC GATTTGTGTG
4561 GATTGAGTGG AGAGAATTCG GAATTAATAG ATAGCTGGTT ACGTACTCCA GCATCCGTGG
4621 GGGGGGGTGG TTGTGCATGG TTACTTGGGG AGGGAAATTT GTGGACGGGA TTAGTACTTA
4681 GGAACAGGA GGAAGCAGAA GTGGTGGAGC GTTCAAATCA CAAAATGATG TCGGATTACG
4741 GGGGGTCAGT TCCTGCCCCG AGATGGATCA AGAGTGTGAC GAAGGGCGCA AAACCGGCTT
4801 TTGGGTTCAA GAGGATAGAG AGGGTAAAGC CACTAGGGAA CTGGTTAAAA GATCTAAGGC
4861 CAGTGGCGGA ATCAATTAAA TCAATTAGTG ACTTAGTCGA AACATCACAG GTACCAAAAT
4921 TCAGATTTAA GGAAATTTAT GATTGGACAC CATTATGTT ACACAAGAGG AACGCGCTCA
4981 TAGAAGAAAA GAACTGGGAC AAATTGTGCT CGCTTTATCA TAATGAAGGG GAAGTGAGAC
5041 TATATAAAAG GATATTACCC AGGTGGATGT GGCTGAGTAT GATAAAAAAT GAGTTAACAT
5101 GGGGGGATAT GCCACCAAAC ATTGCTAATT CGGAAGTTGC TTCATGTGCA AAAGAAAGAG
5161 TGGAGTACAA AGTGTGAGT AAAGTATTGA GGTATCACCG AACCCTCTCA AGAGAAACGC
5221 TCACTCGTTT TTCTATTGGT GGGGAGTATG TACTGAGTGC TAAACTCAAC GACAGGCAAA
5281 GGATATTACA GTAGGACGGT GGTAGCTGAA ACAGGGGCTA CTAGCACAAA GAAGCTATCT
5341 GGAATAGGGG ATAGTGTAAC CTGATCACGG AACTAATACG CACCGTGTAC CTATTAGTAA
5401 GGCGTATATG GTAGGTGCCG GAGGAGGCGA CGATCCCGGG ACTTCACTTA CCGCAGAGGC
5461 AGCTATAGTA GGTTAGGCGT GGAATTCCGG CCATTTTAGA GGACCTATTC GGACCGGCGT
5521 GGTGTGCGAA GTAAGACCCG GATGTCAAAC AAGATGAAGA GTTTTTTACT GGTGCTTTTA
5581 TGTTTGTGTG TAGGGGAAGG GATTGTTCCA ATGTTCAGGC GAGAATGGTG TTTGTGCACT
5641 GCAGGGAATG CGAGAGTACC CTTGGTGGGA GAGGGAAGGG CTGAGAAGAT CGAGCTATTC
5701 AATCAGAGCG CCACATGTGG GAAGAAAGAG CTAATAATAA CGTGAAGGG AAAGAGGTGG
5761 TGCTACGATA TTGAGAGCAA GCGAGGGAAA ATACTAGTAA AGACACTGAG CGGAGGGGGA
5821 CATCTGGAAC GAGAGGGAAA GGGGTACAAG TTAGTTAGGA ACGGGTTCCA TCTTGCTTCA
5881 TTCGGGGGTA AAAAAGAGGA AATACAGGAT TCAAGTCACA TAGAGAAAAGT AAACGGGAAA
5941 GACGCGATAG TTAAGAAAGG GCAACACGTA GAACATCTTC CAGGGGGGAA CGATTTAATT
6001 GTTACAGAGG GCGGGAACCT GTGTGGGAAC GTGGGGTTCT TCGATAATAC GCAGTGATCA
6061 TACAACAGTG TTATAAATAT AGGAGGGGGA AGTGTGACA ATTCACAAGA AGCCAAGAAC
6121 GATAAATCAA ACACAATTGA CAATTTAATT GATATGTTAC CTTTAGTTGT AGGAATAGCC
6181 GGGGGCTGTC TCATAGTGAT AGTAGTCTTA TACTTAACAA TTAAATACTG TAAATGCAAG
6241 AAGAAGAGGA CCAACCCAGA ACCAGCGGAA CCGGAGGAAC ACGAGATGAG GGATCTCAGG
6301 AGAAGGTTAG AGCCACGCCC TCCATACCAA AGACAGATGG GTGTTGAGTT TGAAATAAAT
6361 GAAGCTCTCG AATTCATGGG CGTCGAAGGG AGTGAAAGCC CAGACTCAGG ATGTCAGTCA
6421 GATGAAGAGG GATTCAGAGT AGGAGTCTAG ATGGAACGAA GAGATAGGTC ACCACTATCG
6481 TCCGCATATA ATTTTACACA ATTGGACTAG TACTATTGTA CAGGGGTACC ATTTACCCTG
6541 CCAGACCGAC AAATAGGTCG GGTGTTTAGT GAGAAAGTCG CTAGCACCTG GGCGAAAGAT
6601 GGGGCAACAT ACTGACTCTT GGGAGTGAGA CCAGGGGGAA GTATGTAAGG CATGGTTAGG
6661 CCTGACCCCG TGGGTCAAGG CCGTGCTG

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Figure 2

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445          ATGGAA CCAAACACAT CTGTCATTGC AACGGAGCAG
481 CAGCAGGCTG CCATGAGAGA GGTGGAGGCC GAGGCGGCGG CCAGAGACGA AGTGGTGGAG
541 AAGATCGCAT TCGCTGAAGG AGCGATGATG GTACAGACGA GGAGGTTACC ATCAGGAAAAG
601 TCGTCGGTAG GAGGTTTTCT CGGCGAACTG GCACAGAACA TACGTGCCAT GAATCGGTCA
661 TTGCACACAG ATACCAACAT GCTGACCGAA GGGGCGATGG TGGACAGAGC GAGGGCAAAA
721 GTACACAAAA TCATTAGGGA AGGGAATTTG GACTCTAGGG TATTTTCAAA CACGGGGAGC
781 AACACTATGT TGTCACGTGT GGTACCAGCA GTACCGGGAC CACCGGCAGT ACCGGAGCAT
841 TGGGACGTTG CGCCGTCCTG GTTCGTATGC AGACCGGGGA AAAAGGGGGG GATAAAGATC
901 ACACAAAGCG CATCAATGGC AGCATTA AAC CCACTATTTA GAGGCGCAGA CGTGGGGCCA
961 ATCGGGACAG CAGTCAGGGC GGATGTAAAC GCATTTTCAA TGAATGCAGT TCTGGGAGCA
1021 CTAAGAGCCG GGGGATTTAA CACCGAACAT TCCCTGGTGT CATTCGTTGA ACCACTAATT
1081 CGGATCTTGC TAATGGGGGT ACAAAACAAA GACAGGGGGA CCAGCCCATG GGATTGGGTT
1141 GGAGGGATGA GTTCGCGAAT AGTCAATCCC CTAGTATTCA CAACAAGCCG GAACCTCTTC
1201 CCAGGGGGAC CAAATTTGAG GGTGTGGGGA GCCAACGATA CAGTGGCCAG GATAGTAAAC
1261 GTTGAGGACT ACATGCGCGA GGC GGCGCCGGG GAGGGGAGGT TCGACGCTGG ATGGGGACCG
1321 GAATTCTGGG GTGGGACAGG GGACGACGCA GTGGCGGTGG TACCGATAAG GGCAGTAGAA
1381 GCAGGGCTAG GAGAAGTAAA CGCAGGGTGG ACATTGGCAC ACATGGAATA CCCAGTCAAG
1441 GTTAGACTAC TTGACGTCGA CGACCGAACA ATTGACCAG GGGGGAGCCT GCCCTAAAC
1501 GCAAACAGAG AATACACGGC GGCAGGAGCT ACGCATGTAC CCGGGCCCTA TGCCAGGGTA
1561 CTGTACGTCG TCGTGGACCA AAACGCAGAC AGGTGTGTGG GGGTGAGAGT GCAGGGACAG
1621 GGTGCTGTAA TTGACGTGGA TCCGGCGTTG AATTACGTGA TAGGGGGAGC GGATTTGGGG
1681 ATGTTGCCGT TGATACAGTG GAGTGTAGGG CTGGGGGCCG AGGACATGGC GCAGGGATCG
1741 ATTGCACAGA CGCAGCGATG GGTGAGGATG TATGGAAACG AGGACGATTG GGAATCAGCG
1801 TGGCATCTAG TGTCTAGCGC GTACACAGTG TACAGCCCGG CATTCAGGAG ATCGGGTGTC
1861 GCAGTGGAGG GAGGATTCTG GGCGCAACCA GCTGCAGGGG CAGCACCGTT TCCACTAGGA
1921 GGATTGGCAG GGTGGGTGAG GTACGACAAT CAGGCACGGG CGGCGCAGGT TGCACTTTGC
1981 AGAGAGAGGG CGGATATGGC GGAGTGTCTT TGGGGGGGGT ACAGGGAGAG AGGGGTGAGA
2041 CCGGGGAGTG TGGCAAATG GCAGTACGTA AGGTTTCGATC CCACAGTGGC TGTAGGAGTA
2101 GCTGCTCACT TCTGGTCGGT AGTGAAGGTG ATGGTGGCTC CCGTCCCAGA CAGAGCGGCT
2161 GCTCTGGCGG ACATGGCGTG GGGGAAGGGG AAGGTGCAAG CCATGGGTGA GGATGTGATC
2221 AACGGGCAGA TGGGACAACC TGAGTCCATG ATGAGAGGGG TGGCGCTGAA CGAGAACCAG
2281 GGA CTAGCGG CGGCTACAGT CAGGAGGGTG GTTGGGCTGG AGAACGAGTC GATGCAAACA
2341 ACGCACTGGA GTACAACGGA GGTAGCAATG AACGGGTACT ACGGGAGAGC AGGAGCAACA
2401 GCACACCACG CTGCATTTCC GTTGTCCGAG GGGGGGACAA TGCGAAAACG AATACCAGCT
2461 ATAGAGATGA GGGAGAACGG GGTGGAGGGG GACCTGATGA ACGATGATCT CTATTCAATT
2521 GGAACGGCAG CGGGGTACCT GGCGGTAGAG GGGATGGCAG GTGCGCAGGG GGGTATCTGG
2581 GACGTGGTCC AGTACCAGCT GCCTGGGCCT GACGATGAGG CGAGGGGGGT GATGAACACG
2641 GTGGGGGCGA TGGGGGGATG GACGAGGGCG GTGACACCAG TAGACAATGT GGCCACCATG
2701 AGGGACAACG GGGTTGAGGG GGAACCTTGT GGAATAGTGA TGTCTCTACC AACAAAGTGG
2761 ACCGCTGTGG TGGATAGGTT AGCTAATTTT GGATTACCAC CAGCGAGGGC GGAATTAAGA
2821 GAAGTACCAT TTGGCGGGTA CCAAAGATCA GTCACAAACA CCAACCACAG AGTCAAGGTG
2881 AGTGTGAGTG GGGGGCGAGC AGTTGTTCAA AAAGGGAACA AAGCCGAGAT GAATCCAGTC
2941 TTTGTCAATA GGACACCAGG ACAAACGACC CTAGGCCAAC CAACAACAGA CACTACAGGG
3001 ATGACAAC TG CAGATTTTTT AGATATATAG

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Figure 3

MEPNTSVIATEQQQAAMREVEAEAAARDEVVEKIAFAEGAMMVQTRRLPS 50
GKSSVGGFLGELAQNIRAMNRSLHTDTNMLTEGAMVDRARAKVHKIIREG 100
NLDSRVFSNTGSNTMLSLWVPAVPGPPAVPEHWDVAPSWFVCRPGKKGGI 150
KITQSASMAALNPLFRGADVGPIGTAVRADVNAFSMNAVLGALRAGGFNT 200
EHSLSVFVEPLIRILLMGVQTQDRGTSPWDWVGGMSSRIVNPLVFTTSGN 250
FFPGGPNLRVWGANDTVARIVNVEDYMR EAAGEGRFDAGWGPEFWGGTGD 300
DAVAVVPIRAVEAGLGEVNAGWTLAHMEYPVKVRLLDVDDRTIGPGGSLP 350
LNANREYTAAGATHVPGPYARVLYVVVDQNADRCVGVVRVQGGGAVIDVDP 400
ALNYVIGGADLGMLPLIQWSVGLGAEDMAQGSIAQTQRWVRMYGNEDDWE 450
SAWHLVSSAYTVYSPAFRRSQVAVEGGFWAQPAAGAAPFPLGGLAGWVRY 500
DNQARAAQVALCRERADMAECPWGGYRERGV RPGSVANWQYVRFDPTVAV 550
GVAAHFWSVVKVMVAPVPDRAAALADMAWGKGVQAMGEDVINGQMGPPE 600
SMMRGVALNENQGLAAATVRRVVGLENE SMQTTHWSTTEVAMNGYYGRAG 650
ATAHHAAFPLSEGGTMRKRIPAIE MRENGVEGDLMNDDLYSIGTAAGYLA 700
VEGMAGAQQGGIWDVVQYQLPGPDDEARGVMNTVGAMGGWTRAVTPVDNVA 750
TMRDNGVEGEPCGIVMSLPTSGTAVVDRLANFGLPPARAELREVPFGGYQ 800
RSVTNTNHRVKVSVSGGRAVVQKGNKAEMNPVFVNRTPGQTTLGQPTTDT 850
TGMTTADFLDI *

Figure 4

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3114                                     ATGATAA
3121 CCAAAGCATT GGCCCAGGGA CGAGAAGAAG ATTGGATTAA GATAACCACC AATACTACAA
3181 AGATCAAATT GAAATCACTA AGGCCACTGG ATGGATGGGA GCATTGAGGA TGGGGACTGG
3241 GACTGAGGTT AGCCTTAGAA CTAGTTTCTG AGCCAACGAG AGAGACAATG TCAGGGGTAA
3301 TTAGACAAAT TAGGATAGGG AAGCTAGATC CAACCAAGGT ATTTAAATCA ATTAGTAACC
3361 AGGCGAAAGT TAGCGGGTGC GGGCTATGTA GGGAGTGGAA GGAGTACGTA AATTGGGAAT
3421 TACTAGGGGG GTACCAGATG ATAACAGCCC AACAGATGGG TGAAGAGGTA GCACACCAGG
3481 TGACCGAAAA GGCTAAAAGC GAGGAAGCGG GAAGCGATAG GATAAATAGG GCATTAGAAAT
3541 CAATTTTAGG GCCACTAAGG GGTACATGCA CCGTACCGTG CTCACAAAGG GAATTTTTTAT
3601 TATGTAGGGA TAAATGGGCC AAAAACACGT CCGGTACAT AGGGTTAGGG CAAAATTTAG
3661 GGCGACAGAA GATAGACGTA GCACTTAATT CAGACATCAA GGCCTGGAA AGGATGATGG
3721 AGGGAGAATT CGAGAACAGA CCATTTATTA AAAGTGAACC AGGGAAAAGC AGGCCGGTAG
3781 TTAATAGTAA TATATCGTGT TACCTAAATT TAGAGTATGG GTGGGTAACC ATTA AAAAGA
3841 CACTGAAAAA ATACTTAGGC AAGAGGACAA CAATTTTCGA CGACGCAAGC CAAAAGGTAC
3901 TGTTATGGCA AGAAATGATA ATTGGAGCAG ATAACCATAG GGAGATTAAA GTGCCACTAG
3961 ACTACTCGCG GTTCGATTCA ACAATTGGGA AAACGCAAAAT CATGAAGTGT GTTAGACTGC
4021 TATTAGACAT GGTGCGAGGT AACGACAATT GGAAAAGGTC AGTTACTGAA AGATTCGAGA
4081 GACAAACGGT GTACATTGAC GGTACGGAA AACTCAGGTG GGAAAACAGC GTACTTAGCG
4141 GATGGAGATG GACAAGTATA ATCACATCTG TAATAAATTT GGCCATTCTT AAGGCGACCG
4201 GAGCCGATGC GAGCGGAGTA GGGATTAAAG TCCAGGGAGA TGATGTTAAA ATCAGCTTCC
4261 AAACAATTTC AGAAGCGGAA AGGTGTGTCG AAGAAATTAA CGCTTTAGGG TATGAAATTA
4321 ACCCGGGTAA AGTATTTGCG TCAAGGAAAA GGGACGAGTA TCTCAGGATG GTAGCGGAAG
4381 AGGGGAAAAT GGCAGGTTAT GTAATTAGGT GTCTACCGAA AATTGTATTT ACATCACCBA
4441 CAGAAGAGGT TACCACATGG GAAGAACGAA TTAGGGGAAC AGTGTCAAAA TGGATGAGGG
4501 TGTTATCACG GGGGGGGGAT AGGGAAGTAG CTAAATATTG GATGAAACGC GATTTGTGTG
4561 GATTGAGTGG AGAGAATTTC GAATTAATAG ATAGCTGGTT ACGTACTCCA GCATCCGTGG
4621 GGGGGGGTGG TTGTGCATGG TTACTTGGGG AGGGAAATTT GTGGACGGGA TTAGTACTTA
4681 GGGAACAGGA GGAAGCAGAA GTGGTGGAGC GTTCAAATCA CAAAATGATG TCGGATTACG
4741 GGGGGTTCAG TCCTGCCCCG AGATGGATCA AGAGTGTGAC GAAGGGCGCA AAACCGGCTT
4801 TTGGGTTCAA GAGGATAGAG AGGGTAAAGC CACTAGGGAA CTGGTTAAAA GATCTAAGGC
4861 CAGTGGCGGA ATCAATTAAA TCAATTAGTG ACTTAGTCGA AACATCACAG GTACCAAAAT
4921 TCAGATTTAA GGAAATTTAT GATTGGACAC CATTGATGGT ACACAAGAGG AACCGCTCA
4981 TAGAAGAAAA GAAGTGGGAC AAATTGTGCT CGCTTTATCA TAATGAAGGG GAAGTGAGAC
5041 TATATAAAAG GATATTACCC AGGTGGATGT GGCTGAGTAT GATAAAAAAT GAGTTAACAT
5101 GGGTAAGTAT GCCACCAAAC ATTGCTAATT CGGAAGTTGC TTCACTTGCA AAAGAAAAGAG
5161 TGGAGTACAA AGTGTTGAGT AAAGTATTGA GGTATCACCG AACCGTCTCA AGAGAAAACGC
5221 TCACTCGTTT TTCTATTGGT GGGGAGTATG TACTGAGTGC TAAACTCAAC GACAGGCAAA
5281 GGATATTACA GTAG

```


Figure 5

MITKALAQGREEDWIKITNTNTTKIKLKSRLPLDGWEHSGWGLGLRLALEL 50
VSEPTRETMSGVIRQIRIGKLDPTKVFKSISNQAKVSGCGLCREWKEYVN 100
WELLGGYQMITAQQMGE EVAHQVTEKAKSEEAGSDRINRALESILGPLRG 150
TCTVPCSQREFLLCRDKWAKNTSGYIGLGQNLGRQKIDVALNSDIKALER 200
MMEGEFENRPFKIKSEPGKSRPVVNSNISCYLNLEYGWVTIKKTLKKYL GK 250
RTTIFDDASQKVLLWQEMIIGADNHREIKVPLDYSRFDSTIGKTQIMKCV 300
RLLLDMVRGNDNWKR SVTERFERQTVYIDGYGKL RWENSVLSGWRWTSII 350
TSVINLAILKATGADASGVGIKVQGDDVKISFQTIREAERCVEEINALGY 400
EINPGKVFASRKRDEYLRMVAEEGKMAGYVIRCLPKIVFTSPTEEVTTWE 450
ERIRGTVSKWMRVL SRGGDREVAKYWMKRDLCGLSGENSELIDSWLRTPA 500
SVGGGGCAWLLGEGNLWTGLVLREQEAEVVERS NHKMMSDYGGSPARR 550
WIKSVTKGAKPAFGFKRIERVKPLGNWLKDLRPVAESIKSISDLVETSQV 600
PKFRFKEIYDWTPFMVHKRNALIEEKNWDKLC SLYHNEGEVRLYKRILPR 650
WMWLSMIKNELTWVSMPPNIANSEVASLAKERVEYKVLSKVLRYHRTVSR 700
ETLTRFSIGGEYVLSAKLNDRQRILQ*

Figure 6

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5542          ATGTCAAAC AAGATGAAGA GTTTTTTACT GGTGCTTTTA
5581 TGT TTGTGTG TAGGGGAAGG GATTGTTCCA ATG TTCAGGC GAGAATGGTG TTTGTGCACT
5641 GCAGGGAATG CGAGAGTACC CTTGGTGGGA GAGGGAAGGG CTGAGAAGAT CGAGCTATTC
5701 AATCAGAGCG CCACATGTGG GAAGAAAGAG CTAATAATAA CGTGGAAGGG AAAGAGGTGG
5761 TGCTACGATA TTGAGAGCAA GCGAGGGAAA ATACTAGTAA AGACACTGAG CGGAGGGGGA
5821 CATCTGGAAC GAGAGGGAAA GGGGTACAAG TTAGTTAGGA ACGGGTTCCA TCTTGCTTCA
5881 TTCGGGGGTA AAAAAGAGGA AATACAGGAT TCAAGTCACA TAGAGAAAAGT AAACGGGAAA
5941 GACGCGATAG TTAAGAAAGG GCAACACGTA GAACATCTTC CAGGGGGGAA CGATTTAATT
6001 GTTACAGAGG GCGGGAACCT GTGTGGGAAC GTGGGGTTCT TCGATAATAC GCAGTGTACA
6061 TACAACAGTG TTATAAATAT AGGAGGGGGA AGTGTTGACA ATTCACAAGA AGCCAAGAAC
6121 GATAAATCAA ACACAATTGA CAATTTAATT GATATGTTAC CTTTAGTTGT AGGAATAGCC
6181 GGGGGCTGTC TCATAGTGAT AGTAGTCTTA TACTTAACAA TTAAATACTG TAAATGCAAG
6241 AAGAAGAGGA CCAACCCAGA ACCAGCGGAA CCGGAGGAAC ACGAGATGAG GGATCTCAGG
6301 AGAAGGTTAG AGCCACGCCC TCCATACCAA AGACAGATGG GTGTTGAGTT TGAAATAAAT
6361 GAAGCTCTCG AATTCATGGG CGTCGAAGGG AGTGAAAGCC CAGACTCAGG ATGTCAGTCA
6421 GATGAAGAGG GATTCAGAGT AGGAGTCTAG

```

Figure 7

MSNMKSFLLVLLCLCVGEGIVPMFRREWCLCTAGNARVPLVGEGRAEKI 50
ELFNQSATCGKKELIITWKGKRWCYDIESKRGKILVKTLSGGGHLEREGK 100
GYKLVRNGFHLASFSGGKKEEIQDSSHIEKVNGKDAIVKKGQHVEHLPGGN 150
DLIVTEGGNLCGNVGFFDNTQCTYNVSVINIGGGSVDNSQEAKNDKSNTID 200
NLIDMLPLVVGIAGGCLIVIVVLYLTIKYCKCKKKRTNPEPAEPEEHEMR 250
DLRRRLEPRPPYQRQMGVEFEINEALEFMGVEGSESPDSGCQSDEEGFRV 300
GV*

Figur 8

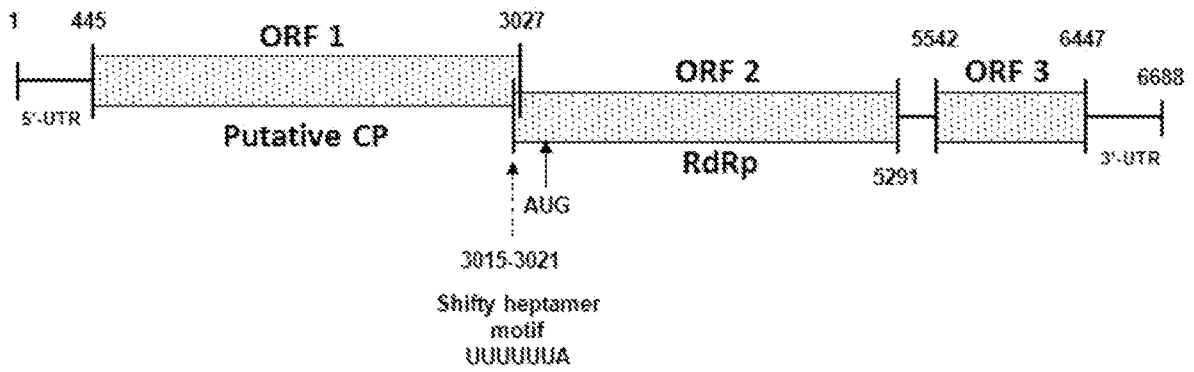


Figure 9A

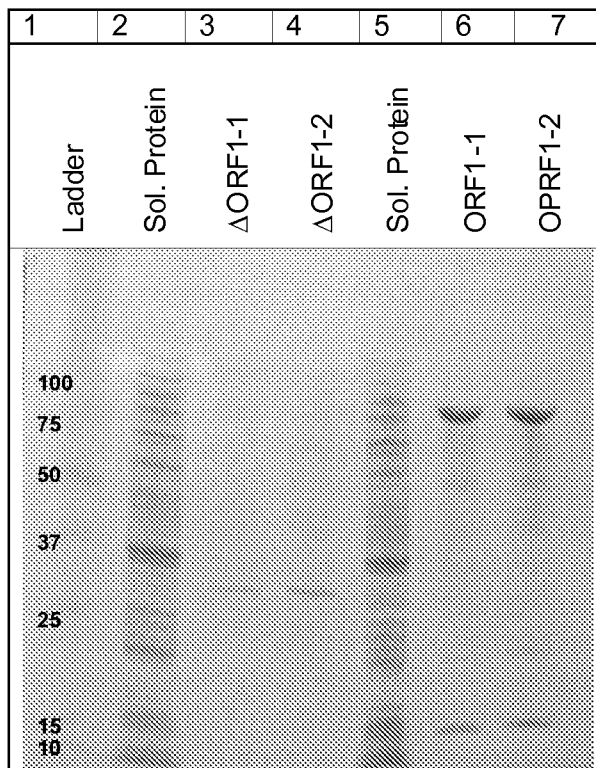


Figure 9B

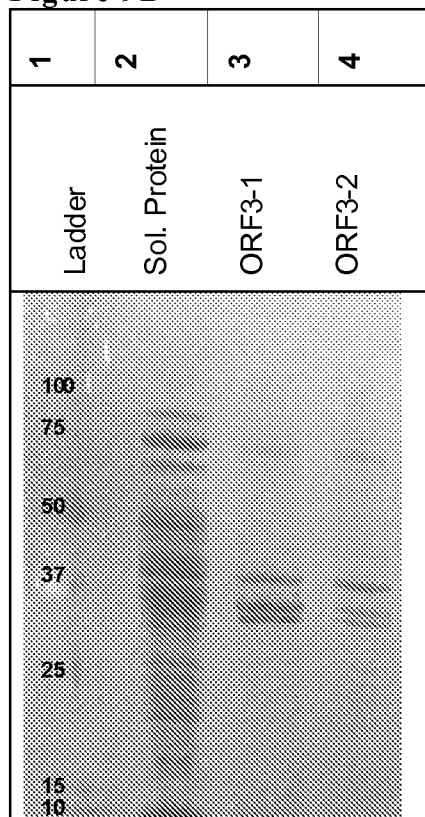
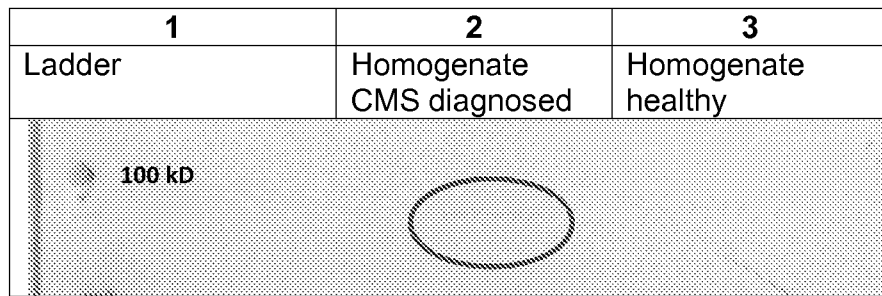


Figure 10**Figure 11**

A.

B.

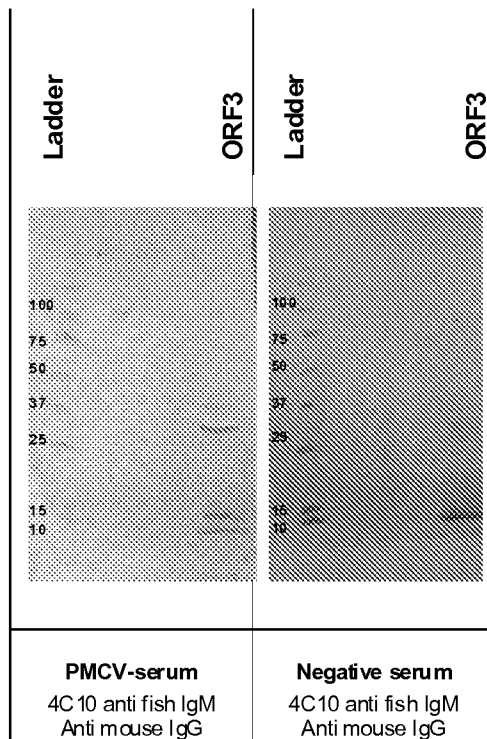


Figure 12

