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(54) **Title**: MEANS AND METHODS FOR DISTINGUISHING FECV AND FIPV

(57) **Abstract**: The invention provides methods and means for distinguishing FECV and FIPV, and methods and means for determining whether FIPV is present in a sample. Further provided are primers and probes for detecting FIPV specific nucleic acid sequences encoding a spike protein, antibodies for detecting a FIPV, and an immunogenic composition and use thereof for eliciting an immune response against a feline coronavirus, preferably a FIPV.

Title: Means and methods for distinguishing FECV and FIPV

The invention relates to the field of veterinary diagnosis, more specifically the invention relates to the field of feline coronaviruses and identification thereof.

Feline coronaviruses (FCoVs) are common pathogens of domestic and non-domestic Felidae, including but not limited to cats, lions, tigers, leopards, jaguars, lynxes, caracals, cheetahs, cougars and servals. In domestic multi-cat environments up to 90% FCoV seropositivity is reached . FCoV are closely related to canine coronavirus (CCoV) and transmissible gastroenteritis virus (TGEV) of swine. Two serotypes, I and II, exist of FCoV of which serotype I predominates, with 80-95% of FCoV infections. Type II FCoV presumably results from RNA recombination in animals doubly infected by serotype I FCoV and CCoV, during which a CCoV spike gene or part thereof is incorporated into the FCoV genome, apparently an infrequently occurring event. Feline enteric coronavirus (FECV) is the most common pathotype of FCoV, for both serotype I and serotype II. FECV is mainly confined to the intestines, spreads via the oral-fecal route, and is highly contagious. FECV infection generally occurs unapparently; sometimes, however, it causes symptoms such as mild enteritis (Haijema et al., 2007).

In the 1970's feline infectious peritonitis (FIP), a (then) rare but serious disease in cats, was reported to be caused by a feline coronavirus, which was called feline infectious peritonitis virus (FIPV). Contrary to FECV, FIPV is highly virulent. FIPV infection can be either granulomatous (dry) or effusive (wet) and is a progressive and usually fatal disease. Symptoms of FIP include failure to thrive in young cats, lameness, fluctuating fever, inappetence and weight loss resulting in death (Pedersen 2009). A dramatic dysregulation of the adaptive immune system accompanies progression of FIP as demonstrated by hypergammaglobulinemia and depletion of lymphoid and peripheral T cells (Haijema et al., 2007). Whereas FECV is confined to the gut,

FIPV is able to infect - and replicate in - monocytes and macrophages causing systemic disease with multiple organs being affected.

Two prevailing theories exist about the origin of FIPV. According to the "mutation hypothesis", FIPV originates from FECV by de novo mutation in infected felines resulting in a highly virulent FIP virus. The mutation giving rise to FIPV has not been identified but has been proposed to be in the non structural 3c, 7a or 7b genes (see figure 1), which encode proteins with unknown function (Vennema et al., 1998, Poland et al., 1996, Kennedy et al., 2001, Pedersen 2009). Therefore it is thought that a mutation in the 3c, 7a or 7b gene or a combination of mutations in these genes alters the biological properties of the virus allowing the enteric coronavirus to infect monocytes and macrophages thereby spreading infection to the organs and causing FIP (Pedersen 2009): the transition of FECV to FIPV. The mutation hypothesis has not been formally proven.

According to another theory, two distinct strains of FECV circulate in natural populations, a virulent and an avirulent strain, and only felines infected by the virulent strain will develop FIP (Brown et al., 2009). Brown et al. (2009) isolated viral sequences from cats suffering from FIP, and from FECV infected but asymptomatic (healthy) cats. Using phylogenetic analyses they found that distinct viral sequences are present in sick cats and healthy cats. Dye and Siddell (2007) compared the viral sequences of feline coronavirus isolated from jejunum and from liver of a cat suffering from FIP. According to the mutation theory, FECV is confined to the intestines, while FIPV, which is able to infect macrophages and monocytes, is present in the liver. Yet, Dye and Siddell found 100% nucleotide identity and thus questioned the mutation hypothesis according to which the liver coronavirus is a mutated jejunum coronavirus. They suggested that in cats suffering from FIP the same virulent feline coronavirus strain was present in both liver and jejunum.

Previously, the present inventors identified a number of differences in the spike protein of tissue culture-adapted serotype II feline coronaviruses FECV 79-1683 and FIPV 79-1146 (Rottier et al., 2005). The FIPV 79-1146 contained several mutations in the C-terminal domain of the spike protein, the S2 domain. However, FECV 79-1683 and FIPV 79-1146 are not prototypical feline coronaviruses and are thus not representative for the serotype I FECV and FIPV most cats are infected with (Pedersen 2009). Firstly, the serotype II feline coronaviruses originate from RNA recombination of canine and feline coronaviruses and contain the canine coronavirus spike protein. Spike proteins of feline and canine coronaviruses have only approximately 45% amino acid sequence identity (Motokawa et al., 1996). Secondly, FECV 79-1683 and FIPV 79-1146 are tissue culture-adapted to cell lines other than macrophages. Because FIPV infects monocytes and macrophages *in vivo*, tropism of these laboratory strains differs from prototypical feline coronaviruses. Thirdly, FIPV 79-1146, unlike serotype I FIPV which infects monocytes and macrophages, is exceptionally virulent by every common route of infection (Pedersen 2009). Fourthly, FECV 79-1683 cannot be qualified as a true FECV as argued extensively by Pedersen in his recent review (Pedersen, 2009). Notably, FECV 79-1683 lacks most of the 7b gene, which is present in non-tissue culture-adapted strains of FECV and has a deleterious mutation in its 3c gene, indicating that it may have originated from an FIPV.

Feline coronavirus infection is generally demonstrated by the presence of antibodies in the blood. An effective treatment or vaccine for FIPV infection does not exist. Cats developing FIP die within days or weeks - in the case of effusive FIP - or months, in the case of dry or granulomatous FIP. A commercially available vaccine consisting of a temperature sensitive mutant of a FIPV strain has not convincingly proven its protective efficacy in a number of immunization studies (McArdle et al., 1995; Fehr et al., 1997). Furthermore, up to date there is no diagnostic test to discriminate between FECV and FIPV.

A further complicating factor is that the clinical picture of FIP is highly variable and, as a consequence, the disease cannot easily be established unequivocally. The diagnosis is often a presumptive one, based on anamnestic, clinical and non-specific laboratory parameters. Because there is no specific diagnostic test for FIPV, it is often also not possible to discriminate between FIP and other diseases with overlapping symptoms. Both diagnostic tests for and vaccines against FIPV are highly needed due to the progressive and debilitating course of FIP.

It is an aim of the present invention to provide means and methods for distinguishing FIPV and FECV.

The present inventors found that FIPV harbours a specific alteration relative to FECV in the spike protein at amino acid position 1049 as depicted in figure 2B.

The invention therefore provides a method for identifying feline infectious peritonitis virus (FIPV) comprising determining the identity of an amino acid of a feline coronavirus spike protein at a position corresponding to amino acid position 1049 as depicted in figure 2B, and identifying the feline coronavirus as FIPV if the determined identity of the amino acid is not a methionine. According to this method of the invention, FECV is identified if the determined identity of the amino acid is methionine.

With identifying FIPV or FECV is meant the identification of a virulent (FIPV) or an avirulent (FECV) type feline coronavirus. Identification is carried out by determining the identity of an amino acid and/or nucleic acid sequence of said feline coronavirus.

A feline coronavirus nucleic acid sequence comprises a chain of nucleotides, preferably (c)DNA or RNA, that is part of a feline coronavirus or

obtained from a feline coronavirus, either directly, or after processing, such as for example by using reverse transcriptase PCR, and/or amplification.

A feline coronavirus spike protein is a feline coronavirus membrane protein comprising an ectodomain. The spike protein is one of the four canonical structural proteins of coronavirus and is responsible for attachment to and entry of the virus into cells during infection.

FIPV from lesions of cats with pathologically confirmed FIP were compared genetically with FECV obtained from asymptomatic cats. Typical lesions of FIP were (pyo)granulomatous lesions presented in different internal organs mainly in spleen, liver, lung, kidney, or mesenteric lymph node. Due to the high mutation rates of RNA viruses, numerous differences were observed between individual FECV and FIPV sequences. However, in all 47 FECV faeces or plasma isolates, the amino acid at position 1049 of the spike protein as depicted in figure 2B is a methionine, whereas in 52 out of 54 FIPV lesion isolates an alteration of the amino acid at position 1049 as depicted in figure 2B is present resulting in an amino acid at this position other than methionine. It was later found that five sequences classified as derived from healthy cats were actually derived from blood samples of cats with confirmed FIP (Q093501030_326B_4546.scf, Q093501032_327B_4546.scf, Q093501036_321S_4546.scf, Q093501038_321A_4546.scf and Q093501046_K11_019.ab1), meaning that the identity of the amino acid at a position corresponding to position 1049 as depicted in figure 2B was determined and demonstrated to be methionine in 42, instead of 47, samples from FECV faeces or plasma isolates from healthy cats.

The nucleic acid sequence encoding the methionine at position 1049 of the spike protein of FECV corresponds to the codon comprising nucleotide positions 3145, 3146 and/or 3147 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A. The nucleotide sequence encoding the methionine in the FECV spike protein at position 1049 as depicted in figure 2A

is adenine-thymine-guanine (a-t-g), which corresponds with the sequence adenine-uridine-guanine (a-u-g) in the viral genomic RNA. Any substitution of at least one nucleotide in this nucleotide codon results in an amino acid other than methionine in the spike protein of FECV at position 1049 as depicted in figure 2B. According to the present invention the identified nucleotide and/or amino acid sequence at nucleotide positions 3145, 3146 and/or 3147 of the gene encoding a feline coronavirus spike protein, and amino acid position 1049 respectively as depicted in figures 2A and 2B is used to discriminate between FIPV and FECV. With the present invention for the first time a polymorphism of the feline coronavirus that enables distinguishing FECV and FIPV has been identified in prototypical serotype I FECV and FIPV.

The present inventors further found that a significant part of the small percentage of FIPV which do not harbour the specific alteration relative to FECV in the spike protein at amino acid position 1049 as depicted in figure 2B, harbours a specific alteration relative to FECV in the spike protein at amino acid position 1051 as depicted in figure 2B. In these cases, a serine at this position appeared to be substituted. Thus, the specific alteration at amino acid position 1051 also provides an approach to identify FIPV.

The invention therefore also provides a method for identifying feline infectious peritonitis virus (FIPV) comprising determining the identity of an amino acid of a feline coronavirus spike protein at a position corresponding to amino acid position 1051 as depicted in figure 2B, and identifying the feline coronavirus as FIPV if the determined identity of the amino acid is not a serine. Also provided is a method for determining whether feline infectious peritonitis virus (FIPV) is present in a sample, comprising determining whether said sample comprises a feline coronavirus, and if a feline coronavirus is present determining the identity of an amino acid in a spike protein of said feline coronavirus at a position corresponding to amino acid position 1051 as

depicted in figure 2B, and determining that FIPV is present if said amino acid is not serine.

In a set of 97 cats with pathologically confirmed FIP, in 87 out of 97 FIPV lesion isolates an alteration of the amino acid at position 1049 as depicted in figure 2B is present resulting in an amino acid at this position other than methionine. In five of the ten FIPV lesion isolates in which a methionine was present at position 1049 as depicted in figure 2B, an alteration of the amino acid at position 1051 as depicted in figure 2B was present resulting in an amino acid at this position other than serine. The nucleic acid sequence encoding the serine at position 1051 of the spike protein of FECV corresponds to the codon comprising nucleotide positions 3151, 3152 and 3153 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A. Serine is encoded by the nucleotide codons t/u-c-t/u, t/u-c-c, t/u-c-a, t/u-c-g, c-g-t/u and c-g-c. Any substitution of one or more nucleotides in this nucleotide codon resulting in a nucleotide sequence other than these codons results in an amino acid other than serine in the spike protein of FECV at position 1051 as depicted in figure 2B. According to the present invention the identified nucleotide and/or amino acid sequence at either nucleotide positions 3151, 3152 and/or 3153 of the gene encoding a feline coronavirus spike protein, and/or amino acid position 1051 of the Spike protein respectively, as depicted in figures 2A and 2B is also used to discriminate between FIPV and FECV. In a preferred embodiment, an alteration of a serine at an amino acid position corresponding to position 1051 as depicted in figure 2B is the result of a replacement of the nucleobase thymine at a position corresponding to nucleotide position 3151 as depicted in figure 2A with the nucleobase guanine.

In one embodiment, the identity of the amino acids in a spike protein of a feline coronavirus at positions corresponding to amino acid positions 1049 and 1051 as depicted in figure 2B are both determined. If the

identity of both these amino acids is determined, a high accuracy in distinguishing FIPV and FECV is obtained. In one embodiment, the identity of the amino acids at positions 1049 and 1051 as depicted in figure 2B is determined in one test. However, it is also possible to determine the identity of these amino acid sequentially. For instance, first the identity of the amino acid at a position corresponding to position 1049 as depicted in figure 2B is determined. If the presence of a methionine is detected at this position, subsequently the identity of the amino acid at a position corresponding to position 1051 as depicted in figure 2B is preferably determined. If the presence of an amino acid other than a methionine is detected at this position, determining the identity of the amino acid at a position corresponding to position 1051 as depicted in figure 2B can be omitted. However, the identity of the amino acid at this position may also be determined in that case.

A nucleic acid sequence of the spike gene (nucleotides 1-4407) of feline coronavirus comprising the nucleotides 20395-24801 as defined in the sequence of gene accession number NC_012955 (Feline coronavirus UU10, complete genome) and nucleotides 20382-24788 as defined in the sequence of gene accession number NC_012952 (Feline coronavirus UU8, complete genome) is presented in figure 2A. Nucleotides 20395-24801 of NC_012955 encode the feline coronavirus spike protein (YP_003038574). Nucleotides 20382-24788 of NC_012952 encode the feline coronavirus spike protein (YP_003038543). Thus, nucleotides 3145, 3146 and 3147 of the gene encoding the spike protein as used throughout the description and as depicted in figure 2A correspond to nucleotides 23539, 23540 and 23541 of the complete genome as defined in the sequence of NC_012955 and/or nucleotides 23526, 23527 and 23528 of the complete genome as defined in the sequence of NC_012952. Nucleotides 3151, 3152 and 3153 of the gene encoding the spike protein as used throughout the description and as depicted in figure 2A correspond to nucleotides 23545, 23546 and 23547 of the complete genome as defined in the

sequence of NC_012955 and/or nucleotides 23532, 23533 and 23534 of the complete genome as defined in the sequence of NC_012952.

The amino acid sequence of a feline coronavirus spike protein referring to the amino acid numbering defined in the sequences of YP_003038574 and YP_003038543 which are partial translations of NC_012955 and NC_012952 respectively is presented in figure 2B. The numbering of amino acid positions as used throughout the description refers to the amino acid positions as defined in YP_003038574 and/or YP_003038543. A skilled person is able to identify the nucleotide and amino acid positions in any given feline coronavirus sequence which correspond to the nucleotide positions 3145, 3146 and/or 3147 and amino acid position 1049 and the nucleotide positions 3151, 3152 and/or 3153 and amino acid position 1051 as depicted in figure 2A or 2B, for instance using alignment software such as "Align 2" or "Bioconductor".

The symptoms of FIP include for instance the accumulation of ascitic fluid within the abdomen (only in effusive FIP), retarded growth, lack of appetite, fever, weight loss and diarrhea. As indicated herein above, similar symptoms are also observed with cats suffering from other diseases, making unequivocal diagnosis of FIP so far impossible. Now that a polymorphism has been identified for feline coronavirus spike protein that allows for determining the presence of FIPV in a sample it can be determined whether a feline, for instance a cat, suffers from FIP. Because currently there is no treatment for FIP, and the course of the disease is progressive and debilitating resulting inevitably in death, it can be decided to euthanize said cat when the animal has been demonstrated to carry FIPV. In addition, the cat or cattery owner can take proper measures to prevent possible spread of the infection and/or reduce predisposing conditions such as stress. However, when FIPV has been demonstrated to be absent in a cat, feline infectious peritonitis can be eliminated as a possible cause of the disease. Therefore, in that case the cat

should not be euthanized but diagnostic approaches could be continued and the animal could be provided with treatment for the possible alternative disease(s) the symptoms of which resemble those of FIP. Such treatment can for instance be further symptomatic treatment or application of antibiotics to counteract a possible bacterial cause of the disease.

Further provided by the invention is therefore a method for determining whether feline infectious peritonitis virus (FIPV) is present in a sample, comprising preferably from a feline or from a substance that has been in contact with a feline, determining whether said sample comprises a feline coronavirus and, if a feline coronavirus is present, determining the identity of an amino acid in a spike protein of said feline coronavirus at a position corresponding to amino acid position 1049 and/or 1051 as depicted in figure 2B, and determining that FIPV is present if said amino acid at amino acid position 1049 is not methionine and/or if said amino acid at amino acid position 1051 is not serine.

A sample comprising feline enteric coronavirus, feline infectious peritonitis virus, feline coronavirus (spike) protein or feline coronavirus nucleic acid can be obtained from any feline directly or indirectly. Such a sample can for instance be obtained from any feline tissue or fluid or excretion product. Feline tissues, fluids or excretion products from which such sample is obtained include but are not limited to FIP lesions, blood, white blood cells, blood plasma, blood serum, saliva, ascites, urine, faeces, skin, muscle, lymph nodes and liver. A sample according to the invention that is obtained indirectly from a feline may comprise any material that contains feline tissue, fluid or excretion product, such as for instance soil or cat litter. In a preferred embodiment of the invention a sample is obtained from a FIP lesion, faeces, blood and/or ascites. In a more preferred embodiment a sample is obtained from white blood cells. Blood samples are relatively easy obtained from an animal, and white blood cells are easily isolated from a blood sample

subsequently. The present inventors found that in 29 out 31 white blood cell samples obtained from cats in which an alteration of the amino acid at a position corresponding to amino acid position 1049 as depicted in figure 2B was detected in a FIP lesion sample, the alteration of said amino acid was also present in the white blood cell sample. Thus, the detection of an alteration of an amino acid is accurately detected in feline white blood cell samples.

When a feline is suspected of suffering from a feline coronavirus infection, a feline coronavirus nucleic acid encoding a spike protein, whereby the nucleic acid comprises the nucleotide positions 3145, 3146 and/or 3147 and/or the nucleotide positions 3151, 3152 and/or 3153 as depicted in figure 2A can be detected in a sample from said feline. A sample from said feline may comprise feline coronavirus nucleic acid, or isolated feline coronavirus nucleic acid. Optionally a feline coronavirus nucleic acid comprising nucleotide positions 3145, 3146 and/or 3147 and/or the nucleotide positions 3151, 3152 and/or 3153 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A is amplified before detection. A sample according to the invention may further comprise feline coronavirus or feline coronavirus proteins, including but not limited to the spike protein.

According to the present invention the presence of methionine at a position corresponding to amino acid position 1049 of a feline coronavirus as depicted in figure 2B is indicative of FECV and the presence of any amino acid other than methionine at said position is indicative of FIPV. Thus the presence of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and/or valine at a position corresponding to amino acid position 1049 of a feline coronavirus as depicted in figure 2B is indicative of FIPV. In a preferred embodiment of the invention said amino acid other than methionine is leucine. The presence of any amino

acid other than serine at a position corresponding to amino acid position 1051 of a feline coronavirus as depicted in figure 2B is indicative of FIPV. Thus the presence of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine and/or valine at a position corresponding to amino acid position 1051 of a feline coronavirus as depicted in figure 2B is indicative of FIPV. In a preferred embodiment of the invention said amino acid other than serine is alanine.

The presence of the nucleobase adenine (a) at a position corresponding to nucleotide position 3145 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A, the nucleobase thymine (t) at a position corresponding to nucleotide position 3146 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A and the nucleobase guanine (g) at the position corresponding to nucleotide position 3147 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A is indicative of FECV and the presence of any nucleobases other than adenine (a) at a position corresponding to nucleotide position 3145 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A, and/or any nucleobase other than thymine (t) at a position corresponding to nucleotide position 3146 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A and/or any nucleobase other than guanine (g) at the position corresponding to nucleotide position 3147 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A is indicative of FIPV. Thus, the presence of nucleobase thymine (t), and/or cytosine (c), and/or guanine (g) at a position corresponding to nucleotide position 3145 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A, and/or nucleobases adenine (a), and/or cytosine (c), and/or guanine (g) at a position corresponding to nucleotide position 3146 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A, and/or nucleobases adenine (a), and/or

thymine (t), and/or cytosine (c) at a position corresponding to nucleotide position 3147 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A is indicative of FIPV. Feline coronavirus is an RNA virus. Therefore, when a nucleotide is identified herein as thymine, a uracil is also encompassed by said term, as is known by a skilled person.

Therefore, the invention provides a method according to the invention, wherein the identity of the amino acid at position 1049 is determined by determining a nucleic acid sequence of a feline coronavirus nucleic acid encoding a spike protein, said nucleic acid comprising a nucleotide at, or corresponding to, position 3145, 3146 and/or 3147 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A. In a preferred embodiment, a cytosine or thymine or guanine at a position corresponding to nucleotide position 3145 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A is indicative of FIPV, and an adenine at a position corresponding to nucleotide position 3145 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A is indicative of FECV. The invention also provides a method according to the invention, wherein the identity of the amino acid at position 1051 is determined by determining a nucleic acid sequence of a feline coronavirus nucleic acid encoding a spike protein, said nucleic acid comprising a nucleotide at, or corresponding to, position 3151, 3152 and/or 3153 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A.

Coronaviruses are RNA viruses. Viral RNA can be isolated and processed with methods known in the art. For example, RNA samples can be freshly prepared from cells or tissues at the moment of harvesting, or they can be prepared from samples that are stored at -70°C until processed for sample preparation. Alternatively, tissues or cell samples can be stored under conditions that preserve the quality of the RNA. Examples of these preservative conditions are fixation using e.g. formalin, RNase inhibitors such

as RNAsin (Pharmingen) or RNasecure (Ambion), aqueous solutions such as RNAlater (Assuragen), Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect (HOPE), and RCL2 (Alphelys), and non-aqueous solutions such as Universal Molecular Fixative (Sakura Finetek USA Inc.). RNA can for instance be isolated according to the method of Chomczynski and Sacchi (1987) or the method of Boom et al. (1990), or commercially available systems (such as RNeasy total RNA isolation kit from QIAGEN, Germany or High-Pure-RNA-Isolation-Kit® from Roche Diagnostics, Basel, Switzerland). Alternatively, or additionally, RNA is reverse transcribed into cDNA. Reverse transcriptase polymerase chain reaction (RT-PCR) is for instance performed using specific primers that hybridize to an RNA sequence of interest and a reverse transcriptase enzyme. Furthermore, RT-PCR can be performed with random primers, such as for instance random hexamers or decamers which hybridize randomly along the RNA, or oligo d(T) which hybridizes to the poly(A) tail of mRNA, and reverse transcriptase enzyme.

Amplification of nucleotides derived from feline coronavirus, either directly or after RT-PCR can be performed using any nucleic acid amplification method, such as the Polymerase Chain Reaction (PCR; Mullis and Faloona, 1987) or by using amplification reactions such as Ligase Chain Reaction (LCR; Barany, 1991), Self- Sustained Sequence Replication (3SR; Guatelli et al., 1990), Strand Displacement Amplification (SDA; Walker et al., 1992), Transcriptional Amplification System (TAS; Kwoh et al, 1989), Q- Beta Replicase (Lizardi et al., 1988), Rolling Circle Amplification (RCA; U.S. Pat. No. 5,871,921), Nucleic Acid Sequence Based Amplification (NASBA; Compton, 1991), Cleavase Fragment Length Polymorphism (U.S. Pat. No. 5,719,028), Isothermal and Chimeric Primer-initiated Amplification of Nucleic Acid (ICAN), Ramification-extension Amplification Method (RAM; U.S. Pat. Nos. 5,719,028 and 5,942,391) or other suitable methods for amplification of nucleic acids.

As used herein, the term “nucleic acid” or “nucleic acid molecule” comprises a chain of nucleotides, preferably DNA and/or RNA.

The term "primer" as used herein refers to an oligonucleotide which is capable of annealing to the amplification target allowing a DNA polymerase to attach, thereby serving as a point of initiation of DNA synthesis when placed under conditions in which synthesis of primer extension product is induced, i.e., in the presence of nucleotides and an agent for polymerization such as DNA polymerase and at a suitable temperature. An amplification primer is preferably single stranded for maximum efficiency in amplification. Preferably, a primer is an oligodeoxyribonucleotide. A primer must be sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact lengths of the primers will depend on many factors, including temperature and composition (A/T en G/C content) of primer. A primer pair consists of one forward and one reverse primer as commonly used in the art of DNA amplification such as in PCR amplification.

The term "probe" refers to a single-stranded oligonucleotide sequence that will recognize and form a hydrogen-bonded duplex with a complementary sequence in a target nucleic acid sequence analyte or its cDNA derivative. To facilitate the detection of binding, a specific amplicon detection probe may comprise a label moiety such as a fluorophore, a chromophore, an enzyme or a radio-label, so as to facilitate monitoring of binding of the probes to the reaction product of the amplification reaction. Such labels are well known to those skilled in the art and include, for example, fluorescein isothiocyanate (FITC), [beta]-galactosidase, horseradish peroxidase, streptavidin, biotin, digoxigenin, <35>S, <14>C, <32>P and <125>I.

A primer or probe according to the invention comprises a nucleic acid sequence, preferably DNA and/or RNA. Said nucleic acid sequence also encompasses other kinds of nucleic acid structures such as for instance a DNA/RNA helix, peptide nucleic acid (PNA), and/or locked nucleic acid (LNA). Hence, the term “nucleic acid sequence” also encompasses a chain comprising

non-natural nucleotides, modified nucleotides and/or non-nucleotide building blocks which exhibit the same function as natural nucleotides.

Hybridization is known in the art and refers to the combining of complementary, single-stranded nucleic acids, preferably under stringent conditions. The term “complementary”, or “sequence complementarity” is also known in the art and refers to two nucleic acid strands that can be non-covalently connected by base-pairing. As used herein, “complementary” or "substantially complementary" means that two nucleic acid sequences have at least about 70%, preferably about 80%, more preferably 90%, and most preferably about 95%, sequence complementarity to each other. This means that primers and probes must exhibit sufficient complementarity to their template and target nucleic acid, respectively, to hybridise under stringent conditions. Therefore, the primer and probe sequences need not reflect the exact complementary sequence of the binding region on the template and degenerate primers can be used. For example, a non-complementary nucleotide fragment may be attached to the 5 '-end of the primer, with the remainder of the primer sequence being complementary to the strand.

The term "stringent conditions" refers to hybridization conditions that affect the stability of hybrids, e.g., temperature, salt concentration, pH, formamid concentration and the like. These conditions are empirically optimised to maximize specific binding and minimize non-specific binding of primer or probe to its target nucleic acid sequence. The term as used includes reference to conditions under which a probe or primer will hybridize to its target sequence, to a detectably greater degree than to other sequences (e.g. at least 2-fold over background). Stringent conditions are sequence dependent and will be different in different circumstances.

The term “% sequence identity” is defined herein as the percentage of residues in a candidate amino acid sequence or candidate nucleic acid sequence that is identical to the residues in a reference sequence after aligning the two sequences and introducing gaps, if necessary, to achieve the maximum

percent identity. Methods and computer programs for the alignment are well known in the art. One computer program which may be used or adapted for purposes of determining whether a candidate sequence falls within this definition is "Align 2", authored by Genentech, Inc., which was filed with user documentation in the United States Copyright Office, Washington, D.C. 20559, on Dec. 10, 1991, or the UWGCG Package which provides the BESTFIT program (Devereux et al., 1984).

A feline coronavirus nucleic acid comprising a nucleotide corresponding to nucleotide position 3145, 3146 or 3147 and/or the nucleotide positions 3151, 3152 or 3153of the gene encoding a feline coronavirus spike protein as depicted in figure 2A may be amplified using primers that are capable of hybridizing to at least part of said feline coronavirus nucleic acid sequence. Said primers for instance hybridize to the feline coronavirus nucleic acid sequence encoding a spike protein between a position corresponding to nucleotide position 3055 and a position corresponding to nucleotide position 3669 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A. Said primers preferably have a length of between 9 and 50 nucleotides, for instance 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or 31 nucleotides. The nucleic acid product obtained with an amplification method using such primers preferably comprises at least 35 nucleotides, more preferably at least 40 nucleotides, even more preferably at least 50 nucleotides.

Therefore, the invention provides a method according to the invention, further comprising amplifying at least part of a feline coronavirus nucleic acid molecule comprising a region including, or corresponding to, nucleotide position 3145, 3146 and 3147 and/or the nucleotide position 3151, 3152 and 3153 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A using at least one primer which is capable of hybridizing to at least part of said nucleic acid sequence between a position corresponding to nucleotide position 3055 and a position corresponding to nucleotide position

3669 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A.

A preferred primer pair for use in a method according to the invention comprises a primer which has at least 70% sequence identity with the nucleic acid sequence 5'-CCCTCGAGTCCCGCAGAAACCATACCTA-3', preferably at least 80% sequence identity with said nucleic acid sequence, more preferably at least 90% sequence identity with said nucleic acid sequence, most preferably at least 95% sequence identity with said nucleic acid sequence, and a primer which has at least 70% sequence identity with the nucleic acid sequence 5'-CAATATTACAATGGCATAATGG-3', preferably at least 80% sequence identity with said nucleic acid sequence, more preferably at least 90% sequence identity with said nucleic acid sequence, most preferably at least 95% sequence identity with said nucleic acid sequence. Another preferred primer pair for use in a method according to the invention comprises a primer which has at least 70% sequence identity with the nucleic acid sequence 5'-GGCATAATGGTTTTACCTGGTG-3', preferably at least 80% sequence identity with said nucleic acid sequence, more preferably at least 90% sequence identity with said nucleic acid sequence, most preferably at least 95% sequence identity with said nucleic acid sequence, and a primer which has at least 70% sequence identity with the nucleic acid sequence 5'-TAATTAAGCCTCGCCTGCACTT-3', preferably at least 80% sequence identity with said nucleic acid sequence, more preferably at least 90% sequence identity with said nucleic acid sequence, most preferably at least 95% sequence identity with said nucleic acid sequence.

In one embodiment a primer pair according to the invention comprises a combination of a nucleic acid sequence 5'-CCCTCGAGTCCCGCAGAAACCATACCTA-3' and a nucleic acid sequence 5'-CAATATTACAATGGCATAATGG-3', or a combination of a nucleic acid

sequence 5'-GGCATAATGGTTTTACCTGGTG-3' and a nucleic acid sequence 5'-TAATTAAGCCTCGCCTGCACTT-3'.

In one embodiment of the invention the primer pairs indicated above are used in a nested PCR reaction. In a nested polymerase chain reaction two primer pairs are used in successive PCR reactions. The second primer pair is used to amplify a nucleic acid product or part thereof obtained in the amplification reaction using the first primer pair. Therefore, in one embodiment at least part of an amplified nucleic acid, amplified using a first primer pair, is further amplified using a second primer pair. A first primer pair according to the invention comprises, for example, a primer which has at least 70% sequence identity with the nucleic acid sequence 5'-CCCTCGAGTCCCGCAGAAACCATACCTA-3' and a primer which has at least 70% sequence identity with the nucleic acid sequence 5'-CAATATTACAATGGCATAATGG-3', and a second primer pair according to the invention comprises, for example, a primer which has at least 70% sequence identity with the nucleic acid sequence 5'-GGCATAATGGTTTTACCTGGTG3'- and a primer which has at least 70% sequence identity with the nucleic acid sequence 5'-TAATTAAGCCTCGCCTGCACTT3'-.

Also provided by the invention is a use of a primer pair according to the invention, preferably for identifying feline enteric coronavirus (FECV) or feline infectious peritonitis virus (FIPV), and/or for determining the presence of FIPV or feline infectious peritonitis (FIP) in an animal suspected of suffering from a feline coronavirus infection.

The identity of a nucleotide at position 3145, 3146 and/or 3147 and/or the nucleotide at position 3151, 3152 and/or 3153 of the gene encoding a feline coronavirus spike protein can be determined by any method known in the art. These methods include, but are not limited to allele specific

oligonucleotides (ASO), sequencing of a nucleic acid sequence (for example tag-array minisequencing [Fan et al., 2000] or pyrosequencing [Fakhrai-Rad et al., 2002]), allele-specific PCR with a blocking reagent (ASB-PCR, Morlan et al., 2009), oligonucleotide ligation assay (OLA, Baron et al., 1996), mass spectrometry (MS, for instance matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS, Crain and McCloskey 1998), quantitative polymerase chain reaction (qPCR), electronic hybridization, fluorescent single-stranded conformation polymorphism (F-SSCP) analysis (Makino et al., 1992), denaturing high-performance liquid chromatography (DHPLC), gel electrophoresis (such as microplate array diagonal gel electrophoresis [MADGE, Day et al., 1998] and denaturing gradient gel electrophoresis [DGGE, Fischer and Lerman 1980]), and microarray analysis.

Allele Specific Oligonucleotides (ASO) are fluorophore-, chromophore-, enzyme- or radio-labelled nucleotide probes which are short and specific for particular RNA or DNA sequences. ASO for instance comprise a nucleotide mutation and only hybridize with nucleic acid sequences comprising this mutation. The nucleic acid sequence of a feline coronavirus nucleic acid sequence encoding a spike protein comprising a nucleotide at, or corresponding to, position 3145, 3146 and/or 3147 and/or nucleotide position 3151, 3152 and/or 3153 as depicted in figure 2A is for instance detected using a probe that is capable of specifically hybridizing to at least part of said feline coronavirus nucleic acid sequence comprising a nucleotide corresponding to nucleotide position 3145, 3146, and/or 3147 and/or nucleotide position 3151, 3152 and/or 3153 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A. Said probe preferably has a length of between 14 and 100 nucleotides, preferably 14, 15, 16, 17, 18, 19, 20, 21, 22, or more nucleotides. Therefore, in one embodiment a feline coronavirus nucleic acid sequence comprising a nucleotide at, or corresponding to, position 3145, 3146 and 3147 and/or nucleotide position 3151, 3152 and 3153 of the gene encoding a feline

coronavirus spike protein as depicted in figure 2A is detected using a probe with a length of at least 14 nucleotides that is capable of specifically hybridizing to at least part of said nucleic acid. In a preferred embodiment a probe is capable of specifically hybridizing to a feline coronavirus nucleic acid comprising cytosine or thymine at a position corresponding to nucleotide position 3145 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A.

A probe used in a method according to the invention is complementary to a feline coronavirus nucleic acid sequence encoding a spike protein comprising a nucleotide corresponding to nucleotide position 3145, 3146, and 3147 and/or nucleotide position 3151, 3152 and 3153 as depicted in figure 2A. Because coronaviruses are RNA viruses they have relatively high rates of mutation as a skilled person will know. Therefore, the sequence of feline coronaviruses may differ in some nucleotides surrounding nucleotide position 3145, 3146, and 3147 and/or nucleotide position 3151, 3152 and 3153 of the gene encoding a feline coronavirus spike protein. A person skilled in the art knows how a probe according to the invention is modified, for instance by nucleic acid substitution, to enable said probe to hybridize to the nucleic acid sequence of a specific feline coronavirus and detect a nucleotide at, or corresponding to, position 3145, 3146, or 3147 and/or position 3151, 3152 or 3153 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A.

A preferred probe comprising a nucleotide corresponding to nucleotide positions 3145, 3146, and 3147 as depicted in figure 2A for use in a method according to the invention comprises a sequence which has at least 70% sequence identity with the nucleic acid sequence 5'-CCCARRGCCATAGG-3', wherein R is A or G, preferably at least 80% sequence identity with said nucleic acid sequence, more preferably at least 90% sequence identity with said nucleic acid sequence, most preferably at least 95% sequence identity with said nucleic acid sequence. In one embodiment the invention provides a

method according to the invention, wherein said probe comprises the sequence CCCARRGCCATAGG. Also provided by the invention is a use of a probe according to the invention, preferably for identifying feline enteric coronavirus (FECV) and/or feline infectious peritonitis virus (FIPV), and/or for determining the presence of feline infectious peritonitis (FIP) in an animal suspected of suffering from a feline coronavirus infection.

Feline coronavirus nucleic sequences may be determined by sequencing methods known to the skilled person, preferably directly after amplification of relevant nucleic acid. These methods comprise for instance direct double-stranded nucleotide sequencing using fluorescently labeled dideoxynucleotide terminators (Smith et al., 1986), tag-array minisequencing or pyrosequencing. In general such sequencing methods include the isolation of the viral genome nucleic acids by nucleic acid isolation procedures, and the determination of the nucleotide sequence of the isolated nucleic acid, for instance by dideoxy chain termination methods (Sanger et al., 1977) optionally preceded by reverse transcription of RNA into DNA, and/or amplification of the target nucleic acid.

In one embodiment at least part of a feline coronavirus nucleic acid sequence comprising a nucleotide corresponding to nucleotide position 3145, 3146 and/or 3147 and/or nucleotide position 3151, 3152 and 3153 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A is sequenced.

Oligonucleotide ligation assay (OLA) is a method for the detection of known single nucleotide polymorphisms. The method is based on the ligation of two adjacent oligonucleotide probes using a DNA ligase while they are annealed to a complementary DNA target. One of the probes is for instance fluorescently labeled and allele-specific. Typically, there are two differently labeled probes, one for each allele. These two probes differ only in sequence at

the last base at the 3' end, thus at the site of the polymorphism. The second probe is a common probe that is complementary to the target DNA sequence immediately downstream (3') of the site containing the polymorphism, and thus complementary to both alleles. This probe does not need to be fluorescently labeled. Allele discrimination occurs by the ability of DNA ligase to join perfectly matched probes; a 3' mismatch in the capture probe will prevent ligation. In a method of the invention, for instance an oligonucleotide ligation assay is used wherein one of the probes is specifically able to hybridize to a feline coronavirus nucleic acid sequence encoding a spike protein comprising an adenine at nucleotide position 3145 as depicted in figure 2A which is indicative of FECV. Thus the first nucleotide of a right probe or the last nucleotide of a left probe is a thymine. The second probe is a common probe, which is able to hybridize to both FECV and FIPV nucleic acid sequence starting next to position 3145 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A, for example starting at position 3146 if said second probe is a right probe. In the presence of FECV, ligation of said two probes is possible, whereas in the presence of FIPV, ligation of said two probes is not possible. In one embodiment of the invention a feline coronavirus nucleic acid sequence encoding a spike protein, comprising nucleotide position 3145, 3146 and/or 3147 is determined using an oligonucleotide ligation assay (OLA).

Real time PCR technology can be used to detect one specific allele of a gene when a blocking reagent is used. This technology is called allele specific PCR with a blocking reagent (ASB-PCR, Morlan et al., 2009). During the PCR reaction a blocking agent is added to the reaction mixture to prevent amplification of one allele. One of the primers, for example the forward primer, is designed as mutant allele specific primer. The other primer is a common primer, which is able to hybridize to both alleles. A blocking agent, which is phosphorylated at the 3‘ end to prevent amplification, is then designed to bind specifically to the wildtype allele. During the PCR reaction the blocking agent

prevents hybridization of the mutant specific primer to the wildtype allele. In the presence of only the wildtype allele no amplification product is obtained, whereas in the presence of only the mutant allele an amplification product is obtained. With ASB-PCR it is for instance possible to discriminate between a feline coronavirus nucleic acid sequence comprising an adenine at nucleotide position 3145 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A and which is indicative of FECV, and a feline coronavirus nucleic acid sequence comprising a cytosine or thymine at said position, which is indicative of FIPV. For example, a primer set is used consisting of a common reverse primer and two FIPV nucleic acid specific primers from which the 3' end nucleotide is complementary to nucleotide position 3145 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A. One of these FIPV specific primers has an adenine at its 3' end and the other primer has a guanine at its 3' end which enables said primers to hybridize to a feline coronavirus nucleic acid sequence encoding a spike protein containing a thymine or a cytosine at nucleotide position 3145 respectively. A blocking agent comprising a thymine at the 3' end can be used, which is able to hybridize to an adenine at nucleotide position 3145. Using said primer set, amplification will occur when FIPV nucleic acid is present whereas amplification will not occur when only FECV nucleic acid is present. In a preferred embodiment of the invention a feline coronavirus nucleic acid sequence encoding a spike protein, comprising nucleotide position 3145, 3146 and/or 3147 and/or nucleotide position 3151, 3152 and/or 3153 is determined using allele-specific PCR with a blocking reagent (ASB-PCR).

Using MALDI-TOF MS the detection of low (femtomole) quantities of DNA can be achieved. Nucleic acids ranging from 2 to 2000 nucleotides can be detected by using MALDI-TOF MS. MS can be used to analyze mixtures of different nucleic acid fragments without the use of any label because of the mass differences of the nucleobases. Thus, in most cases, separation of nucleic

acid fragments is not necessary before MS measurements. Using MALDI-TOF MS it is for instance possible to determine whether a nucleotide of a feline coronavirus nucleic acid sequence encoding a spike protein at nucleotide position 3145 is an adenine, which is indicative of FECV, or a cytosine or thymine, which is indicative of FIPV. In one embodiment of the invention the mass of at least part of a feline coronavirus nucleic acid sequence, said part comprising a nucleotide corresponding to nucleotide position 3145, 3146, and/or 3147 and/or nucleotide position 3151, 3152 and/or 3153 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A is determined. In a preferred embodiment the mass of said nucleic acid sequence is determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

The identity of an amino acid in a feline coronavirus amino acid sequence encoding a spike protein at amino acid position 1049 and/or amino acid position 1051 as depicted in figure 2B can be detected using any method known in the art. Such amino acid is for instance detected using antibodies or functional equivalents thereof, mass spectrometry or Edman degradation reactions. Optionally, a coronaviral protein can be purified with methods known in the art. For instance, coronaviral protein can be purified using gel electrophoresis or chromatography methods, such as affinity chromatography.

A functional equivalent of an antibody is defined as a compound which has at least one same property as said antibody in kind, not necessarily in amount. Said functional equivalent is capable of binding the same antigen as said antibody, albeit not necessarily to the same extent. A functional equivalent of an antibody preferably comprises a single domain antibody, a single chain antibody, a nanobody, a unibody or a single chain variable fragment (scFv). A functional equivalent of an antibody is for instance produced by altering an antibody such that at least one property - preferably

an antigen-binding property - of the resulting compound is essentially the same in kind, not necessarily in amount. This is done in many ways, for instance through conservative amino acid substitution, whereby an amino acid residue is substituted by another residue with generally similar properties (size, hydrophobicity, etc), such that the overall functioning is likely not to be seriously affected.

An immunogenic part of a feline coronavirus comprising a feline coronavirus spike protein is defined as a part which has at least one property in common with a feline coronavirus comprising a feline coronavirus spike protein in kind, though not necessarily in amount. An immunogenic part of a feline coronavirus spike protein is defined as a part which has at least one same property as a feline coronavirus spike protein in kind, not necessarily in amount. Said immunogenic part, is preferably capable of eliciting an immune response against a feline coronavirus, preferably a feline infectious peritonitis virus (FIPV), in an animal.

An amino acid of a feline coronavirus spike protein amino acid sequence, corresponding to amino acid position 1049 and/or amino acid position 1051 as depicted in figure 2B is for instance detected using an antibody or functional equivalent that is specifically directed against an epitope of a feline coronavirus spike protein that comprises amino acid position 1049 and/or amino acid position 1051 as depicted in figure 2B. Said amino acid position 1049 and/or amino acid position 1051 as depicted in figure 2B enables discrimination between FECV and FIPV. A methionine at amino acid position 1049 is indicative of FECV, whereas any amino acid other than methionine at this position, preferably leucine, is indicative of FIPV and any amino acid other than serine at amino acid position 1051, preferably alanine, in indicative of FIPV. Therefore, the invention provides a method according to the invention, wherein an amino acid of a feline coronavirus spike protein at a position corresponding to amino acid position 1049 and/or amino acid position

1051 as depicted in figure 2B is detected by using an antibody or functional equivalent thereof specifically directed against an epitope of a FIPV spike protein encompassing amino acid 1049 and/or amino acid 1051. In one embodiment said epitope comprises an amino acid other than methionine at a position corresponding to amino acid position 1049 and/or said epitope comprises an amino acid other than serine at a position corresponding to amino acid position 1051 as depicted in figure 2B.

An antibody or functional equivalent thereof specifically directed against an epitope of a FIPV spike protein, which epitope comprises an amino acid corresponding to amino acid position 1049 and/or amino acid position 1051 as depicted in figure 2B can be detected with any method known in the art. For instance, said antibody or functional equivalent thereof is fluorophore-, chromophore- or enzyme-labelled, and can thus be detected with for instance fluorescence microscopy or spectrophotometry. An antibody or functional equivalent can also be detected using a second antibody which is for instance fluorophore-, chromophore- or enzyme-labelled. Such labels are well known to those skilled in the art and include, for example, fluorescein isothiocyanate (FITC), [beta]-galactosidase, horseradish peroxidase, streptavidin, biotin or digoxigenin.

Also provided by the invention is an antibody or functional equivalent specifically directed against an epitope of a FIPV spike protein, which epitope comprises an amino acid other than methionine at a position corresponding to amino acid position 1049 as depicted in figure 2B and a kit of parts comprising an antibody according to the invention and means for detecting said antibody.

Using MALDI-TOF MS the detection of low quantities of amino acid sequences can be achieved. MS can be used to analyze mixtures of different amino acid sequences without the use of any label because of the mass differences of the amino acid sequences. Thus, in most cases, separation of

amino acid sequences is not necessary before MS measurements. Using MALDI-TOF MS it is for instance possible to discriminate between a feline coronavirus amino acid sequence comprising a methionine at amino acid position 1049 as depicted in figure 2B, and a feline coronavirus amino acid sequence comprising an amino acid other than methionine at amino acid position 1049 as depicted in figure 2B. In one embodiment of the invention the mass of at least part of a feline coronavirus amino acid sequence, said part comprising an amino acid corresponding to amino acid position 1049 as depicted in figure 2B is determined. In a preferred embodiment the mass of said amino acid sequence is determined using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

The development of vaccines against FIPV has been unsuccessful to date. Various approaches have failed to provide a vaccine that induces protection against FIPV. These approaches include vaccination with closely related heterologous live coronaviruses, sublethal amounts of virulent FIPV, low-virulence FIPV, and (recombinant) feline coronavirus subunits or proteins. Some of these approaches provided some protection but the results were inconsistent. Occasionally, vaccination even resulted in enhanced disease progression and death. The only currently available vaccine is based on a temperature-sensitive strain of FIPV of which the efficacy is questionable (McArdle et al., 1995; Fehr et al., 1997).

Now that a polymorphism in the spike protein of feline coronavirus has been identified that allows discrimination between FECV and FIPV it is possible to develop immunogenic compositions comprising feline coronaviruses comprising the identified nucleic acid or amino acid indicative for FECV. Using an immunogenic composition comprising a feline coronavirus with a nucleic acid or amino acid representative of a FECV there is no risk of disease and/or death because said immunogenic composition does not comprise the virulent FIPV or part thereof. It is now also possible to develop an immunogenic

composition comprising feline coronavirus spike protein comprising the identified amino acid indicative for FIPV. Using an immunogenic composition comprising FIPV spike protein or immunogenic part thereof a better immune response against FIPV can be elicited, without the risk of enhanced disease progression and/or death because only isolated viral proteins are used.

Therefore in one embodiment the invention provides an immunogenic composition comprising a feline coronavirus spike protein or immunogenic part thereof comprising an amino acid other than methionine at a position corresponding to amino acid position 1049, and/or an amino acid other than serine at a position corresponding to amino acid position 1051 as depicted in figure 2B, or a spike protein encoding feline coronavirus nucleic acid, comprising a cytosine or thymine at a position corresponding to nucleotide position 3145, and/or a guanine at a position corresponding to nucleotide position 3151 as depicted in figure 2A, or a feline coronavirus comprising a nucleic acid comprising an adenine at a position corresponding to nucleotide position 3145, and/or a thymine at a position corresponding to position 3151 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A, or a feline coronavirus comprising a feline coronavirus spike protein or immunogenic part thereof comprising a methionine at a position corresponding to amino acid position 1049, and/or a serine at a position corresponding to amino acid position 1051 as depicted in figure 2B, or any combination thereof. In a preferred embodiment an immunogenic composition according to the invention is used as a vaccine.

Further provided is a feline coronavirus spike protein or immunogenic part thereof comprising an amino acid other than methionine at a position corresponding to amino acid position 1049, and/or an amino acid other than serine at a position corresponding to amino acid position 1051 as depicted in figure 2B, or a spike protein encoding feline coronavirus nucleic acid comprising a cytosine or thymine at a position corresponding to nucleotide position 3145, and/or a guanine at a position corresponding to nucleotide

position 3151 as depicted in figure 2A, or a feline coronavirus comprising a nucleic acid comprising an adenine at a position corresponding to nucleotide position 3145, and/or a thymine at a position corresponding to position 3151 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A, or a feline coronavirus comprising a feline coronavirus spike protein or immunogenic part thereof comprising a methionine at a position corresponding to amino acid position 1049, and/or a serine at a position corresponding to amino acid position 1051 as depicted in figure 2B, or any combination thereof, for eliciting an immune response against a feline coronavirus, preferably a feline infectious peritonitis virus (FIPV), in a feline.

One embodiment provides a use of a feline coronavirus spike protein or immunogenic part thereof comprising an amino acid other than methionine at a position corresponding to amino acid position 1049, and/or an amino acid other than serine at a position corresponding to amino acid position 1051 as depicted in figure 2B, or a spike protein encoding feline coronavirus nucleic acid comprising a cytosine or thymine at a position corresponding to nucleotide position 3145, and/or a guanine at a position corresponding to nucleotide position 3151 as depicted in figure 2A, or a feline coronavirus comprising a nucleic acid comprising an adenine at a position corresponding to nucleotide position 3145, and/or a thymine at a position corresponding to position 3151 of the gene encoding a feline coronavirus spike protein as depicted in figure 2A, or a feline coronavirus comprising a feline coronavirus spike protein or immunogenic part thereof comprising a methionine at a position corresponding to amino acid position 1049, and/or a serine at a position corresponding to amino acid position 1051 as depicted in figure 2B, or any combination thereof, for the preparation of an immunogenic composition or prophylactic agent for eliciting an immune response against a feline coronavirus, preferably a feline infectious peritonitis virus (FIPV), in a feline.

The invention is further explained in the following examples. These examples do not limit the scope of the invention, but merely serve to clarify the invention.

Figure legends

Figure 1. Schematic representation of the feline coronavirus RNA genome. The 5' part (left) specifies the precursors encoding the replication and transcription functions derived from open reading frames (ORFs) 1a and 1b. Downstream thereof, towards the 3' end, the genes for the structural proteins S (spike protein), E (envelope protein), M (membrane protein) and N (nucleocapsid protein), and for the accessory proteins 3a, 3b, 3c, 7a and 7b are located.

Figure 2. A) Nucleotide sequences of the feline coronavirus spike gene (nucleotides 1-4407), corresponding to nucleotides 20395-24801 of a feline coronavirus as defined in the nucleotide sequence of NC_012955 (Feline coronavirus UU10, complete genome) and nucleotides 20382-24788 of a feline coronavirus as defined in the nucleotide sequence of NC_012952 (Feline coronavirus UU8, complete genome). B) Amino acid sequences of feline coronavirus spike protein, as defined in the amino acid sequence of YP_003038574 and YP_003038543.

Figure 3. Agarose gel electrophoresis of amplified RNA from 6 clinical samples obtained from faeces of infected cats. Lane M is a molecular size standard, lanes 1-6 are the clinical samples and lane 7 is a negative control.

Figure 4. A) Alignment of partial sequences of faeces or plasma derived FCoV RNA isolated from 42 healthy cats and five partial sequences derived from samples of FIP-confirmed cats, i.e. Q093501030_326B_4546.scf (white blood cell derived), Q093501032_327B_4546.scf (white blood cell derived), Q093501036_321S_4546.scf (serum derived), Q093501038_321A_4546.scf (ascites derived) and Q093501046_K11_019.ab1

(white blood cell derived); B) Alignment of partial sequences of lesion-derived FCoV RNA isolated from 54 FIP-confirmed cats; C) Alignment of partial sequences of faeces-derived FCoV RNA isolated from FIP-confirmed cats. On the right of figures 4A, B and C the identity code of the analysed feline coronavirus is indicated. The targeted nucleotides and predicted amino acid is indicated by an arrow.

Examples

Example 1

In this example 6 clinical samples (faeces) were analyzed. RNA was extracted from the clinical samples, RT-PCR was applied to the extracted RNAs and the products were analyzed by agarose gel electrophoresis (see figure 3) after the first PCR (1st run) and after the nested PCR (2nd run).

Materials and Methods

A nested RT-PCR was used to amplify the FCoV spike gene region containing the target point mutation. Genomic RNA was extracted from faeces of 6 healthy cats using the QIAamp Viral R*NA* Mini Kit and Qiagen RNeasy Mini Kit (Qiagen, Inc.) according to the manufacturer's instructions. cDNA synthesis was performed with M-MLV reverse transcriptase (RT) and followed by polymerase chain reaction (PCR) amplification with Taq DNA polymerase. All enzymes were used according to the manufacturer's instructions (Promega Corp., Madison, WI). Both reactions were primed with specific primers (see primers table 1). Primers were designed using the FCoV genome sequences with accession numbers of NC_012955 and NC_012952. Amplifications was performed using 30 cycles of 94°C for 60 s, 50°C for 30 s, and 72°C for 1 min and additional extension at 72°C for 7 min at the end of amplification. The PCR fragments were isolated and purified from agarose gel after electrophoresis using the Qiagen gel Extraction kit (Qiagen Benelux B.V., Venlo, The Netherlands). Sequencing was performed by BaseClear Holding B.V. (Leiden, The Netherlands).

Results

After the first PCR, a 601-bp fragment was obtained only in one clinical sample, as is seen in lane 2 of figure 3. After the second round of PCR, a 139-bp fragment was amplified when the nested primers were applied on the products of the 1st run RT-PCR. Now a product was seen not only in lane 2, but also with the amplified RNA's shown in lanes 3, 5 and 6.

Example 2

In this example faecal or plasma samples of 47 healthy cats, clinical samples of 54 FIP-confirmed cats and faecal samples of 14 FIP-confirmed cats were analyzed.

Material and methods

Genomic RNA extraction, cDNA synthesis, amplification and sequencing were performed according to the materials and methods of example 1.

Results

The nucleic acid sequence encoding a methionine at amino acid position 1049 was detected in all (47/47) faeces or plasma derived FCoVs from healthy cats (figure 4A). It was later found that figure 4A contains five sequences derived from samples of cats with confirmed FIP (Q093501030_326B_4546.scf, Q093501032_327B_4546.scf, Q093501036_321S_4546.scf, Q093501038_321A_4546.scf and Q093501046_K11_019.ab1), meaning that in 42/42 faeces or plasma derived FCoVs from healthy cats a methionine was present at amino acid position 1049. This sequence was also observed in 2/54 lesion-derived (figure 4B)and 12/14 faeces-derived (figure 4C) RNAs amplified from FIP-confirmed cats. Importantly, 52/54 (96%) lesion-derived RNAs from FIP-confirmed cats had an alteration of A to C or T at position 3145, leading to

an amino acid alteration at position 1049 that changes a methionine into a leucine (figure 4B).

Example 3

We continued collecting samples and cats through veterinarians and owners in the Netherlands. In this example the following samples were analyzed:
- faecal samples of 352 healthy cats,
- white blood cell samples of 89 healthy or non-FIP suspected cats,
- plasma samples of 89 healthy or non-FIP suspected cats,
- serum samples of 56 healthy or non-FIP suspected cats,
- FIP lesion samples (mesenteric lymph node (LN) and/or kidney and/or spleen and/or omentum and/or lung and/or LN and/or liver and/or ascites) of 97 FIP-confirmed cats,
- white blood cell samples of 34 FIP-confirmed cats,
- plasma samples of 34 FIP-confirmed cats, and
- serum samples of 15 FIP-confirmed cats.

Material and methods

Genomic RNA extraction, cDNA synthesis, amplification and sequencing were performed according to the materials and methods of example 1.

Results

Samples from healthy cats

137/352 (39%) of faeces samples were FCoV positive. A nucleic acid sequence encoding a methionine at amino acid position 1049 and a serine at amino acid position 1051 was detected in all (137) faeces-derived FCoVs from healthy cats.

Samples from healthy or non-FIP suspected cats

EDTA-blood samples from 89 healthy or non-FIP suspected cats were obtained and separated into white blood cells (WBC) and plasma. Serum samples from 56 healthy or non-FIP suspected cats were obtained.

20/89 white blood cells samples, 4/89 plasma samples and 8/56 serum samples were FCoV positive. All 4 plasma-positive samples were also positive in the WBC fraction and in each animal the sequence in plasma was 100% identical to that in WBC. A nucleic acid sequence encoding a methionine at amino acid position 1049 and a serine at amino acid position 1051 was detected in all samples tested positive for FCoV.

Samples from FIP-confirmed cats

A total of 97 FIP-confirmed cats were studied. 97/97 organs with typical FIP lesions (including mesenteric LN and/or kidney and/or spleen and/or omentum and/or lung and/or LN and/or liver and/or ascites) tested positive for FCoV. 87/97 (90%) of lesion-derived RNAs from FIP-confirmed cats had an amino acid alteration at position 1049 that changes a methionine into a leucine. 5/97 (5%) of lesion-derived RNAs from FIP-confirmed cats had an amino acid alteration at position 1051 that changes a serine into an alanine. In all 5 samples in which an alanine was present at position 1051, a methionine was present at position 1049. Thus, 92 out of 97 (95%) lesion-derived RNA's from FIP confirmed cats had an amino acid alteration indicative for FIP, whereas 5 out of 97 (5%) did not have an amino acid alteration indicative for FIP.

From 34 of the 97 FIP-confirmed cats blood was obtained before euthanizing the animal. Blood samples were separated into white blood cells (buffy coat) and plasma. Serum samples from 15 FIP-confirmed cats were obtained of which EDTA-blood had also been obtained.

WBC:

34/34 (100%) of WBC samples were FCoV positive. In 29/34 (85%) of WBC-derived RNAs from FIP-confirmed cats a leucine was present at position 1049 and a serine was present at position 1051; for all 29 a leucine was present also at position 1049 in the organ samples. Of the 5 cats with a methionine at position 1049 in WBC samples, 2 had a leucine at this position in organ(s) containing FIP-lesions, the other 3 had none of the amino acid alterations indicative for FIPV. Thus, from the 31/34 (90%) FIP cats in which a leucine was detected at position 1049 in organ material, leucine was also detected at this position in WBC in 29/31 (94%) cases.

Plasma:

14/34 (41%) of plasma samples were FCoV positive. In 11/34 (32%) plasma-derived RNA from FIP-confirmed cats a leucine was present at position 1049 and a serine was present at position 1051. Of the 3 FCoV positive cats with a methionine at position 1049 in plasma, 1 had a leucine at this position in FCoV RNA of organ(s) containing FIP-lesions, the other 2 had none of the amino acid alterations indicative for FIPV. Thus, from the 31/34 (90%) FIP cats in which a leucine was detected at position 1049 in organ material, leucine was also detected in plasma in 11/31 (35%) cases.

Serum:

4/15 (27%) serum samples were FCoV positive. In 2/15 (13%) serum-derived RNA from FIP-confirmed cats a leucine was present at position 1049 and a serine was present at position 1051. 15/15 (100%) of these cats had a leucine at position 1049 in organ derived FCoV RNA.

Table 1. Primers used for amplification of the FCoV spike gene target region.

Primers 5'-3'	Position in spike gene	
CCCTCGAGTCCCGCAGAAACCATACCTA	3642-3656	Reverse primer for 1st run RT-PCR
CAATATTACAATGGCATAATGG	3055-3076	Forward primer for 1st run RT-PCR
GGCATAATGGTTTTACCTGGTG	3067-3088	Forward primer for 2nd run RT-PCR
TAATTAAGCCTCGCCTGCACTT	3188-3206	Reverse primer for 2nd run RT-PCR

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Claims

1. A method for determining whether feline infectious peritonitis virus (FIPV) is present in a sample, comprising determining whether said sample comprises a feline coronavirus, and if a feline coronavirus is present determining the identity of an amino acid in a spike protein of said feline coronavirus at a position corresponding to amino acid position 1049 as depicted in figure 2B, and determining that FIPV is present if said amino acid is not methionine.

2. A method according to claim 1, wherein the identity of the amino acid at position 1049 is determined by determining a nucleic acid sequence of a feline coronavirus nucleic acid encoding a spike protein, said nucleic acid comprising a nucleotide at, or corresponding to, position 3145, 3146 and/or 3147 as depicted in figure 2A.

3. A method according to claim 1 or 2 wherein said amino acid other than methionine is leucine.

4. A method according to claim 2, further comprising amplifying at least part of a feline coronavirus nucleic acid molecule comprising a region including, or corresponding to, nucleotide position 3145, 3146 and 3147 as depicted in figure 2A using at least one primer which is capable of hybridizing to at least part of said nucleic acid sequence between a position corresponding to nucleotide position 3055 and a position corresponding to nucleotide position 3669 as depicted in figure 2A.

5. A method for determining whether feline infectious peritonitis virus (FIPV) is present in a sample, comprising determining whether said sample comprises a feline coronavirus, and if a feline coronavirus is present

determining the identity of an amino acid in a spike protein of said feline coronavirus at a position corresponding to amino acid position 1051 as depicted in figure 2B, and determining that FIPV is present if said amino acid is not serine.

6. A method according to any one of claims 1-4, further comprising determining the identity of an amino acid in a spike protein of said feline coronavirus at a position corresponding to amino acid position 1051 as depicted in figure 2B, and determining that FIPV is present if said amino acid is not serine.

7. A method according to claim 5 or 6, wherein the identity of the amino acid at position 1051 is determined by determining a nucleic acid sequence of a feline coronavirus nucleic acid encoding a spike protein, said nucleic acid comprising a nucleotide at, or corresponding to, position 3151, 3152 and/or 3153 as depicted in figure 2A.

8. A method according to any one of claims 5-7 wherein said amino acid other than serine is alanine.

9. A method according to claim 7, further comprising amplifying at least part of a feline coronavirus nucleic acid molecule comprising a region including, or corresponding to, nucleotide position 3151, 3152 and 3153 as depicted in figure 2A using at least one primer which is capable of hybridizing to at least part of said nucleic acid sequence between a position corresponding to nucleotide position 3055 and a position corresponding to nucleotide position 3669 as depicted in figure 2A.

10. A method according to claim 4 or 9, wherein said at least one primer is selected from the primers listed in table 1.

11. A primer pair comprising an isolated or recombinant nucleic acid sequence comprising a sequence which has at least 70% sequence identity with the sequence 5'-CCCTCGAGTCCCGCAGAAACCATACCTA-3' and an isolated or recombinant nucleic acid sequence comprising a sequence which has at least 70% sequence identity with the sequence 5'-CAATATTACAATGGCATAATGG-3'.

12. A primer pair comprising an isolated or recombinant nucleic acid sequence comprising a sequence which has at least 70% sequence identity with the sequence 5'-GGCATAATGGTTTTACCTGGTG-3' and an isolated or recombinant nucleic acid sequence comprising a sequence which has at least 70% sequence identity with the sequence 5'-TAATTAAGCCTCGCCTGCACTT-3'.

13. A method according to any one of claims 2-10, wherein said nucleic acid sequence is detected using a probe with a length of at least 14 nucleotides that is capable of specifically hybridizing to at least part of a feline coronavirus nucleic acid comprising a nucleotide at, or corresponding to, position 3145, 3146 and 3147 as depicted in figure 2A, or that is capable of specifically hybridizing to at least part of a feline coronavirus nucleic acid comprising a nucleotide at, or corresponding to, position 3151, 3152 and 3153 as depicted in figure 2A, said part having a length of at least 14 nucleotides.

14. A probe with a length of between 14 and 100 nucleotides, comprising a nucleic acid sequence which has at least 70% sequence identity with the sequence 5'-CCCARRGCCATAGG-3'.

15. A method according to any one of claims 2-10 or 13, further comprising sequencing at least part of a feline coronavirus nucleic acid sequence, said part

comprising a nucleotide corresponding to nucleotide position 3145, 3146 and/or 3147, or a nucleotide corresponding to nucleotide position 3151, 3152 and/or 3153 as depicted in figure 2A.

16. A method according to claim 1, 3, 5, 6 or 8, wherein an amino acid of a feline coronavirus spike protein at a position corresponding to amino acid position 1049 or 1051 as depicted in figure 2B is detected by using an antibody or functional equivalent thereof specifically directed against an epitope of a FIPV spike protein, which epitope comprises an amino acid other than methionine at a position corresponding to amino acid position 1049 or an amino acid other than serine at a position corresponding to amino acid position 1051 as depicted in figure 2B.

17. An antibody or functional equivalent specifically directed against an epitope of a FIPV spike protein, which epitope comprises an amino acid other than methionine at a position corresponding to amino acid position 1049 as depicted in figure 2B or which epitope comprises an amino acid other than serine at a position corresponding to amino acid position 1051 as depicted in figure 2B.

18. An immunogenic composition comprising:
- a feline coronavirus spike protein or immunogenic part thereof comprising an amino acid other than methionine at a position corresponding to amino acid position 1049, and/or an amino acid other than serine at a position corresponding to amino acid position 1051 as depicted in figure 2B, or
- a spike protein encoding feline coronavirus nucleic acid, comprising a cytosine or thymine at a position corresponding to nucleotide position 3145, and/or a guanine at a position corresponding to nucleotide position 3151 as depicted in figure 2A, or
- a feline coronavirus comprising a nucleic acid comprising an adenine at a

position corresponding to nucleotide position 3145, and/or a thymine at a position corresponding to nucleotide position 3151 as depicted in figure 2A, or
- a feline coronavirus comprising a feline coronavirus spike protein or immunogenic part thereof comprising a methionine at a position corresponding to amino acid position 1049, and/or a serine at a position corresponding to amino acid position 1051 as depicted in figure 2B,
or any combination thereof.

19. Use of:
- a feline coronavirus spike protein or immunogenic part thereof comprising an amino acid other than methionine at a position corresponding to amino acid position 1049, and/or an amino acid other than serine at a position corresponding to amino acid position 1051 as depicted in figure 2B, or
- a spike protein encoding feline coronavirus nucleic acid, comprising a cytosine or thymine at a position corresponding to nucleotide position 3145, and/or a guanine at a position corresponding to nucleotide position 3151 as depicted in figure 2A, or
- a feline coronavirus comprising a nucleic acid comprising an adenine at a position corresponding to nucleotide position 3145, and/or a thymine at a position corresponding to nucleotide position 3151 as depicted in figure 2A, or
- a feline coronavirus comprising a feline coronavirus spike protein or immunogenic part thereof comprising a methionine at a position corresponding to amino acid position 1049, and/or a serine at a position corresponding to amino acid position 1051 as depicted in figure 2B,
or any combination thereof, for the preparation of an immunogenic composition or prophylactic agent for eliciting an immune response against a feline coronavirus, preferably a feline infectious peritonitis virus (FIPV), in a feline.

Figure 1

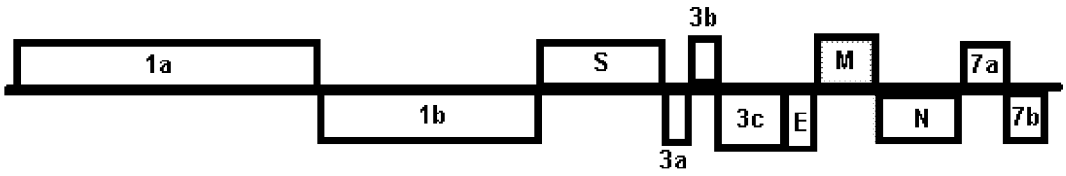

Figure 2A

Nucleotide sequence of feline coronavirus spike gene derived from the sequence of NC_012955

```
ATGATGTTGT TAATACTTGC GCTTCTTAGT ACCGCTCACT CTGAAGATGC GCCTCATGGT GTTACATTAC CACAATTTAA CACTTCCCAT GACAATGCCA   100
AGTTTGAACT TAATTTTTAC AATTTCTTAC AAACTTGGGA TATACCACCA AACACTGAAA CTATTCTTGG TGGCTATCTA CCATATTGTG CTGAAGGTGT   200
CAATTGTGGG TGGCATAATT TTGCTTTCCA GCAACATGAT GCCCTAAATG GTAAGTATGC CTACATAAAT TCGCAAAACT TGGGTATACC GAATGTCCAC   300
GGCGTCTACT TTGACGTACG AGAACGCTAT TATGATGACG GCGTATGGGA GGCAGTCGAT AGAGTTGGCC TATTGATAKC TATACATGGC AAATCTCACT   400
ACAGTTTACT AATGGTTTTA CAAGACAACK TTGAGGAGAA TCAGCCTCAT GTTGCCGTTA AAATCTGCCA TTGGAAACCA GGTAACATAA GTTCTTACCA   500
TCAATTTAGT GTTAATCTAG GAGATAGTGG TCAGTGCGTG TTTAACCGGA GGTTCTCATT GGACACCAAG TTGACAGCTG ATGATTTCTA TGGCTTCCAG   600
TGGACTGATA CATCTGTAGA CATATATTTA GGTGGCACTA TTACTAAAGT GTGGGTAGAC AACGATTGGA GTGTTGTTGA AGCTAGTATT TCCCATTATT   700
GGAGTGGTGC TAGCTATGGC TATTACATGC AATTTGTCAA CCGCACCACT TATTATGCCT ACAATAATAC TGGTGGTTCA AATTATACTC ATTTACAGTT   800
AAGTGAGTGC AGCAGTGACT ACTGTGCTGG TTATGCTAAG AATGTCTTTG TGCCAATTGA TGGCAAAATA CCAGAAAGCT TCTCTTTTAG TAACTGGTTT   900
CTGTTATCAG ATAAATCCAC TTTAGTGCAA GGACGTGTTC TTAGTAAACA ACCTGTTCTT GTACAGTGCC TTAGGCCCGT GCCAACGTGG TCTAACAATA  1000
CTGCTGTGGT GCATTTTAAA AATGATGTCT TCTGCCCCAA CGTTACGGCG GAAGTTTTGA GGTTCAACTT GAACTTTAGT GACAGTGATG TTTACACAGA  1100
GTCAAGCATA GATGATCAGT TGTATTTTAC ATTTGAAGAT AACACAAATG CATCCATAGC CTGTTATAGC AGTGCTAATG TCACTGATTT TCAACCCGCA  1200
AATCAAAGCG TCTCTCACAT CCCATTTGGA AAAACTGATC ACGCTTATTT TTGTTTTGCC ACTTTTTCTA GTTCTGTTGT GGGTAGACAG TTCTTGGGTA  1300
TACTGCCACC AACTGTCCGA GAGTTTGCAT TCGGCAGAGA TGGATCCATT TTTGTTAATG GTTATAAATA TTTCAGTTTA TCACCTATCA AGAGTGTTAA  1400
CTTCTCCATC AGTTCAGTAG AGAATTATGG CTTTTGGACC ATAGCTTACA CCAACTATAC AGATGTAATG GTGGATGTTA ATGGCACTGG TATCACTAGG  1500
TTATTCTATT GCGACTCACC CCTCAATAGA ATCAAGTGTC AACAATTGAA GCATGAGCTA CCAGATGGAT TTTATTCTGC TAGCATGCTT GTTAAAAAGG  1600
ATCTACCTAA GACATTTGTA ACTATGCCAC AGTTTTATGA TTGGATGAAT GTCACGTTAC ATGTCGTGTT GAATGATACT GAAAAGGGGA AGGATATCAT  1700
TTTGGCTAAG GCTGCCGAAC TAGCATCACT TGCTAATGTA CATTTTGAAA TAGCCCAGGC TAATGGCAGT GTAACTAATG TTACTAGCCT GTGTGTGCAA  1800
ACAAGACAAT TGGCTCTATT CTATAAGTAT ACTAGCGTAC AAGGTTTGTA TACTTATTCC AATTTAGTAG AGTTACAAAA TTATGACTGC CCTTTTTCAC  1900
CACAACAGTT TAATAATTAT CTGCAGTTCG AAACTTTGTG TTTTGATGTG AACCCATCTG TCGCAGGCTG TAAGTGGTCG TTAGTTCATG ATGTTAAGTG  2000
GCGCACACAG TTCGCTACTA TTACTGTTTC TTACAAGGAG GGTGCTATGA TAACGACCAT GCCGAAGGCG CAGCTGGGTT TTCAAGATAT TTCCAATTTA  2100
GTAAAAGATG AATGCACTGA TTACAATATA TATGGATTTC AGGGCACAGG CATTATTAGA AATACCACAT CAAGATTGGT CGCTGGCCTT TACTACACGT  2200
CAGTTAGTGG TGAYCTTCTT GCGTTTAAGA ATAGCACTAC AGGTGAAATT TTCACCGTAG TGCCATGTGA CTTAACAGCT CAAGCAGCAG TGATTAATGA  2300
CGAGATAGTG GGAGCTATAA CAGCCATCAA TCAAACTGAT CTGTTTGAGT TTGTAAATCA CACAAGTTCA AAAAGATCAC GCAGATCAGC ACCAATAACA  2400
CCAACCACCT ATACTATGCC ACAATTCTAT TACATAACAA AGTGGAATAA TGACACTTCG TCTAATTGTA CGTCTACCAT CACTTATTCT TCCTTTGCTA  2500
TTTGTAATAC TGGTGAAATT AGATATGTTA ATGTCACTAA GGTTGAAATT GTGGATGATA GTATAGGAGT TATCAAACCT GTTTCAACAG GCAACATATC  2600
AATACCTAAA AATTTCACTG TTGCAGTGCA GGCCGAATAC ATTCAGATTC AAGTCAAACC TGTCGTTGTG GATTGTGCCA AGTATGTCTG CAATGGTAAC  2700
AGACATTGCC TTAACTTGTT AACACAATAC ACTTCAGCTT GTCAGACAAT TGAAAATGCC CTTAACCTTG GTGCACGCCT TGAATCTTTA ATGCTTAAGG  2800
ATATGATTAC AGTATCAGAT CACAGTTTAG AGCTTGCAAC TGTTGAAAAG TTTAACAGTA CTGTTGTAGG TGGTGAAAGG CTTGGTGGTT TCTATTTTGA  2900
CGGTTTGAGA AATTTGTTAC CAACTAGCAT TGGTAAGAGG TCAGCTATTG AAGATCTATT GTTCAACAAA GTTGTGACCA GCGGTCTTGG CACTGTTGAC  3000
GATGACTATA AAAAGTGCTC TTCTGGCACT GATGTTGCAG ATCTAGTTTG TGCCCAATAT TACAATGGCA TAATGGTTTT ACCTGGTGTT GTGGATGACA  3100
ATAAGATGGC CATGTACACT GCCTCTTTAA TAGGAGGTAT GGCTATGGGC TCTATTACAT CCGCTGTAGC TGTTCCTTTT GCCATGCAAG TGCAGGCTAG  3200
```

Figure 2A continued

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ACTTAACTAT GTCGCATTAC AAACTGATGT ACTACAGGAA AACCAGAAAA TACTTGCTAA TGCTTTTAAT AATGCCATTG GTAACATCAC ACTAGCGCTT  3300
GGAAAAGTTT CCAATGCTAT TACAACCATC TCAGATGGTT TTAATAGTAT GGCCTCAGCA TTGACTAAGA TTCAGAGTGT AGTTAATCAA CAGGGTGAAG  3400
CGTTGAGTCA ACTTACCAGT CAGTTGCAGA AAAATTTCCA GGCCATTAGT AGTTCTATTG CTGAAATTTA TAATAGACTG GAAAAAGTAG AAGCTGATGC  3500
TCAAGTTGAC CGTCTCATTA CTGGTAGATT GGCAGCACTT AATGCTTATG TGTCTCAAAC TTTAACTCAG TATGCTGAAG TTAAGGCTAG TAGGCAACTG  3600
GCAATGGAGA AAGTTAATGA GTGTGTTAAA TCTCAGTCGG ATAGGTATGG GTTCTGTGGA AATGGAACAC ACCTATTCTC ACTTGTCAAT TCTGCACCTG  3700
ATGGTTTACT TTTCTTTCAC ACAGTGTTAC TTCCTACGGA ATGGGAAGAG GTGACGGCAT GGTCAGGAAT ATGTGTTAAT GACACATATG CATATGTGTT  3800
GAAAGACTTT GAATATTCTA TTTTTAGCTA TAATGGCACG TATATGGTAA CTCCTCGTAA TATGTTTCAA CCTAGAAAAC CTCAGATGAG TGATTTCGTG  3900
CAAATTACGA GTTGTGAGGT GACTTTTCTG AACACTACAT ATACGAAATT TCAAGAGATT GTGATTGATT ATATTGACAT CAACAAGACT ATCGTTGATA  4000
TGCTTGAACA ATATAATCCT AATTACACAA CACCTGAATT ACATCTACAG CTGGAAATCT TTAATCAGAC AAAGCTAAAC CTCACTGCAG AAATAGACCA  4100
ATTAGAACAA AGAGCAGACA ACCTTACTAA TATAGCGCAT GAGCTACAGC AGTACATTGA CAACCTTAAT AAGACGCTTG TTGACCTTGA ATGGCTCAAC  4200
AGGGTTGAAA CTTATGTAAA ATGGCCTTGG TATGTGTGGC TACTAATCGG ATTAGTAGTA GTATTCTGCA TACCATTGTT ACTGTTTTGC TGTCTGAGTA  4300
CTGGCTGTTG TGGGTGTTTT GGTTGTCTTG GAAGTTGTTG CAATTCTCTT TGTAGTAGAA GACAATTTGA AAGTTACGAA CCCATCGAAA AGGTTCACAT  4400
TCATTAA                                                                                                       4407
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Nucleotide sequence of feline coronavirus spike gene derived from the sequence of NC_012952

```
ATGATGTTGT TAATACTTGC GCTTCTTAGT ACCGCTCACT CTGAAGATGC GCCTCATGGT GTTACATTAC CACAATTTAA CACTTCCCAT GGCAATGACA   100
AGTTTGAACT TAATTTTTAC AATTTCTTAC AAACTTGGGA TATACCACCA AACACTGAAA CTATTTTTGG TGGCTATCTA CCATATTGTG CTGAAGGTGT   200
CAATTGTGGG TGGCATAATT TTGCTTCCCA GCAACATGAT GCCCTAAATG GTAAGTATGC CTACATAAAT TCGCAAAACT TGGGTATACC GAATGTCCAC   300
GGCGTCTACT TTGACGTACG AGAACGCTAT TATGATGACG GCGTATGGGA TGCAGTCGAT AGAGTTGGCC TATTGATAGC TATACATGGC AAATCTCACT   400
ACAGTTTACT AATGGTTTTA CAAGACAACG TTGAGGAGAA TCAGCCTCAT GTTGCCGTTA AAATCTGCCA TTGGAAACCA GGTAACATAA GTTCTTACCA   500
TCAATTTAGT GTTAATCTAG GAGATAGTGG TCAGTGCGTG TTTAACCGGA GGTTCTCATT GGACACCAAG TTGACAGCTG ATGGTTTCTA TGGCTTCCAG   600
TGGACTGATA CATCTGTAGA CATATATTTA GGTGGCACTA TTACTAAAGT GTGGGTTGAC AACGATTGGA GTGTTGTTGA AGCTAGTATT TCCCATTTTT   700
GGAGTGGTAC TAGCTATGGC TATTACATGC AATTTGTCAA CCGCACAACC TATTATAYTT ACAATAATAC ACTTGGCTCA AATTATACAC ATTTGCAGTT   800
AAGCGAGTGC AGTAGTGATT ATTGTGCTGG TTATGCTAAA AATGTCTTTG TGCCAGTTGG TGGCAAGATA CCAGAGAGTT ATTCTTTTAG TAACTGGTTT   900
CTGTTATCAG ACAAATCCAC TTTGGTGCAA GGACGTGTTC TTAGTAAACA ACCTGTTCTT GTACAGTGCC TTAGGCCCGT GCCAACGTGG TCTAACAATA  1000
CTGCTGTGGT GCATTTTAAA AATGACGTCT TCTGCCCCAA CGTTACGGCG GAAGTTTTGA GGTTCAACTT GAACTTTAGT GACAGTGATG TTTACACAGA  1100
GTCAAGCATA GATGATCAGT TGTATTTTAC ATTTGAAGAT AACACAAATG CATCCATAGC CTGTTATAGC AGTGCTAATG TCACTGATCT TCAACCCGCA  1200
AATCAAAGCG TCTCTCACAT CCCATTTGGA AAAACTGATT ACGCTTATTT TTGTTTTGCC ACTTTTTCTA GTTCTGTTGT GGGTAGACAG TTCTTGGGTA  1300
TACTKCCACC AACTGTCCGA GAGTTTGCAT TCGGCAGAGA TGGATCCATT TTTGTTAATG GTTATAAATA TTTCAGTTTA CCACCTATCA AGAGTGTTAA  1400
```

Figure 2A continued

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CTTCTCCATC AGTTCAGTAG AGAATTATGG CTTTTGGACC ATAGCTTACA CCAACTATAC AGATGTAATG GTGGATGTTA ATGGCACTGG TATCACTAGT  1500
TTATTCTATT GCGACTCACC CCTCAATAGA ATCAAGTGTC AACAATTGAA GCATGAGCTA CCAGATGGAT TTTATTCTGC TAGCATGCTT GTTAAAAAGG  1600
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TTTGGCTAAG GCTGCCGAAC TAGCATCACT TGCTAATGTA CATTTTGAAA TAGCCCAGGC TAATGGCAGT GTAACTAATG TTACTAGCCT GTGTGTGCAA  1800
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```

Figure 2B

YP_003038574

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 241 yymqfvnrtt yyaynntggs nythlqlsec ssdycagyak nvfvpidgki pesfsfsnwf
 301 llsdkstlvq grvlskqpvl vqclrpvptw snntavvhfk ndvfcpnvta evlrfnlnfs
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 421 tfsssvvgrq flgilpptvr efafgrdgsi fvngykyfsl spiksvnfsi ssvenygfwt
 481 iaytnytdvm vdvngtgitr lfycdsplnr ikcqqlkhel pdgfysasml vkkdlpktfv
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1321 vidyidinkt ivdmleqynp nyttpelhlq leifnqtkln ltaeidqleq radnltniah
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YP_003038543

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Figure 2B continued

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Figure 3

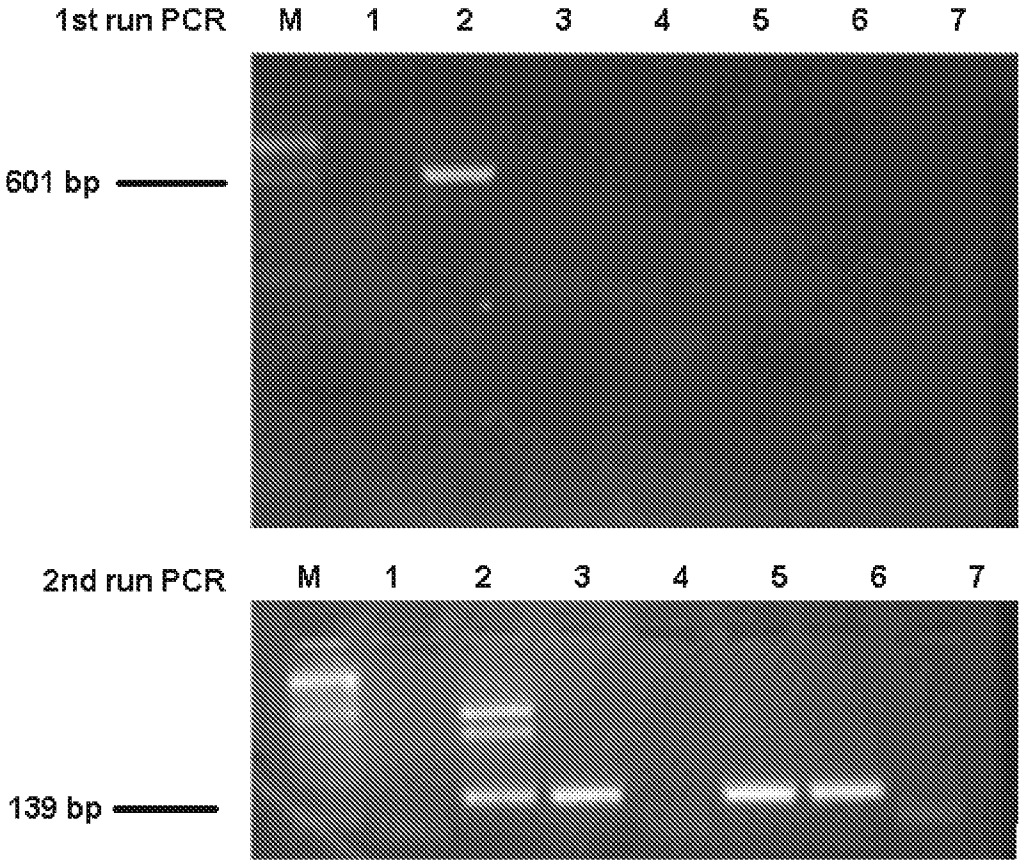
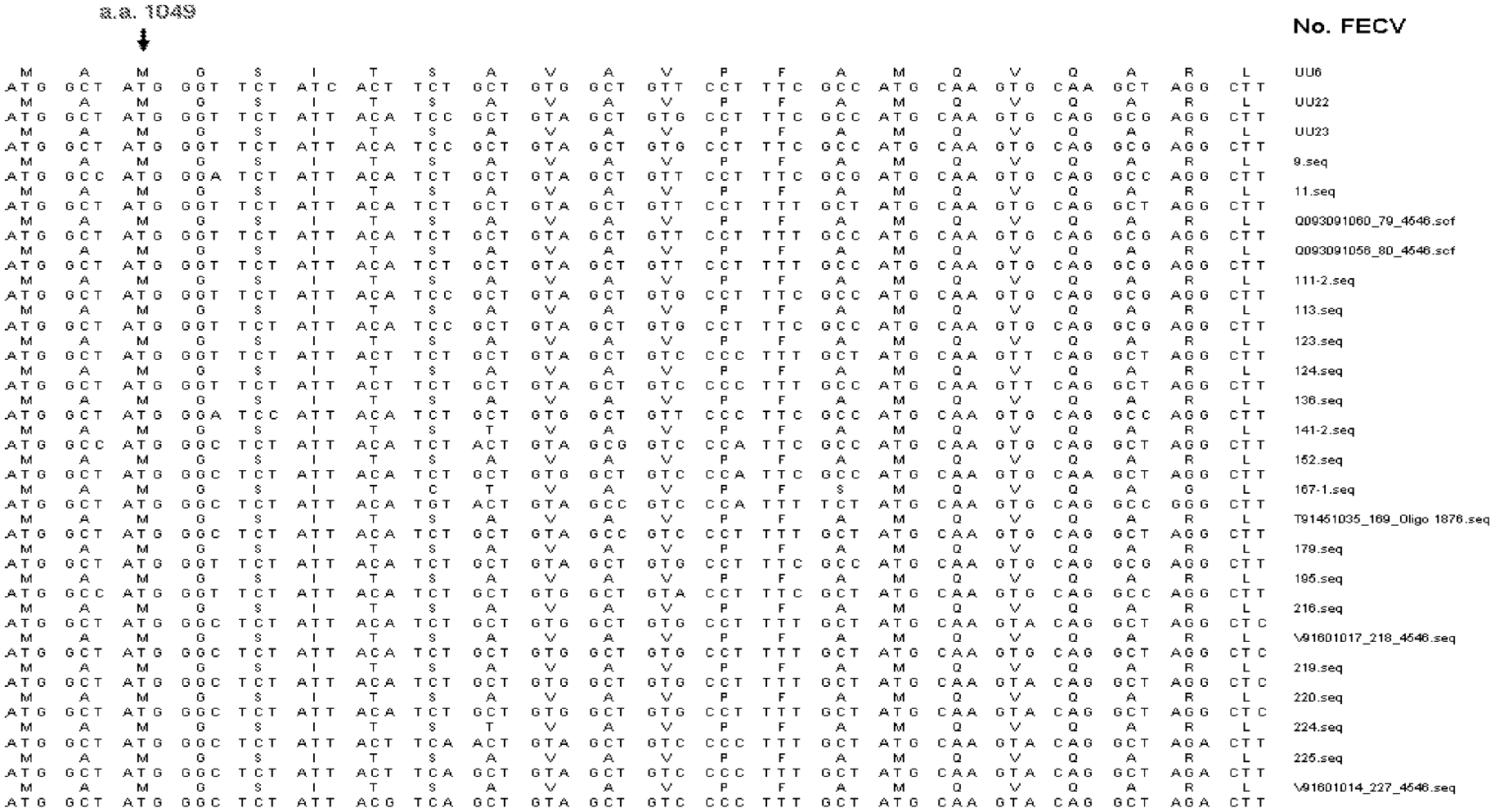


Figure 4A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No. FECV
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Figure 4A continued

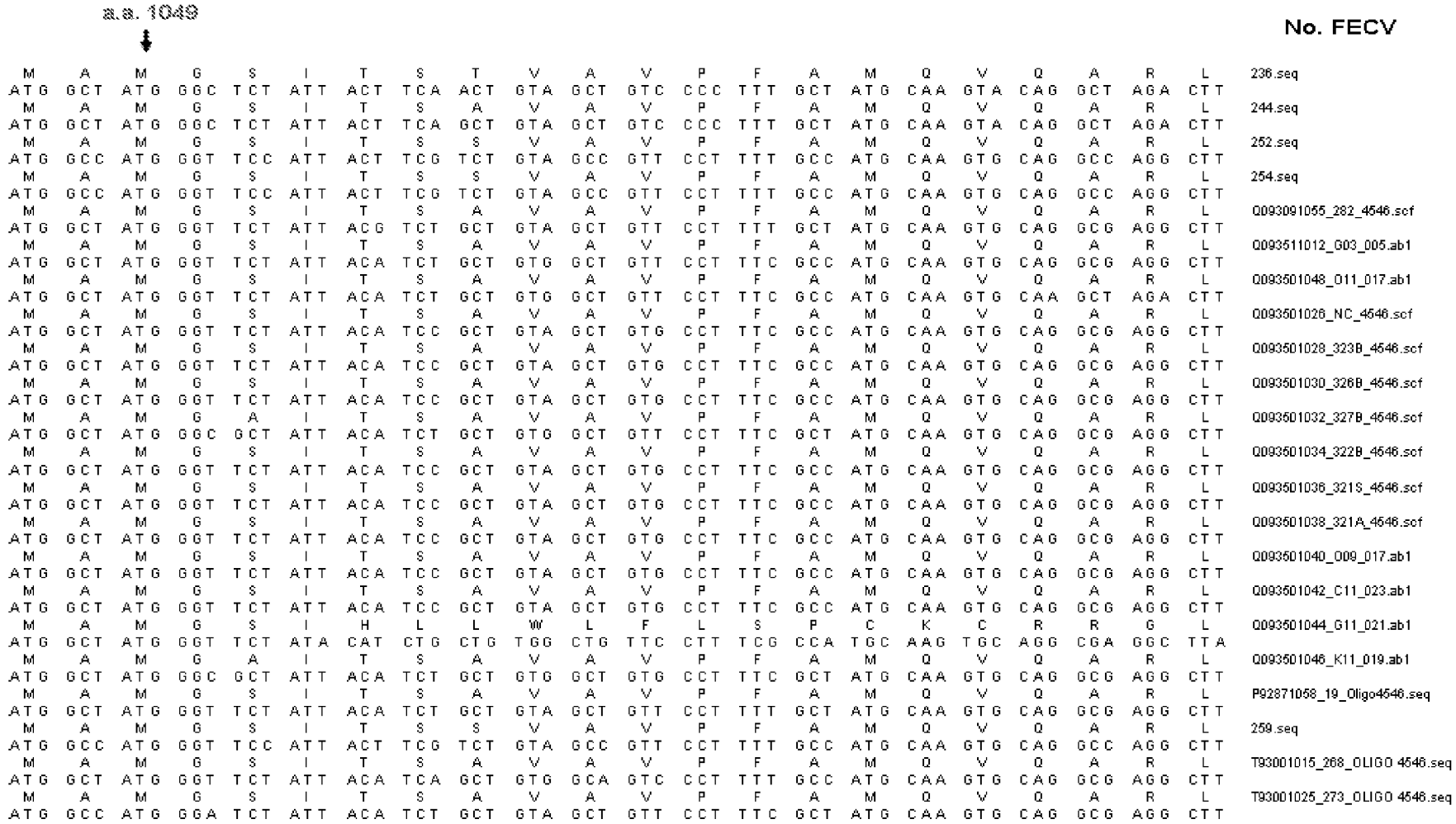

Figure 4B

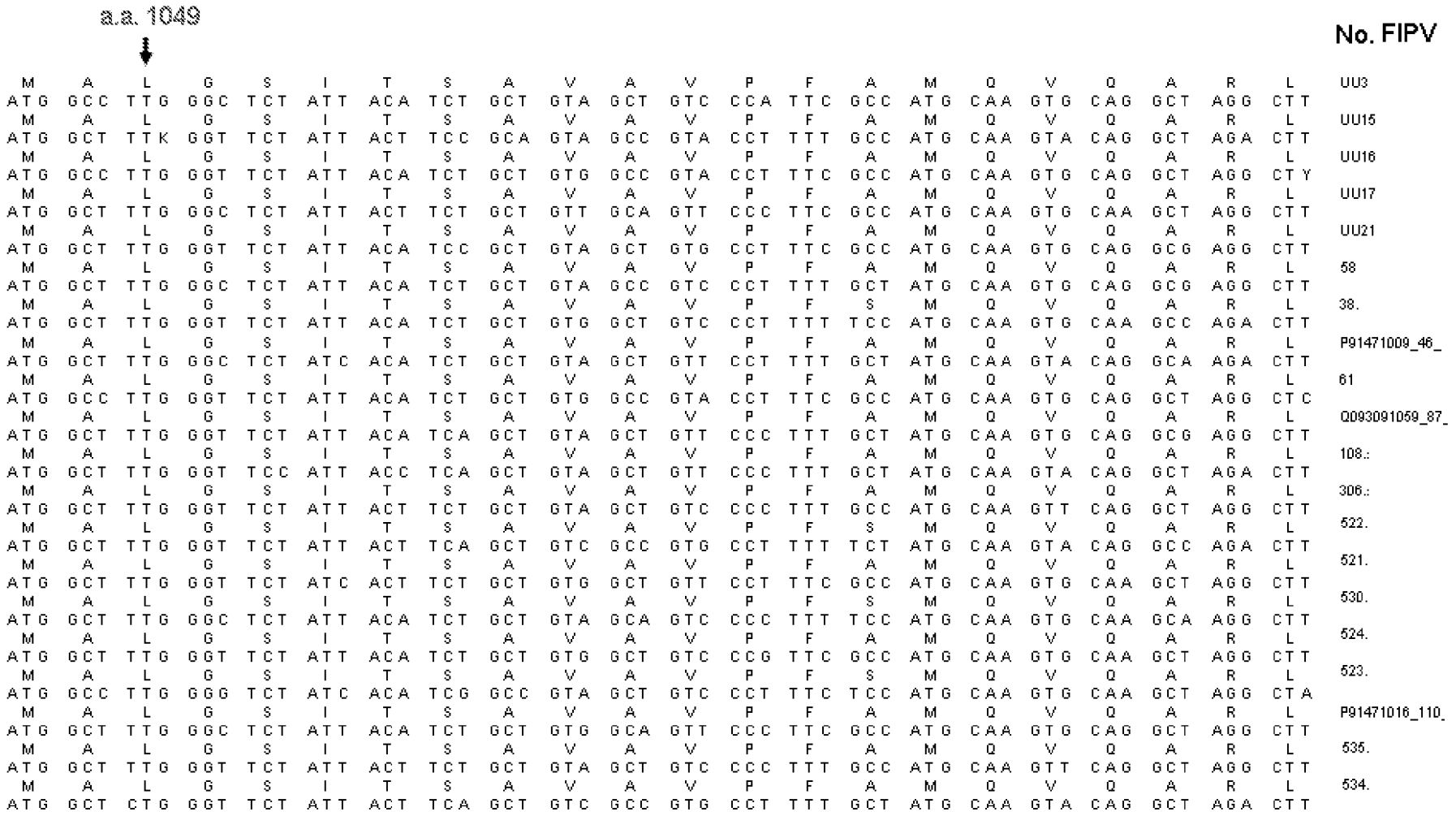
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Figure 4B continued

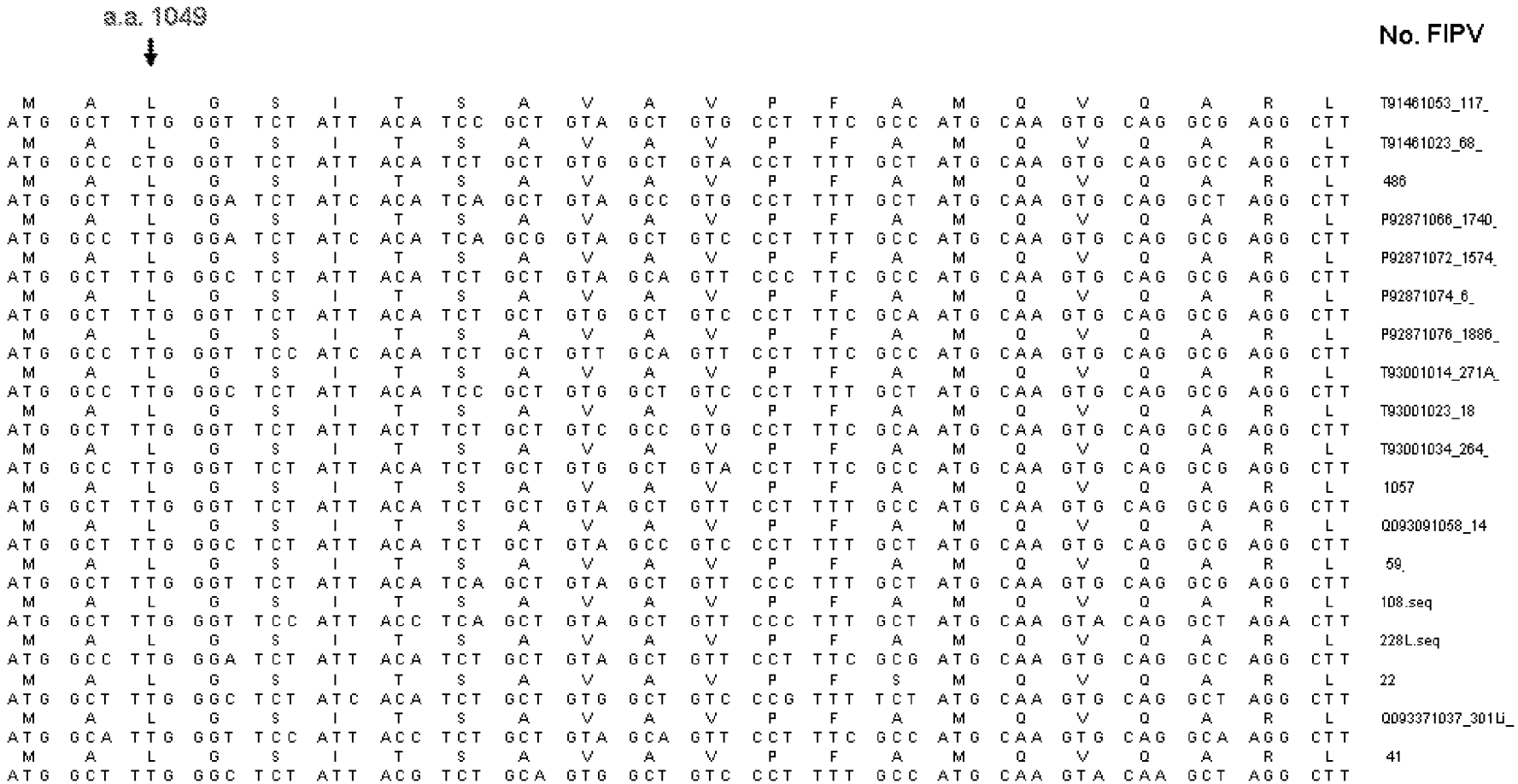


Figure 4B continued

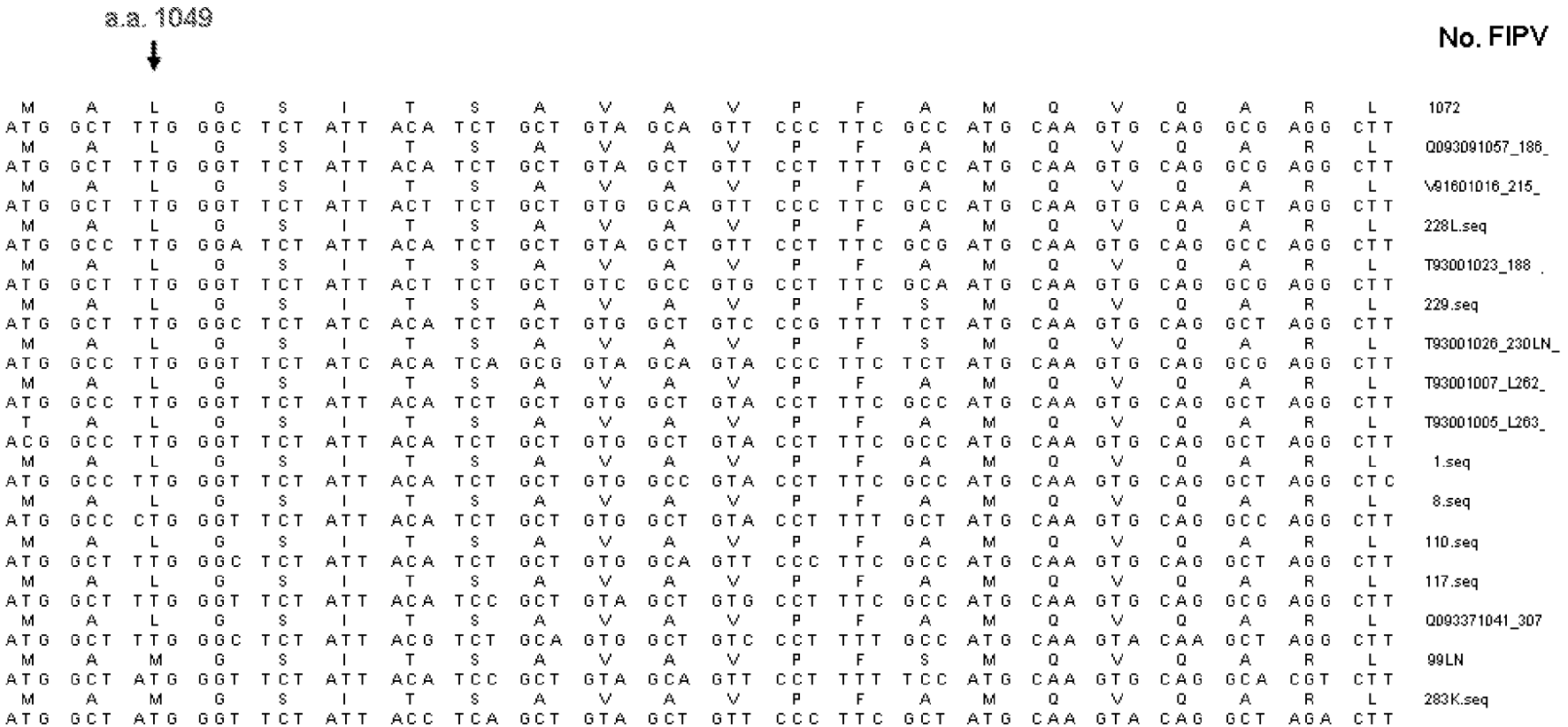
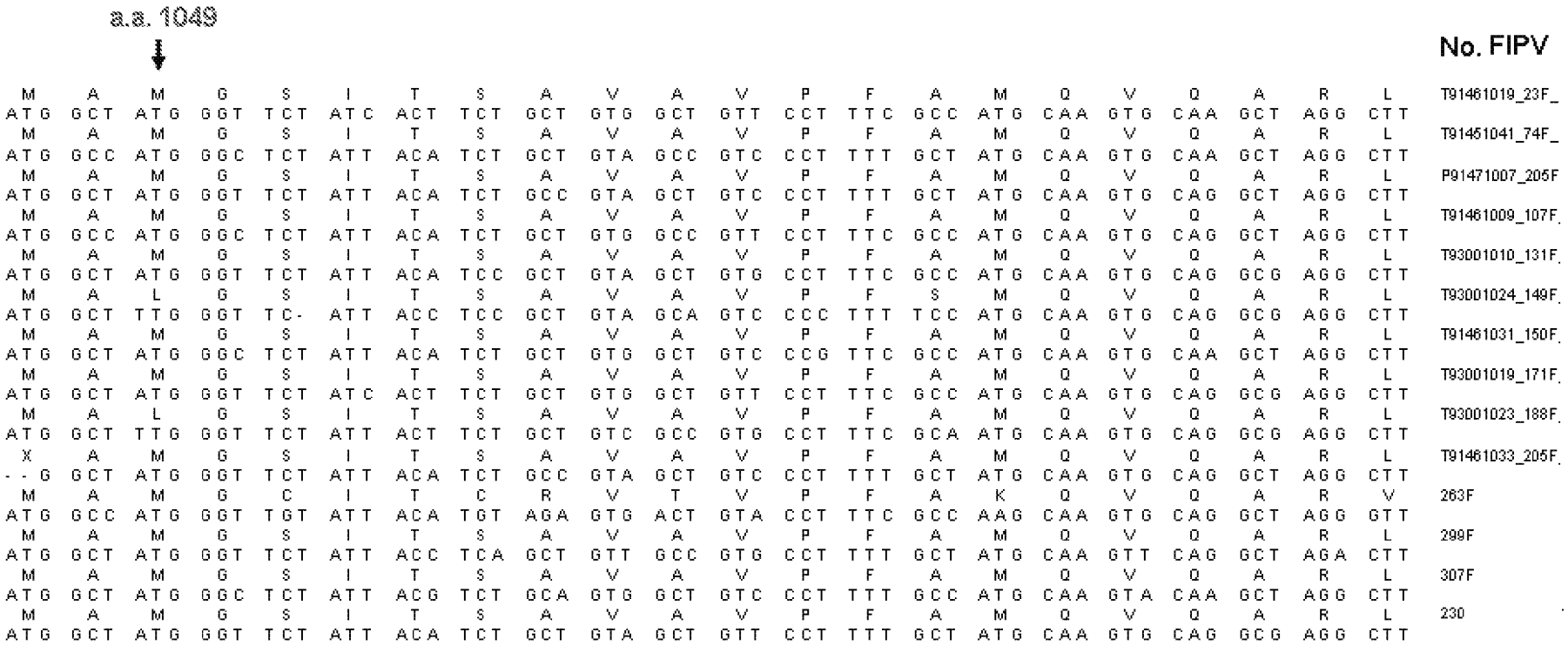


Figure 4C



WO 2011/087366 PCT/NL2011/050027

INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050027

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12Q1/70
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Q

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, Sequence Search, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JP 7 327683 A (KITASATO INST) 19 December 1995 (1995-12-19) the whole document	18,19
X	WO 94/13836 A1 (SYNBIOTICS CORP [US]) 23 June 1994 (1994-06-23) the whole document	17
X	WO 95/08575 A1 (CORNELL RES FOUNDATION INC [US]) 30 March 1995 (1995-03-30) claims 1-3	17
X	WO 02/066686 A1 (ID LELYSTAD INST DIERHOUDERIJ [NL]; EGBERINK HERMANUS FRANSISCUS [NL];) 29 August 2002 (2002-08-29)	11,12,14
A	claim 6	1

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
4 April 2011	13/04/2011

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

2

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2004/063093 A1 (MILLER TIMOTHY J [US] ET AL) 1 April 2004 (2004-04-01) claim 1	1
A	ROTTIER PETER J M ET AL: "Acquisition of macrophage tropism during the pathogenesis of feline infectious peritonitis is determined by mutations in the feline coronavirus spike protein", JOURNAL OF VIROLOGY, vol. 79, no. 22, November 2005 (2005-11), pages 14122-14130, XP002583147, ISSN: 0022-538X cited in the application the whole document	1
A	GAMBLE D ET AL: "Development of a nested PCR assay for the detection of feline infectious peritonitis virus in clinical specimens", JOURNAL OF CLINICAL MICROBIOLOGY, AMERICAN SOCIETY FOR MICROBIOLOGY, WASHINGTON, DC, US, vol. 35, no. 3, 1 March 1997 (1997-03-01), pages 673-675, XP002171735, ISSN: 0095-1137 the whole document	1
A	WO 97/20054 A1 (VIROGENETICS CORP [US]) 5 June 1997 (1997-06-05) claims 1,7	1
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A	PEDERSEN NIELS C: "A review of feline infectious peritonitis virus infection: 1963-2008.", JOURNAL OF FELINE MEDICINE AND SURGERY APR 2009 LNKD- PUBMED:19254859, vol. 11, no. 4, April 2009 (2009-04), pages 225-258, XP002583148, ISSN: 1098-612X the whole document	1

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/NL2011/050027

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