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(54) Title **Pancreas Disease Virus Markers**

(57) Abstract

The present invention relates to methods for detection of the Pancreatic Disease (PD) virus used for production of PD vaccines, use of primers and probes to differentiate PD virus used in a vaccine and infectious virus, and a kit for detection of PD virus used in a vaccine. The method includes nine SNP's and related methods that can be used to identify the virus in the vaccine.

Pancreas Disease Virus Markers

The present invention relates to methods for detection of the Pancreatic Disease (PD) virus used for production of PD vaccines, use of primers and probes to differentiate PD virus used in a vaccine and infectious virus, and a kit for detection of PD virus used in a vaccine.

Background

Pancreas disease has become a serious disease in farmed salmonids in several geographical areas. The disease was first reported from Scotland, and has later been reported from Ireland, Norway and North America. The causative agent of pancreas disease is salmon pancreas disease virus (SPDV), also called salmonid alphavirus (SAV). Pancreas disease has caused severe production losses within the Scottish, Norwegian, Irish and Canadian salmonid farming industries, mainly due to reduced growth and reduced quality at slaughter.

In Norway the disease has spread over the years with 58 confirmed infected sites in 2006, 88 in 2010, and 178 confirmed sited in 2017. Salmonid alphavirus is now a major health problem for the aquaculture industry in Norway.

The first alphavirus was isolated from fish in 1995. Alphaviruses have been isolated from both Atlantic salmon suffering from pancreas disease, and rainbow trout suffering from sleeping disease. The salmonid alphaviruses (SAV) are spherical (approximately 65 nm in diameter) enveloped RNA viruses, and they contain a single stranded positive-sense RNA genome of 11-12 kB. In EP patent 712,926 it is described as being sensitive to chloroform, rapidly inactivated at pH=3 and at 50°C and with a buoyant density of 1.20 g/ml. Nucleotide sequencing studies assigns the salmonid alphaviruses to three genetically different subtypes.

According to the Norwegian Veterinary Institute, SAV1 causes pancreas disease in Scotland and Ireland, SAV2 is known from freshwater production of rainbow trout in France and Scotland, but a marine type of SAV2 has also been observed in Atlantic

salmon, both in Scotland and in Norway, SAV3 appear endemic in salmon and rainbow trout in the West coast of Norway, SAV4 is detected in Ireland and Scotland, SAV5 in Ireland and SAV6 is detected in Ireland. SAV2 and SAV3 is most prominent in Norway. The DNA of Salmonid alphavirus 3 (SAV3) was deposited in GenBank in 2012 with acc no KC122925, and a modification of acc no KC 122925 with cut of 27 nt at 5-end is enclosed herein as SEQ ID NO 1.

Salmonid alphavirus is a Listed disease (OIE) and presence or assumptions of the disease shall be reported to the Food Safety Authorities. The criteria for assumption of PD is according to the National veterinary Institute: Detection of the disease by PCR or propagation, clinical or pathological changes related to pancreas disease, or detection of antibodies against pancreas disease. Thus the consequence of false positive results may be dramatic for the fish farmer.

The current diagnostic methods for SAV3 are histology, immunohistochemistry, virus propagation and PCR. The PCR gives the fastest result and is the preferred method.

Farmed Atlantic salmon are generally vaccinated against the most common bacterial diseases and very often against infectious viral diseases as for example infectious salmon anaemia virus (ISAV), infectious pancreatic necrosis virus (IPNV) and salmonid alphavirus (SAV) before transfer to sea. Vaccination programs are certainly desirable in order to avoid economic losses and further dissipation of infectious diseases. There are several vaccines to immunize against SAV on the market, for example ALPHA JECT micro 1 PD, Aquavac PD vet., Aquavac PD 7 vet. and Norvax Compact PF vet.

Vaccines for domestic animals are often prepared with markers to allow for immunological differentiation (or segregation) of infected animals and vaccinated animals, also referred to as a DIVA vaccine in veterinary medicine. The current vaccines used for PD have not taken this challenge into consideration and there is a confusion of analysis and interpretation of testing as the virus causing disease and the virus in the vaccine cannot be differentiated by current diagnostic tools. This may

result in false interpretation and wrong conclusion, wherein infected fish is considered healthy but vaccinated, or healthy fish being considered infected.

The genomes of all organisms undergo spontaneous mutations during their continuing evolution, forming variant forms of progenitor genetic sequences. A mutation may result in an evolutionary advantage or disadvantage relative to a progenitor form or may be neutral. A variant that result in an evolutionary advantage may eventually be incorporated in many members of the species and may thus effectively become the progenitor form. Furthermore, often various variant forms survive and coexist in a species population. The coexistence of multiple forms of a genetic sequence gives rise to genetic polymorphism, including single-nucleotide polymorphisms (SNPs).

A single-nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide — A, T, C or G — in the genome (or other shared sequence such as RNA) differs between members of a biological species or paired chromosomes in an organism. For example, two DNA fragments from different individuals, AAGCCTA to AAGCTTA, contain a difference in a single nucleotide, commonly referred as two alleles. Almost all common SNPs have only two alleles. The genomic distribution of SNPs is not homogenous; SNPs usually occur in non-coding regions more frequently than in coding regions or, in general, where natural selection is acting and fixating the allele of the SNP that constitutes the most favorable genetic adaptation.

The present invention described below solves some of the issues set out above.

Summary of invention

The problems above is solved by methods, use of primers and probes and a kit according to the characterizing part of the enclosed independent claims. Further advantageous features are stated in the dependent claims.

The present invention is based on the surprising finding that unique mutations in the DNA of salmonid alphavirus 3 (SAV3) can be used to differentiate virus extracted from a vaccine and a virus causing the disease in diagnostic surveys. More particularly, the present invention is based on the identification of novel single-nucleotide polymorphisms (SNPs) in the DNA of the SAV3 shown to be unique for the Salmon pancreas disease virus extracted from a vaccine, for example from ALPHA JECT micro 1 PD vaccine. The nucleic acid sequence of the virus extracted from the vaccine is given in SEQ ID No 2. Specifically, the present inventors have identified nine unique SNPs located in the SAV3 DNA (T3064C, T4113C, G5085A, C5243T, G5553A, G7356A, G8093A, A9123G, G11559A).

The present invention thus provides an in vitro method to specify if a SAV3 virus detected in fish, originates from disease causing virus or from a vaccine produced on the extracted virus. Disease causing virus is also referred to as infectious virus or infectious SAV3, and virus identical to virus extracted from the vaccine is referred to as vaccine virus or vaccine SAV3. The method is comprising the steps of detecting at least one single nucleotide polymorphism (SNP) associated with vaccine virus in the sample from fish to be analysed. In a preferred embodiment, the fish is teleost fish, such as salmonids, basses, breams.

In particular, the said method is useful for uniquely identifying viruses from vaccines prepared on a virus comprising SEQ ID NO 2, for example ALPHA JECT micro 1 PD.

According to one embodiment, the present invention provides an in vitro method for detection of vaccine SAV3, in a collected biological sample, comprising the following steps

a) isolating genomic material from the biological sample;

b) confirming that sample contains a SAV3 virus by standard methods,

c) detecting whether any of the following single nucleotide polymorphisms (SNPs) are present in the genomic material

i. C in position 3064;

ii. C in position 4113;
iii. A in position 5085;
iv. T in position 5243;
v. A in position 5553;
vi. A in position 7356;
vii. A in position 8093;
viii. G in position 9123;
ix. A in position 11559;

or the complementary oligonucleotides thereof, wherein the numbering of said positions is in accordance with sequence depicted in SEQ ID No. 1, and

wherein presence of at least one SNP indicates that vaccine SAV3 is present in the biological sample.

According to another embodiment, the invention provides an in vitro method for detecting vaccine SAV3 in a collected biological sample, the method comprises the following steps:

a) preparing the sample comprising nucleic acid sequences for a reverse transcription reaction,

b) subjecting the mixture of a) to polymerase chain reaction with at least one primer or probe comprising

i. C in position 3064;
ii. C in position 4113;
iii. A in position 5085;
iv. T in position 5243;
v. A in position 5553;
vi. A in position 7356;
vii. A in position 8093;
viii. G in position 9123;
ix. A in position 11559;

or the complementary oligonucleotides thereof, wherein the numbering of said positions is in accordance with sequence depicted in SEQ ID No. 1, and

c) determining whether binding of the primers and/or probes to nucleotide sequences in the sample and amplification of the sequences between them have occurred, indicating the presence of SAV3 isolated from the vaccine in the biological sample.

According to another embodiment, the invention relates to an in vitro method for detection of SAV3 different from vaccine SAV3 in a collected biological sample. The method comprises the following steps

a) isolating genomic material from the biological sample;

b) confirming that sample contains a SAV3 virus by standard methods,

c) detecting whether any of the following nucleotides are present in the genomic material

i. T, A, or G in position 3064;
ii. T, A or G in position 4113;
iii. G, T or C in position 5085;
iv. C, G or A in position 5243;
v. G, T or C in position 5553;
vi. G, T or C in position 7356;
vii. G, T or C in position 8093;
viii. T, A or C in position 9123;
ix. G, T or C in position 11559;

or the complementary oligonucleotides thereof, wherein the numbering of said positions is in accordance with sequence depicted in SEQ ID No. 1, and

wherein presence of one of the nucleotides in each position indicates that SAV3 different from vaccine SAV3 is present in the biological sample.

According to another embodiment, the invention relates to another method for detecting SAV3 in a collected biological sample, wherein the SAV3 is different from vaccine SAV3, the method comprises the following steps:

a) preparing the sample comprising nucleic acid sequences for a reverse transcription reaction,

b) subjecting the mixture of a) to polymerase chain reaction with primers or probes comprising

i. T, A, or G in position 3064;
ii. T, A or G in position 4113;
iii. G, T or C in position 5085;
iv. C, G or A in position 5243;
v. G, T or C in position 5553;
vi. G, T or C in position 7356;
vii. G, T or C in position 8093;
viii. T, A or C in position 9123;
ix. G, T or C in position 11559;

or the complementary oligonucleotides thereof, wherein the numbering of said positions is in accordance with sequence depicted in SEQ ID No. 1, and

c) determining whether binding of the primers and/or probes to nucleotide sequences in the sample at all positions have occurred, indicating the presence of SAV3 different from vaccine SAV3 in the sample tested.

The skilled person is well aware of the fact that nucleic acid molecules may be double-stranded or single-stranded, and that reference to a particular site of one strand refers, as well, to the corresponding site on a complementary strand. Thus, when defining a SNP position, reference to an adenine (A), a thymine (T) (uridine (U)), a cytosine (C) or a guanine (G) at a particular site on one strand of a nucleic acid is also to be understood to define a thymine (uridine), adenine, guanine, or cytosine, respectively, at the corresponding site on a complementary strand of the nucleic acid molecule. Thus, reference may be made to either strand in order to refer to a particular SNP position, and the expression "complementary oligonucleotide thereof" of a SNP as used herein, should be interpreted as the complementary strand of the nucleic acid molecule, having a complementary SNP. All sequences mentioned in relation to this application are written 5-3, and will be read forward 5-3 or revers complementary 3-5. The oligonucleotide probes and oligonucleotide primers according to the present invention may be designed to hybridize to either strand,

and SNP detection methods disclosed herein may thus also in general target either strand.

By "standard methods" it is herein meant any method well known to the skilled person which may be used to confirm that the sample contains SAV3. This may be histology, immunohistochemistry, virus propagation and PCR, wherein PCR gives the fastest result. This is thus not described any further in this application.

According to one embodiment, the collected biological sample mentioned above is from a fish.

According to one embodiment, the method of step b) above, being confirming that the sample contains a SAV3 virus, comprises sequencing.

According to a further embodiment, the SAV3 detected in the sample is the vaccine virus if the two, three, four, five, six, seven, eight or nine SNPs alone or in any combination, or the complementary oligonucleotides thereof is present in the sample to be analysed, and wherein the numbering of said positions is in accordance with sequence depicted in SEQ ID No. 1.

According to yet another aspect, a method is provided, comprising the steps of:
a) collecting biological material from fish for example kidney, liver, heart or spleen from the fish ;
b) isolating genomic material of collected fish sample;
c) confirming that the virus is SAV3 by standard PCR
d) determining the nucleotide polymorphic site at the positions 3064, 4113, 5085, 5243, 5553, 7356, 8093, 9123 and/or 11559 of the isolated DNA, and detecting whether any of the following single nucleotide polymorphisms (SNPs) are present in the genomic material

i. C in position 3064;
ii. C in position 4113;
iii. A in position 5085;

iv. T in position 5243;
v. A in position 5553;
vi. A in position 7356;
vii. A in position 8093;
viii. G in position 9123;
ix. A in position 11559;

or the complementary oligonucleotides thereof, wherein the numbering of said positions is in accordance with sequence depicted in SEQ ID No. 1, and

wherein presence of at least one SNP indicates that vaccine SAV3 is present in the biological sample.

According to one embodiment of the above method, the nucleotide polymorphic site at the any combinations of two positions, three positions, 4 positions, 5 positions, 6 positions, 7 positions, 8 positions or all positions among 3064, 4113, 5085, 5243, 5553, 7356, 8093, 9123 and 11559 of the isolated DNA compared with the nucleic acid sequence SEQ ID No. 1 is determined , and wherein said virus from the sample is vaccine virus (SEQ ID NO 2) if any combinations of

i. C in position 3064;
ii. C in position 4113;
iii. A in position 5085;
iv. T in position 5243;
v. A in position 5553;
vi. A in position 7356;
vii. A in position 8093;
viii. G in position 9123;
ix. A in position 11559;

or the complementary oligonucleotides thereof is present.

According to one embodiment, the methods according to the invention is performed using at least one primer pair, each primer being selected from the group consisting of SEQ ID Nos 3-14.

According to yet another embodiment, the methods according to the invention for detecting vaccine SAV3 is performed using at least one probe selected from the group consisting of SEQ ID Nos 15-20.

In a method according to yet another embodiment,
primers according to SEQ ID NO 3 and 4 and a probe according to SEQ ID NO 15,
primers according to SEQ ID NO 5 and 6 and a probe according to SEQ ID NO 16,
primers according to SEQ ID NO 7 and 8 and a probe according to SEQ ID NO 17,
primers according to SEQ ID NO 9 and 10 and a probe according to SEQ ID NO 18,
primers according to SEQ ID NO 11 and 12 and a probe according to SEQ ID NO 19, and/or
primers according to SEQ ID NO 13 and 14 and a probe according to SEQ ID NO 20,
is used in the above said methods, for detecting vaccine SAV3.

In a method according to yet another embodiment,
primers according to SEQ ID NO 3 and 4 and a probe according to SEQ ID NO 21,
primers according to SEQ ID NO 5 and 6 and a probe according to SEQ ID NO 22,
primers according to SEQ ID NO 7 and 8 and a probe according to SEQ ID NO 23,
primers according to SEQ ID NO 9 and 10 and a probe according to SEQ ID NO 24,
primers according to SEQ ID NO 11 and 12 and a probe according to SEQ ID NO 25, and/or
primers according to SEQ ID NO 13 and 14 and a probe according to SEQ ID NO 26,
is used in the above said methods, for detecting SAV3 different form vaccine SAV3.

According to yet another embodiment, the methods according to the invention comprises nucleic acid amplification, e.g. using polymerase chain reaction.

According to yet another embodiment, the methods according to the invention is performed by comparing the sequence of the SAV3 of the sample with a detection

reagent, and determining which nucleotide is present in positions 3064, 4113, 5085, 5243, 5553, 7356, 8093, 9123 and 11559.

According to yet another embodiment, said detection reagent is an oligonucleotide probe.

According to yet another embodiment, the methods according to the invention is performed using SNP specific probe hybridization, SNP specific primer extension, SPN specific amplification, sequencing, 5' nuclease digestion, molecular beacon assay, oligonucleotide ligation assay, size analysis, single-stranded conformation polymorphism analysis, denaturing gradient gel electrophoresis.

According to one embodiment, an oligonucleotide probe or oligonucleotide primer is provided comprising a oligonucleotide sequence being homologous to a fragment of an extracted oligonucleotide sequence from a vaccine, said probe or primer being specific for a DNA sequence of the vaccine SAV3, for example SAV3 extracted from the vaccine ALPHA JECT micro 1 PD. The probe or primer comprises at least one SNP selected from the group consisting of T3064C, T4113C, G5085A, C5243T, G5553A , G7356A, G8093A A9123G and G11559A of SEQ ID Nos 2, and wherein the numbering of said positions is in accordance with the sequence depicted in SEQ ID No. 1. In a preferred embodiment, the probe or primer comprises at least one SNPs selected from the group consisting of T3064C, G5085A, C5243T, G7356A, A9123G and G11559A of SEQ ID Nos 2, and wherein the numbering of said positions is in accordance with the sequence depicted in SEQ ID No. 1.

According to yet another embodiment, the probe or primer according to the present invention are comprising at least 8 contiguous nucleotides of SEQ ID No. 1, including at least one of the following

i. C in position 3064;
ii. A in position 5085;
iii. T in position 5243;
iv. A in position 7356;

v. G in position 9123;
vi. A in position 11559;

or a complementary oligonucleotide thereof.

The probe or primer according to the present invention and as described above, are comprising at least 8, 10, 12, 15 or 20 (or any other number in-between) contiguous nucleotides of SEQ ID No. 1, including at least one SNP selected from the group consisting of T3064C, T4113C, G5085A, C5243T, G5553A , G7356A, G8093A A9123G and G11559A, wherein the numbering of said positions is in accordance with the sequence depicted in SEQ ID No. 1.

The invention further relates to use of primers and probes as described above, to differentiate infectious SAV3 and vaccine SAV3.

Furthermore, the present invention provides a kit for detection of vaccine SAV3, comprising a probe or primer according to the present invention.

In a preferred embodiment, the kit comprises a primer pair and a probe, more preferably the kit comprises
primers according to SEQ ID NO 3 and 4 and a probe according to SEQ ID NO 15,
primers according to SEQ ID NO 5 and 6 and a probe according to SEQ ID NO 16,
primers according to SEQ ID NO 7 and 8 and a probe according to SEQ ID NO 17,
primers according to SEQ ID NO 9 and 10 and a probe according to SEQ ID NO 18,
primers according to SEQ ID NO 11 and 12 and a probe according to SEQ ID NO 19, and/or
primers according to SEQ ID NO 13 and 14 and a probe according to SEQ ID NO 20.

Finally, the present invention provides use of an oligonucleotide sequence comprising at least 8 contiguous nucleotides of the sequence selected from SEQ ID NO 2, and wherein said sequence comprises at least one of the following

i. C in position 3064;

ii. C in position 4113;
iii. A in position 5085;
iv. T in position 5243;
v. A in position 5553;
vi. A in position 7356;
vii. A in position 8093;
viii. G in position 9123;
ix. A in position 11559;

or a complementary oligonucleotide thereof, and wherein the numbering of said positions is in accordance with the sequence depicted in SEQ ID No. 1, and wherein the sequences are used for detection of vaccine SAV3. According to one embodiment of the above use, two, three, four, five, six, seven, eight or nine sequences may be used alone or in any combination.

The present invention provides an in vitro method for determination of whether a SAV3 virus detected in fish, originates from disease causing virus or the virus used for manufacturing of vaccine, particularly a virus comprising SEQ ID No. 2 for example used in the vaccine ALPHA JECT micro 1 PD. The method is based on the surprising findings of SNPs in the DNA in virus extracted from the vaccine.

Although diagnosis related to differentiating vaccine virus from disease causing virus is based on SNPs identified in the SAV3 virus extracted from ALPHA JECT micro 1 PD, the skilled person will acknowledge, based on the teaching herein, that the present method and the present oligonucleotides may be used to determine if SAV3 virus from ALPHA JECT micro 1 PD, particular the virus comprising SEQ ID No. 2 is present in fish, in particular teleosts, in particular teleosts belonging to the family *Salmonidae*. According to one embodiment, the present method and present oligonucleotides are useful for detection of the vaccine virus in biological material from fish.

Throughout the application, the term "vaccine" is to be understood to mean any vaccine produced on a virus comprising SEQ ID NO 2, or a virus strain comprising at least one of the above said SNPs and being at least 80% similar to SEQ ID NO 2, such as vaccine ALPHA JECT micro 1 PD (Pharmaq AS, ATCvet-nr.:QI10A A01).

The nine of the SNPs uniquely associated with the vaccine strain of SAV3 are found in gene positions 3064 (T3064C), 4113 (T4113C), 5085 (G5085A), 5243 (C5243T), 5553 (G5553A), 7356 (G7356A), 8093 (G8093A), 9123 (A9123G) and 11559 (G11559A), see also table 1.

The numbering of the positions of the identified SNPs are based on SEQ ID No. 1. It is to be understood that whenever referring to the positions of the SNPs identified according to the present invention, the numbering is throughout the present description made according to the numbering of SEQ ID No.1 if not otherwise stated.

Based on the identification of the nine SNPs responsible for the unique identification of the vaccine strain, a method for detection of vaccine strain is provided comprising the steps of detecting single nucleotide polymorphism (SNP) associated with vaccine strain in the DNA of SAV3 to be analyzed, wherein said vaccine strain is identified if at least one of the following nucleotides:

1. C in position 3064;
2. C in position 4113;
3. A in position 5085;
4. T in position 5243;
5. A in position 5553;
6. A in position 7356;
7. A in position 8093;
8. G in position 9123;
9. A in position 11559;

or the complementary oligonucleotide thereof, is present in the DNA sequence of the SAV3, and wherein the numbering of said positions is in accordance with the sequence depicted in SEQ ID No. 1 (see also table 1).

Table 1 The nine SNPs related to the invention

SNP	Position in DNA	Virus extracted from vaccine	Infectious SAV3
T3064C	3064	C	T
T4113C	4113	C	T
G5085A	5085	A	G
C5243T	5243	T	C
G5553A	5553	A	G
G7356A	7356	A	G
G8093A	8093	A	G
A9123G	9123	G	A
G11559A	11559	A	G

According to the present invention, “single-nucleotide polymorphisms (SNP)” is to be understood to refer to a nucleotide sequence variation occurring when a single nucleotide, A, T (U), C or G in the genome, or other shared sequences (e.g. RNA) or fragments thereof, differs between vaccine SAV3 and SAV3 of other origin for example disease causing virus. The nine SNPs identified according to the present invention is in the genome.

Furthermore, as used herein, an “oligonucleotide sequence” or “nucleic acid sequence” is generally an oligonucleotide sequence or a nucleic acid sequence containing a SNP described herein, or one that hybridizes to such molecule such as a nucleic acid sequence with a complementary sequence.

An “isolated nucleic acid” as used herein is generally one that contains at least one of the SNPs described herein or one that hybridizes to such molecule, e.g. a nucleic acid with a complementary sequence, and is separated from most other nucleic acids present in the natural source of the nucleic acid, and is thus substantially free of other cellular material.

Oligonucleotide probes and oligonucleotide primers

The present invention provides oligonucleotide probes and oligonucleotide primers that may be used for detection of the presence of the SNPs in DNA of a a biological sample from fish, and thus for determination of whether the virus in the sample is of vaccine origin. The detection of nucleic acids present in a biological sample is widely applied in both human and veterinary diagnosis, and are well known to a skilled person. The nucleic acids from e.g. pathogens present in biological samples are isolated and hybridized to one or more hybridizing probes or primers used in order to amplify a target sequence.

One or more of the oligonucleotide probes according to the invention may be used in hybridization based detection methods where upon the binding of the oligonucleotide probes to the target sequence enables detection of the presence of at least one of the SNPs described herein, if present in the sample to be tested.

The skilled person will acknowledge that an oligonucleotide probe according to the present invention may be a fragment of DNA or RNA of variable length used herein in order to detect an SNP in a target sequence, e.g. single-stranded DNA or RNA, upon hybridization of the oligonucleotide probe to complementary sequence(s) of the said target sequence to be analyzed. The oligonucleotide probe according to the present invention may furthermore be labeled with a molecular marker in order to easily visualize that hybridization, and thus detection of the SNPs disclosed herein, have been achieved. Molecular markers commonly known to the skilled person may be used, e.g. a radiolabel, and more preferably, a luminescent molecule or a fluorescent molecule enabling the visualisation of the binding of the probe(s) to a target sequence.

An oligonucleotide probe according to the present invention is able to hybridize to another nucleic acid molecule, such as the single strand of DNA or RNA originating from a fish sample or a vaccine sample to be analysed, 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 recognise as the "stringency" of the hybridization. The suitable stringency for hybridisation of a probe to target nucleic acids depends on inter alia the length of the probe and the degree of complementation, variables well known to the skilled person. A oligonucleotide probe according to the present invention typically comprises a nucleotide sequence which under stringent conditions hybridize to at least 8, 10, 12, 16, 20, 22, 25, 30, 40, 50 (or any other number in-between) or more consecutive nucleotides in a target nucleic acid molecule, e.g. single-stranded DNA or RNA isolated from the fish or extracted from a vaccine sample to be analysed according to the present invention.

According to one embodiment, the oligonucleotide probe according to the present invention comprises about 8 to 25 consecutive nucleotides. It is to be understood that the oligonucleotide probe according one embodiment comprise one of the SNPs described herein or the complement thereof. New technology like specific Locked Nucleic Acid (LNA) hybridization probes allows for the use of extremely short oligonucleotide probes (You Y.; Moreira B.G.; Behlke M.A. and Owczarzy R. (2006), "Design of LNA probes that improve mismatch discrimination, *Nucleic Acids Res.* **34** (8): e60). According to one embodiment, probes are provided which are selected from the group consisting of SEQ ID Nos 15-20.

The present invention furthermore provides oligonucleotide primers useful for amplification of any given region of a nucleotide sequence, in particular a region containing one of the SNPs described herein. An oligonucleotide primer according to the present invention typically comprises a nucleotide sequence at least 8, 10, 12, 16, 20, 22, 25, 30, 40, 50 (or any other number in-between) or more consecutive nucleotides. According to one embodiment, the oligonucleotide primer according to the present invention comprises about 8 - 25 consecutive nucleotides.

As used herein, the term "oligonucleotide primer" is to be understood to refer to a nucleic acid sequence suitable for directing an activity to a region of a nucleic acid, e.g. for amplification of a target nucleic acid sequence by polymerase chain reaction (PCR). According to one embodiment of the present invention, "oligonucleotide primer pairs" is provided suitable for amplification of a region of mitochondrial genome material comprising the SNPs according to the present invention.

The skilled person will acknowledge that an oligonucleotide primer according to the present invention may be a fragment of DNA or RNA of variable length used herein in order to detect an SNP in a target sequence, e.g. single-stranded DNA or RNA, upon alignment of the oligonucleotide probe to complementary sequence(s) of the said target sequence to be analysed. An oligonucleotide primer according to the present invention may furthermore be labelled with a molecular marker in order to enable visualization of the results obtained. Various molecular markers or labels are available, dependent on the SNP detection method used.

An oligonucleotide primer according to the present invention typically comprises the appropriate number of nucleotides allowing that said primer align with the target sequence to be analysed. It is to be understood that an oligonucleotide primer according to one embodiment of the present invention, comprises one SNP described herein or the complement thereof.

According to one embodiment of the present invention, primer pairs are provided selected from the group consisting of SEQ ID Nos 3-14. (see also tables 2 and 3).

According to one embodiment of the present invention, probes are provided selected from the group consisting of SEQ ID Nos 15-20. (see also tables 2 and 3).

Table 2 Primer pairs and probes for detection of the SNPs related to the invention					
SEQ	**Assay name**	**SNP**	**Sequence 5'-3'**	**Detects**	**Primer/ Probe**
3	3064- Forward		TGGAGGATTGGGAGGATGAG	T3064C	Primer
4	3064- Revers		TGGGACACACGCTTCTCTGA		Primer
15	3064- Vaccine	C	ACAATGGTATC**C**TGGCG		Probe
21	3064- Infectious	T	ACAATGGTATC**T**TGGCG		Probe
5	5085- Forward		CCGGTCAACGACATACCTGTAG	T5085C	Primer
6	5085- Revers		GGCCTTTGCTTCAGCACTGT		Primer
16	5085- Vaccine	A	CAGACC**A**CCTTCTAG		Probe
22	5085- Infectious	G	CAGACC**G**CCTTCT		Probe
7	5243- Forward		GCCGCTCACTAACATCCAAGAT	G5243A	Primer
8	5243- Revers		GCGCACGGGTCTAGGTTCT		Primer
17	5243- Vaccine	T	TGAGAG**T**ACCTGCAG		Probe
23	5243-Infectious	C	TGAGAG**C**ACCTGCAG		Probe
9	7356- Forward		GCGCCGCGTTTATTGG	G7356A	Primer
10	7356- Revers		CCTGGCCACCATCATATCATC		Primer
18	7356 - Vaccine	A	TCATAACCGGAGT**A**GTGT		Probe
24	7356 - Infectious	G	TCATAACCGGAGT**G**GTGT		Probe
11	9123- Forward		GGTGAAACATCTCTGCGATACCT	A9123G	Primer
12	9123- Revers		CAGCACATCGCACTTTGCA		Primer
19	9123- Vaccine	G	CGGAC**G**ACACGCGG		Probe
25	9123 – Infectious	A	CGGAC**A**ACACGCGG		Probe
13	11559- Forward		GTAGAGATCTGTTCGGCAATGGT	G11559A	Primer
14	11559- Revers		TGGTTGCGACTACATGTTCCTT		Primer
20	11559 - Vaccine	A	CGCCAGT**A**AGTGCA		Probe
26	11559 - Infectious	G	CGCCAGT**G**AGTGCA		

When the primer pair and probes should be used to identify virus identical to the virus extracted from the vaccine, at least one of the following primers pairs and probe should be used

SEQ ID NO 3, 4 and 15 for SNP at 3064

SEQ ID NO 5, 6 and 16 for SNP at 3085,

SEQ ID NO 7, 8 and 17 for SNP at 5243,

SEQ ID NO 9, 10 and 18 for SNP at 7356,

SEQ ID NO 11, 12 and 19 for SNP at 9123,

SEQ ID NO 13, 14 and 20 for SNP at 11559

When the probes should be used to identify infectious SAV3, different from virus extracted from the vaccine, the following primer pairs and probe should be used

SEQ ID NO 3, 4 and 21 for SNP at 3064

SEQ ID NO 5, 6 and 22 for SNP at 3085,
SEQ ID NO 7, 8 and 23 for SNP at 5243,
SEQ ID NO 9, 10 and 24 for SNP at 7356,
SEQ ID NO 11, 12 and 25 for SNP at 9123,
SEQ ID NO 13, 14 and 26 for SNP at 11559

Oligonucleotide probes and oligonucleotide primers according to the present invention may be synthesized according to methods well known to the skilled person.

SNP identification methods

Upon the identification of the SNPs associated with the virus extracted from the vaccine, according to the present invention, the skilled person will acknowledge that various methods commonly used in order to detect polymorphisms may be used. Methods based on genome sequencing, hybridization and enzyme based methods are applicable for determining whether a SNP is present or not.

Various enzyme based methods are available for the skilled person, of which a number of polymerase chain reaction (PCR) based methods are available.

For example, Perkin Elmer Life Sciences provides SNP detection kit that may be used in order to determine whether a virus origin from a vaccine or other places, as for example from the environment, or in the fish (AcycloPrime^{TM}-FP SNP Detection). In said method, a thermostable polymerase is used which extends an oligonucleotide primer according to the present invention by one base, then ending the oligonucleotide primer one nucleotide immediately upstream of the relevant SNP position by the incorporation of fluorescent dye-labeled terminators. The identity of the base added is then determined by the increase fluorescence polarization of its linked dye.

Oligonucleotide primers according to the present invention useful in such a method would thus be constructed in order to facilitate the extension of the

primer by one base in the position selected from the group 3064, 4113, 5085, 5243, 5553, 7356, 8093, 9123 and 11559 relative to SEQ ID No. 1.

Another enzyme based method that may be used is restriction fragment length polymorphism (RLFP), utilizing that various, highly specific endonuclease upon digestion of the target sample, i.e. mitochondrial genome, results in different fragments that may be separated by gel electrophoresis.

Yet another enzyme based method that may be utilized in accordance with the present invention is the flap endocuclease (FEN) method. In said method, a structure-specific endonuclease is used to cleave a three-dimensional complex formed by hybridization with the target DNA, and where annealing with a target sequence comprising the SNP of interest triggers cleavage by the endonuclease.

Yet another method applicable in respect of the present invention is based on the use of TaqMan® Assays (Invitrogen). In said assay the oligonucleotide primers used in order to detect an SNP is labeled in both the 5'- and the 3' end, i.e. with a fluorophore at the 5'end of the oligonucleotide primer, and a quencher at the 3'-end of the oligonucleotide primer. Upon annealing of the oligonucleotide primer with a target sequence, the Taq polymerase will extend the oligonucleotide primer and form a nascent strand, followed by degradation of the oligonucleotide primer being annealed to the target, said degradation eventually resulting in the release of the fluorophore and provide a cleavage close to the quencher. The fluorescence signal produced is proportional to the fluorophore released. Various fluorophore labels may be used, such as e.g. 6-carboxyfluorescein, tetrafluorofluorescein. As quenchers, tetramethylrhodamine or dihydrocyclopyrroloindol may be used.

Several hybridization methods for detection of SNPs are available to the skilled person, and which may be utilized in accordance with the method of the present

invention. For example, the SNPs according to the present invention may be detected utilizing molecular beacon technology. According to this aspect of the present invention, oligonucleotide primers may be synthesized comprising complementary regions at each end allowing the formation of a hairpin loop, and wherein a fluorophore is attached at one end of the oligonucleotide primer, and a quenching agent is attached to the other end, and wherein fluorescence signal is produced upon binding to a DNA target of interest, i.e. genomic material isolated from the sample to be analyzed.

Yet another method applicable in respect of the present invention is based on DNA or RNA sequencing, which is the process of determining the precise order of nucleotides within a molecule. It includes any method or technology that is used to determine the order of the four bases (adenine, guanine, cytosine, and thymine) in a strand of DNA. The skilled person is well known with the various commonly known DNA and RNA sequencing methods that may be used according to the present invention, such as e.g. shotgun sequencing or bridge PCR sequencing.

Isolation of genomic material

The method according to the present invention may according to one embodiment involve the isolation of a biological sample from a fish, confirming SAV3 by standard PCR and testing for the presence of a SNP associated with vaccine virus in the genome. The skilled person will acknowledge that the SNPs identified according to the present invention may be detected by analyzing DNA as well as RNA, dependent upon the detection method used.

In order to determine whether a SAV3 originate from the vaccine or from infection/environment in accordance with the present invention, genomic material may be extracted. Various methods for obtaining genomic material well known to the skilled person are available. The skilled person will acknowledge that any tissue (i.e. any part of the fish) may be used in order to extract genomic material. Furthermore, the genomic material to be analyzed according to the

present invention may be obtained from fish of any life stages, e.g. egg, juvenile, smolt or ongrowing fish. Further it may be obtained from all parts of the fish, such as kidney, liver, heart or spleen. According to one embodiment, tissue removed from fish to be tested is maintained in 70% ethanol or other conservation liquid prior to further isolation of genomic material. DNA may be extracted from the obtained tissue using commonly available DNA extraction/isolation methods, such as e.g. DNeasy DNA Tissue Kit according to the protocol of the manufacturer.

Still another method applicable for detecting SNP is High Resolution Melting Analysis (HRM) enabling rapid detection of SNPs and determination of genetic variation within a population. The first step of a HRM protocol consist often of amplification of the region of interest, using standard nucleotide sequence amplification techniques well known to the skilled person, and wherein the amplification is performed in the presence of a specialized double-stranded DNA binding dye being highly fluorescent when bound to dsDNA and poorly fluorescent in unbound state. This difference provides for the monitoring of the DNA amplification. After amplification, the target is gradually denatured by increasing the temperature in small increments, resulting in a characteristic melting profile. As the amplified DNA is denatured gradually, dye is released, thus resulting in a drop in fluorescence.

SNP detection Kits

Based on the teaching herein, the skilled person will acknowledge that, based on the identified SNPs and associated sequence information disclosed herein, detection reagents can be developed and used to determine any SNP described herein individually or in combination, and that such detection reagents can be readily incorporated into kits used for SNP detection known in the art.

The term “kit” as used herein in the context of SNP detection reagents are intended to cover e.g. combinations of multiple SNP detection reagents, or one or more SNP detection reagents, such as oligonucleotide probe(s) and

oligonucleotide primer(s) or primer sets, arrays/microarrays of nucleic acid molecules, and beads that contain one or more oligonucleotide probe(s), oligonucleotide primer(s) or other detection reagents useful in the method of the present invention.

It is furthermore to be understood that the SNP detection reagents in a kit according to the present invention may furthermore include other components commonly included in such kits, e.g. such as various types of biochemical reagents (buffers, DNA polymerase, ligase, deoxynucleotide triphosphates for chain extension/amplification, etc.), containers, packages, substrates to which SNP detection reagents are attached., etc. necessary to carry out the method according to the present invention.

According to one embodiment of the present invention, a kit is provided which comprises the necessary reagents to carry out one or more assays in order to detect one or more of the SNP disclosed herein according to the method of the present invention. A kit according to the present invention may preferably comprise one or more oligonucleotide probes that hybridize to a nucleic acid target molecule enabling detection of each target SNP position if present in the material analyzed. Multiple pairs of probes may be included in the kit to simultaneously analyze for the presence of the SNP disclosed herein at the same time. The probes contained in the kit according to the present invention may according to one embodiment be immobilized on a carrier, such as e.g. an array or a bead.

According to one embodiment, the oligonucleotide probes are suitable for the detection of the SNP T3064C, such as SEQ ID NO 15. According to yet another embodiment, the oligonucleotide probes are suitable for the detection of the SNP G5085A, such as SEQ ID NO 16. According to yet another embodiment, the oligonucleotide probes are suitable for the detection of the SNP C5243T, such as SEQ ID NO 17. According to yet another embodiment, the oligonucleotide probes are suitable for the detection of the SNP G7356A, such

as SEQ ID NO 18. According to yet another embodiment, the oligonucleotide probes are suitable for the detection of the SNP A9123G, such as SEQ ID NO 19. According to yet another embodiment, the oligonucleotide probes are suitable for the detection of the SNP G11559A, such as SEQ ID NO 20.

According to yet another embodiment, the kit according to the present invention comprises oligonucleotide probes suitable for detection of all the SNPs described herein.

According to one embodiment, a kit according to the present invention comprises oligonucleotide primer(s) and optionally further SNP detection reagents useful in SNP detection methods utilizing oligonucleotide primers or primer pair(s).

According to one embodiment, the kit according to the present invention comprises at least one forward primer and reverse primer for amplifying a region containing a SNP selected from the group of SNPs consisting of T3064C (U3064C in case of RNA), T4113C (U4113C in case of RNA), G5085A, C5243T (C5243U in case of RNA), G5553A, G7356A, G8093A or A9123G, G11559A. The kit may also comprise probes for detecting the SNP. Said kit may furthermore optionally comprise further SNP detection reagents (enzymes and nucleotide triphosphates) necessary for conducting PCR or real time PCR.

According to one embodiment, the primer pairs are suitable for the detection of the SNP T3064C. According to yet another embodiment, the primer pairs are suitable for the detection of the SNP G5085A. According to yet another embodiment, the primer pairs are suitable for the detection of the SNP C5243T. According to yet another embodiment, the primer pairs are suitable for the detection of the SNP G7356A. According to yet another embodiment, the primer pairs are suitable for the detection of the SNP A9123G. According to yet another embodiment, the primer pairs are suitable for the detection of the SNP G11559A.

According to the invention, if the SNP is present in one or any combinations of two positions, three positions, 4 positions, 5 positions, 6 positions, 7 positions, 8 positions or all positions among 3064, 4113, 5085, 5243, 5553, 7356, 8093, 9123 and 11559 of the isolated DNA from the sample, when compared with the nucleic acid sequence SEQ ID No. 1 said virus from the sample originate from the vaccine, for example ALPHA JECT micro 1 PD.

Table 3 Sequences related to the invention

SEQ ID No.	Description of sequence
1	DNA of Salmonid Alphavirus 3 from fish (Modification of GenBank acc.KC122925, with cut of 27 nucleotides at 5 end)
2	DNA of Salmonid Alphavirus 3 in the vaccine ALPHA JECT micro 1 PD
3	Oligonucleotide (forward 1) primer for detecting SNP T3064C
4	Oligonucleotide (reverse 1) primer for detecting SNP T3064C
15	Oligonucleotide probe for detecting SNP T3064C
21	Oligonucleotide probe for detecting lack of SNP T3064C
3	Oligonucleotide (forward 1) primer for detecting SNP G5085A
4	Oligonucleotide (reverse 1) primer for detecting SNP G5085A
16	Oligonucleotide probe for detecting SNPs G5085A
22	Oligonucleotide probe for detecting lack of SNP G5085A
5	Oligonucleotide (forward 1) primer for detecting SNP C5243T
6	Oligonucleotide (reverse1) primer for detecting SNP C5243T
17	Oligonucleotide probe for detecting SNPs C5243T
23	Oligonucleotide probe for detecting lack of SNP C5243T
7	Oligonucleotide (forward 1) primer for detecting SNP G7356A
8	Oligonucleotide (reverse 1) primer for detecting SNP G7356A
18	Oligonucleotide probe for detecting SNP G7356A
24	Oligonucleotide probe for detecting lack of SNP G7356A
9	Oligonucleotide (forward 1) primer for detecting SNP A9123G
10	Oligonucleotide (reverse 1) primer for detecting SNP A9123G
19	Oligonucleotide probe for detecting SNP A9123G
25	Oligonucleotide probe for detecting lack of SNP A9123G
11	Oligonucleotide (forward 1) primer for detecting SNP G11559A
12	Oligonucleotide (reverse 1) primer for detecting SNP G11559A
20	Oligonucleotide probe for detecting SNP G11559A
26	Oligonucleotide probe for detecting lack of SNP G11559A

The skilled person will thus acknowledge, based on the teaching of the present invention, that the method according to the invention may be used to

differentiate the virus from a vaccine with the SAV3 causing disease, particularly virus from the vaccine ALPHA JECT micro a PD.

Figures

The present invention and its various embodiments will be described in more detail in the following, where the enclosed Figures shows

Fig. 1 an alignment of SEQ ID NO 1 and SEQ ID NO 2

and the enclosed nucleotid sequences are

SEQ ID NO 1; DNA of Salmonid Alphavirus 3 from fish (Modification of GenBank acc.KC122925, with cut of 27 nucleotides at 5 end)

SEQ ID NO 2; DNA extracted from vaccine ALPHA JECT micro 1 PD

SEQ ID NO 3; Oligonucleotide (forward 1) primer for detecting SNP T3064C

SEQ ID NO 4 Oligonucleotide (reverse 1) primer for detecting SNP T3064C

SEQ ID NO 15 Oligonucleotide probe for detecting SNP T3064C

SEQ ID NO 21 Oligonucleotide probe for detecting lack of SNP T3064C

SEQ ID NO 5. Oligonucleotide (forward 1) primer for detecting SNP G5085A

SEQ ID NO 6 Oligonucleotide (reverse 1) primer for detecting SNP G5085A

SEQ ID NO 16 Oligonucleotide probe for detecting SNPs G5085A

SEQ ID NO 22 Oligonucleotide probe for detecting lack of SNP G5085A

SEQ ID NO 7 Oligonucleotide (forward 1) primer for detecting SNP C5243T

SEQ ID NO 8 Oligonucleotide (reverse1) primer for detecting SNP C5243T

SEQ ID NO 17 Oligonucleotide probe for detecting SNPs C5243T

SEQ ID NO 23 Oligonucleotide probe for detecting lack of SNP C5243T

SEQ ID NO 9 Oligonucleotide (forward 1) primer for detecting SNP G7356A

SEQ ID NO 10 Oligonucleotide (reverse 1) primer for detecting SNP G7356A

SEQ ID NO 18 Oligonucleotide probe for detecting SNP G7356A

SEQ ID NO 24 Oligonucleotide probe for detecting lack of SNP G7356A

SEQ ID NO 11 Oligonucleotide (forward 1) primer for detecting SNP A9123G
SEQ ID NO 12 Oligonucleotide (reverse 1) primer for detecting SNP A9123G
SEQ ID NO 19 Oligonucleotide probe for detecting SNP A9123G
SEQ ID NO 25 Oligonucleotide probe for detecting lack of SNP A9123G

SEQ ID NO 13 Oligonucleotide (forward 1) primer for detecting SNP G11559A
SEQ ID NO 14 Oligonucleotide (reverse 1) primer for detecting SNP G11559A
SEQ ID NO 20 Oligonucleotide probe for detecting SNP G11559A
SEQ ID NO 26 Oligonucleotide probe for detecting lack of SNP G11559A

Examples:

Reference throughout the description to "an embodiment" signifies that a particular feature, structure or property specified in connection with an embodiment is included in the least in one embodiment. The expressions "in one embodiment", "in a preferred embodiment" or "in an alternative embodiment" different places in the description does not necessarily point to the same embodiment. Further, the different features, structures or properties may be combined in any suitable way in one or more of the embodiments.

Example 1:

A vaccine ALPHA JECT micro 1 PD was collected and the presence of SAV3 virus was confirmed by standard PCR. DNA was extracted and sequenced using Next Generation Sequencing. The sequence is enclosed as SEQ ID NO 2. The vaccine sequence was aligned and compared against a reference sequences of SAV3 (SEQ ID No. 1 which is a modification of GenBank acc.KC122925, with cut of 27 nucleotides at 5 end). From this alignment the vaccine strain, herein referred to as vaccine SAV3, were found to have 9 unique single mutations. The alignment is shown in Figure 1.

The following mutations were observed:

1. C in position 3064;
2. C in position 4113;
3. A in position 5085;

4. T in position 5243;
5. A in position 5553;
6. A in position 7356;
7. A in position 8093;
8. G in position 9123;
9. A in position 11559;

Figure 1 also shows more mutations, but these were not unique for the vaccine strain.

Example 2: Test of virus from ALPHA JECT micro 1 PD vaccine

RNA was extracted from vaccine sample of ALPHA JECT micro 1 PD and from a Atlantic salmon (*Salmo salar*) documented positive to SAV3. These two samples were tested on Real time assay using SNP 5085, primers and probe shown in Table 1. The result shows that the 5085 assay detect virus in the SAV3 field sample and gives not detected (ND) on the sample form vaccine. The vaccine sample tested positive in vaccine and not detected ND on the virus sample from field (Table 2).

Table 4: Primers and probes used to differentiate between virus from the vaccine and virus from the fish.

SEQ	Assay name	SNP	Sequence 5'-3'	Detects
5	5085-F		CCGGTCAACGACATACCTGTAG	Primer
6	5085-R		GGCCTTTGCTTCAGCACTGT	Primer
22	5085-infectious	G	CAGACC**G**CCTTCT	Probe
16	5085- Vaccine	A	CAGACC**A**CCTTCTAG	Probe

Table 5: Ct values from PCR analysis of SAV3 virus and the vaccine ALPHA JECT micro 1 PD, using Real time assay (SNP 5085).

Assay	SAV3 assay	5085 - Vaccine	5085 - Infectious	Conclusion
Biological sample	17,8	ND	25,6	Infectious Virus
Vaccine	28,8	30,4	ND	Virus from the vaccine

By "SAV3 assay" any suitable assay may be used. As said above, based on prior art and the sequence of SAV3, it would be obvious to a skilled person to develop a suitable assay. The Ct values will depend on the assay used, but the relative proportion between the samples will be the same.

Example 3: Differentiating the fish virus and the vaccine virus in fish tissue.

Fish were sampled from a farm with fish vaccinated with Alpha Ject micro 1 PD. Biological material from the heart were aseptically collected, and stored on RNA later. The samples were sent to PatoGen lab. The genomic material was extracted and analysed using Real Time PCR assay to confirm the presence of SAV3. When positive to SAV3, the nucleotide polymorphic site at the position 5085 of the extracted RNA, was determined and compared with of the nucleic acid sequence SEQ ID No. 1. The SAV3 was similar to the virus of Vaccine ALPHA JECT micro 1 PD if the nucleotides A or a complementary oligonucleotide thereof, was present in position 5085.

Controls included were Alpha Ject micro PD 1, sample confirmed positive to SAV3 and sample confirmed negative to SAV3 (Table 5).

Table 6 Real-time RT-PCR results from field samples using SAV3 assay and 5085 assay, in fish vaccinated with ALPHA JECT micro PD 1.

Sample Name	SAV3-assay	5085 - infectious	5085 - vaccine	Conclusion
FR17821533	24,5	ND	28,480	ALPHA JECT micro 1 PD
FR17821537	32,9	ND	37,419	ALPHA JECT micro 1 PD
FR17821535	33,8	ND	36,679	ALPHA JECT micro 1 PD
FR17821540	35,1	ND	38,887	ALPHA JECT micro 1 PD
FR17821532	24,5	ND	27,707	ALPHA JECT micro 1 PD
Control confirmed negative to SAV3	ND	ND	ND	No virus
Control Alpha Ject micro 1 PD	29,1	ND	34,3	ALPHA JECT micro 1 PD
Control confirmed positive to SAV3	26,8	29,9	ND	Infectious Virus

All tested fish were positive to SAV3 originating from ALPHA JECT micro 1 PD, and negative to infectious virus. The fish was thus vaccinated, and not infected.

Patent claims

1. An in vitro method for detection of a vaccine SAV3, in a collected biological sample, the method comprises the following steps

a) isolating genomic material from the biological sample;

b) confirming that sample contains a SAV3 virus by standard methods,

c) detecting whether any of the following single nucleotide polymorphisms (SNPs) are present in the genomic material

i. C in position 3064;
ii. C in position 4113;
iii. A in position 5085;
iv. T in position 5243;
v. A in position 5553;
vi. A in position 7356;
vii. A in position 8093;
viii. G in position 9123;
ix. A in position 11559;

or the complementary oligonucleotides thereof, wherein the numbering of said positions is in accordance with sequence depicted in SEQ ID No. 1, and

wherein presence of at least one SNP indicates that vaccine SAV3 is present in the biological sample.

2. Method according to claim 1, wherein presence of SNPs at any combinations of two positions, three positions, 4 positions, 5 positions, 6 positions, 7 positions, 8 positions or all positions among 3064, 4113, 5085, 5243, 5553, 7356, 8093, 9123 and 11559 of the isolated DNA from the sample compared with the nucleic acid sequence SEQ ID No. 1, indicate that vaccine SAV3 is present in the biological sample.

3. Method according to claim 1, wherein the method of step b) includes sequencing.

4. An in vitro method for detecting vaccine SAV3, in a collected biological sample, the method comprises the following steps:

a) preparing the sample comprising nucleic acid sequences for a reverse transcription reaction,

b) subjecting the mixture of a) to polymerase chain reaction with at least one primer or probe comprising

i. C in position 3064;
ii. C in position 4113;
iii. A in position 5085;
iv. T in position 5243;
v. A in position 5553;
vi. A in position 7356;
vii. A in position 8093;
viii. G in position 9123;
ix. A in position 11559;

or the complementary oligonucleotides thereof, wherein the numbering of said positions is in accordance with sequence depicted in SEQ ID No. 1, and

c) determining whether binding of the primers and/or probes to nucleotide sequences in the sample and amplification of the sequences between them have occurred, indicating the presence of vaccine SAV3 in the biological sample.

5. Method according to any of the claims 1- 4, **characterized** in that primers and probes are selected from the group comprising seq id no 3-20.

6. Method according to claim 5, **characterized** in that the primers and probes are selected from the group comprising the following primer pairs and probes:
primers according to seq id no 3 and 4 and a probe according to seq id no 15,
primers according to seq id no 5 and 6 and a probe according to seq id no 16,
primers according to seq id no 7 and 8 and a probe according to seq id no 17,
primers according to seq id no 9 and 10 and a probe according to seq id no 18,
primers according to seq id no 11 and 12 and a probe according to seq id no 19, and
primers according to seq id no 13 and 14 and a probe according to seq id no 20.

7. An in vitro method for detection of SAV3 different from vaccine SAV3, in a collected biological sample, the method comprises the following steps

a) isolating genomic material from the biological sample;

b) confirming that sample contains a SAV3 virus by standard methods,

c) detecting whether any of the following nucleotides are present in the genomic material

i. T, A, or G in position 3064;

ii. T, A or G in position 4113;

iii. G, T or C in position 5085;

iv. C, G or A in position 5243;

v. G, T or C in position 5553;

vi. G, T or C in position 7356;

vii. G, T or C in position 8093;

viii. T, A or C in position 9123;

ix. G, T or C in position 11559;

or the complementary oligonucleotides thereof, wherein the numbering of said positions is in accordance with sequence depicted in SEQ ID No. 1, and

wherein presence of one of the nucleotides at each position indicates that SAV3 different from vaccine SAV3 is present in the biological sample.

8. Method for detecting SAV3 in a collected biological sample, wherein the SAV3 is different from vaccine SAV3, the method comprises the following steps:

a) preparing the sample comprising nucleic acid sequences for a reverse transcription reaction,

b) subjecting the mixture of a) to polymerase chain reaction with primers or probes comprising

i. T, A, or G in position 3064;

ii. T, A or G in position 4113;

iii. G, T or C in position 5085;

iv. C, G or A in position 5243;

v. G, T or C in position 5553;

vi. G, T or C in position 7356;

vii. G, T or C in position 8093;

viii. T, A or C in position 9123;

ix. G, T or C in position 11559;

or the complementary oligonucleotides thereof, wherein the numbering of said positions is in accordance with sequence depicted in SEQ ID No. 1, and

c) determining whether binding of the primers and/or probes to nucleotide sequences in the sample at all positions have occurred, indicating the presence of SAV3 different from vaccine SAV3 in the sample tested.

9. Method according to claim 8, **characterized** in that the primers are selected from the group comprising seq id no 3-15 and the probes are selected from the group comprising SEQ ID NO 21-26.

10. Method according to any one of claims 1-9, **characterized** in that the sample to be analysed is from a teleost fish, such as salmonides, basses, breams among others.

11. Method according to any one of the preceding claims, **characterized** in that vaccine SAV3, is extracted from vaccine ALPHA JECT micro 1 PD, used to vaccinate teleost against PD.

12. Use of primers and probes according to SEQ ID NO 3-26 to differentiate infectious SAV3 from vaccine SAV3.

13. Kit for detection of vaccine SAV3 virus, comprising at least one primer or probe according to claims 5 or 6.

P17313NO00 seq id.txt

SEQUENCE LISTING

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