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DESCRIPTION

[0001] The invention relates to live attenuated parvoviruses, their uses, vaccines comprising such live attenuated parvoviruses, as well as methods for their production.

[0002] Parvovirus belongs to the family of single stranded DNA viruses. Parvoviruses can cause disease in various animals such as cats, dogs and pigs. Because the viruses require actively dividing cells in order to replicate, the type of tissue infected varies with the age of the animal. The gastrointestinal tract and lymphatic system can be affected at any age, leading to vomiting, diarrhea and immunosuppression, but cerebellar hypoplasia is only seen in cats that were infected in the womb or at less than two weeks of age, and disease of the myocardium is seen in puppies infected between the ages of three and eight weeks.

[0003] Canine parvovirus (CPV) is a particularly deadly disease in puppies, about 80% fatal, causing gastrointestinal tract damage and dehydration as well as a cardiac syndrome in very young pups. It is spread by contact with infected dog's feces. Symptoms include lethargy, severe diarrhea, fever, vomiting, loss of appetite, and dehydration. Porcine parvovirus causes a reproductive disease in swine known as SMEDI, which stands for stillbirth, mummification, embryonic death, and infertility. Feline panleukopenia, commonly known as feline distemper, is a viral infection affecting cats, caused by feline parvovirus (FPV), a close relative of canine parvovirus. Feline panleukopenia is common in kittens and causes fever, low white blood cell count, diarrhea, and death. Infection of the cat fetus and kittens less than two weeks old causes cerebellar hypoplasia. Mink enteritis virus is similar in effect to feline panleukopenia, except that it does not cause cerebellar hypoplasia. A different parvovirus causes Aleutian Disease in minks and other mustelids, characterized by lymphadenopathy, splenomegaly, glomerulonephritis, anemia, and death. The most accurate diagnosis of parvovirus is by ELISA. Dogs, cats and swine are commonly vaccinated against parvovirus.

[0004] At the DNA level, canine, feline and porcine parvoviruses are known to have a highly homologous genome. Canine parvovirus CPV2 is a virus which is responsible for an acute and sometimes fatal enteritis in dogs (Kelly, Aust. Vet. J. 54; 593, 1978; Appel et al., Vet. Rec. 105; 156-159, 1979). The virus, which first appeared around 1977, probably arose from a very closely related virus in cats, feline panleukopenia virus (FPLV) through a small number of mutations in the single capsid protein; a species jump which may have involved intermediate passage in other carnivores such as mink or raccoons (Truyen et al., Virology 215, 186-189, 1996).

[0005] As early as 1979 the first variants of CPV2 appeared, termed CPV2a, and they were quickly followed by the appearance of CPV2b in 1984. (Parrish et al., Science 230, 1046-1048, 1985, and J. Virol. 65; 6544-6552, 1991).

[0006] The original type 2 virus has now disappeared from the field having been replaced by the 2a and 2b types, although the relative proportions of these two types varies from country to country (Truyen et al., supra; Chinchkar et al., Arch. Virol. 151, 1881-1887, 2006; Pereira et al., Infect. Genet. Evol. 3, 399-409, 2007). The amino acid changes in the capsid protein (VP2), which characterize the shift from 2 to 2a and to 2b, are very limited. Substitutions at positions 87 (Met to Leu), 300 (Ala to Gly), 305 (Asp to Tyr) and 555 (Val to Ile) occurred in the evolution of 2 to 2a and 426 (Asn to Asp) and 555 (Ile to Val) in the emergence of 2b from 2a (Parrish et al., supra; Truyen et al., J. Virol. 69, 4702-4710, 1995). Recently, 2a strains lacking the Val to Ile substitution at position 555 have been reported (Wang et al., Virus Genes 31, 171-174, 2005; Martella et al., Virus Genes 33, 11-13, 2006). It appeared that a single amino acid change can differentiate the CPV2a and CPV2b VP2 sequences.

[0007] More recently strains have emerged in Italy in which the amino acid at position 426 (Asn in 2a and Asp in 2b) has become a glutamic acid (Glu) residue (Buonavoglia et al., J. Gen. Virol. 82, 3021-3025, 2001; Martella et al., J. Clin. Microbiol. 42, 1333-1336, 2004). The fact that these Glu 426 variants, termed CPV2c viruses, are circulating and co-existing with other CPV types in Italy and other European countries (Decaro et al., J. Vet. Med. B. Infect. Dis. Vet. Public Health 53, 468-472, 2006) and have also been isolated in countries as geographically diverse as Vietnam and Scotland (Nakamura et al., Arch Virol. 149, 2261-2269, 2004; Spibey et al., Vet. Microbiol 128, 48-55, 2008) suggests that they have an advantage in at least a proportion of the dog population.

[0008] The relatively rapid evolution of canine parvovirus has resulted in the loss and then re-gaining of the feline host range (Truyen et al., 1996 supra), and this regained ability to replicate in cats may well account for the replacement of the original type 2 virus with the 2a, 2b and 2c variants. In the late 1970s and early 1980s both live and inactivated FPL vaccines were used to protect dogs against CPV disease due to the shared antigens which stimulated cross-protection, however the level of protection they afforded was poor and duration of immunity was short. These vaccines were replaced by live attenuated CPV vaccines, which provided good protection and longer duration of immunity. Currently the live attenuated vaccines are derived from either CPV2b isolates or the original type 2 virus. Since the type 2 virus has been entirely replaced in the field by 2a, 2b and now 2c

viruses there has been concern over the level of protection afforded by attenuated type 2 vaccines (Pratelli et al., Clin. Diag. Lab. Immunol. 8, 612-615, 2001; Truyen, Vet. Microbiol. 69, 47-50, 1999).

[0009] However, based on studies with available monoclonal antibodies each new antigenic variant has lost at least one neutralizing epitope compared with the former variant (Strassheim et al., Virology 198, 175-184, 1994; Pereira et al., supra). Previously it has been demonstrated that the live attenuated CPV2 vaccine is able to protect dogs against 2a and 2b field challenges (Greenwood et al., Vet. Record. 136, 63-67, 1995) even though cross-neutralization studies conducted in vitro using sera raised against the various antigenic types do show marked differences (Pratelli et al., supra).

[0010] Recently, it was shown that live attenuated type 2 vaccine (Nobivac-Intervet) was able to protect dogs from challenge with the most recent CPV variant, CPV2c (Spibey et al., Vet. Microbiol 128, 48-55, 2008).

[0011] Nevertheless there exists a need in the field for vaccines that combine the induction of a sufficient level of immunity in animals, in particular cats, dogs and pigs against infection with parvoviruses with a highly attenuated behavior. A high level of attenuation is synonymous with safety, especially in young and old animals.

[0012] It is an objective of the present invention to provide new live parvoviruses that are attenuated while still immunogenic. Such viruses provide a basis for safe vaccines.

[0013] The invention in its broadest sense is as defined in the independent claims.

[0014] In this respect, one embodiment of the present invention relates to live attenuated CPV2 parvovirus, characterised in that said CPV2 comprises a capsid gene of CPV2 serotype 2a, 2b or 2c coding for an amino acid other than Isoleucine at amino acid position 219 of the capsid protein and/or an amino acid other than Glutamine at amino acid position 386 of the capsid protein, and characterised in that a DNA fragment of a part of the non-capsid region of said CPV2 is replaced by a homologous DNA fragment of a part of the non-capsid region derived from a second parvovirus, wherein said homologous DNA fragment of said second parvovirus carries an attenuating mutation.

[0015] The live attenuated CPV2 parvovirus of the invention is not the CPV that is present in canine parvovirus vaccine Nobivac Parvo C. A sequence comprised in the CPV of canine parvovirus vaccine Nobivac Parvo C is given in SEQ ID NO: 1.

[0016] It was surprisingly found, that these two sites, at amino acid position 219 and 386 of the capsid gene, play an important role in the attenuation of the virus. Until now it was assumed that mainly amino acids outside the capsid region are involved in the virulence/attenuation of the virus.

[0017] The location of the Isoleucine at amino acid position 219 of the capsid protein and a Glutamine at amino acid position 386 of the capsid protein is identical in both canine and feline parvoviruses, regardless of the serotype.

[0018] The prior art describes the CPV that is present in canine parvovirus vaccine Nobivac Parvo C (Intervet Schering-Plough Animal Health) that comprises the sequence as given in SEQ ID NO: 1.

[0019] Merely to indicate the location of the Isoleucine at amino acid position 219 and the Glutamine at amino acid position 386, the two amino acids are shown below (in bold characters) in an example of the sequential context found in most CPV and FPV strains.

yfqwdrtlipshgtgsg (Isoleucine 219 = bold)

yafgrqhgqktttget (Glutamine 386 = bold)

[0020] Depending upon the strain that is used as the starting material for the substitution of one or both amino acids according to the invention, it may be that a single substitution of the amino acid at position 219 or 386 is not sufficient to e.g. make the virus safe in very young animals. If a further attenuation is required, the substitution of both the amino acid at position 219 and 386 is preferred.

[0021] Therefore, a preferred form of this embodiment relates to a live attenuated CPV2 according to the invention that comprises a capsid gene coding for an amino acid other than Isoleucine at amino acid position 219 of the capsid protein and an amino acid other than Glutamine at amino acid position 386 of the capsid protein.

[0022] A more preferred form of this embodiment relates to a live attenuated CPV2 according to the invention, that comprises a capsid gene coding for a Valine at amino acid position 219 of the capsid protein and/or a Lysine at amino acid position 386 of the capsid protein.

[0023] An even more preferred form of this embodiment relates to a live attenuated CPV2 according to the invention, that comprises a capsid gene coding for a Valine at amino acid position 219 of the capsid protein and a Lysine at amino acid position 386 of the capsid protein.

[0024] A parvovirus that already has an attenuating mutation located outside the capsid region may be used as the starting material for the introduction of an amino acid substitution in order to prepare a live attenuated CPV2 according to the invention. An alternative may be the replacement of a DNA fragment of a part of the non-capsid region of a virus comprising a capsid gene of CPV2 serotype 2a, 2b or 2c coding for an amino acid other than Isoleucine at amino acid position 219 of the capsid protein and/or an amino acid other than Glutamine at amino acid position 386 of the capsid protein, with a homologous non-capsid region of a parvovirus strain that carries an attenuation in that region. Parvoviruses carrying an attenuation in a part of the non-capsid region are e.g. the commercially available canine parvovirus vaccine Nobivac Parvo C (Intervet Schering-Plough Animal Health).

[0025] The advantage of such an approach is, that such viruses have an even higher attenuation level than a CPV2 parvovirus comprising said attenuating mutation in the non-capsid region but a capsid gene of CPV2 serotype 2a, 2b or 2c coding for Isoleucine at amino acid position 219 and Glutamine at amino acid position 386 of the capsid protein.

[0026] A homologous DNA fragment of a part of the non-capsid region from a second parvovirus is a DNA fragment that has the same function as the DNA fragment of the CPV2 parvovirus comprising a capsid gene of CPV2 serotype 2a, 2b or 2c according to the invention, but differs from that DNA fragment in that it carries a mutation that leads to attenuated behavior of the virus. Merely as an example, if a DNA fragment comprises an attenuating mutation in a DNA-fragment between two specific restriction sites, and these two restriction sites are also present at the same location in a virus not having that mutation, the restriction fragment carrying the mutation would be considered homologous with the same fragment from the virus not having that mutation.

[0027] A highly preferred form of this embodiment relates to a live attenuated CPV2 parvovirus according to the invention wherein the homologous DNA fragment of said second parvovirus carries an attenuating mutation in the nonstructural region, in the region from position 2061 to 2070 of SeqIdNo.1.

[0028] The capsid gene of the virus according to the invention encodes a capsid protein of CPV serotype 2a, 2b or 2c comprising an amino acid other than Isoleucine at amino acid position 219 and/or an amino acid other than Glutamine at amino acid position 386.

[0029] Another embodiment of the present invention relates to vaccines for the protection of animals against infection with CPV2 parvovirus, wherein such vaccines comprise a live attenuated CPV2 parvovirus according to the invention and a pharmaceutically acceptable carrier.

[0030] A suitable amount of parvovirus according to the invention, for use in a vaccine would in many cases be within the range of 10^3 - 10^9 TCID₅₀, depending on the level of attenuation and the replication characteristics of the virus. An infectious dose of virus that is below 10^3 TCID₅₀ would in many cases be considered to be too low, since it would take too much time for the virus to replicate to a sufficiently high level to trigger the immune system. Amounts that exceed 10^9 TCID₅₀ would be unattractive, if only for commercial reasons.

[0031] A very suitable dose would be in the range of 10^5 - 10^8 TCID₅₀, even better between 10^6 - 10^8 TCID₅₀.

[0032] Pharmaceutically acceptable carriers are well-known in the art. Merely as an example; such a carrier can be as simple as sterile water or a buffer solution such as PBS.

[0033] Vaccines according to the invention can be administered in several ways. Since the vaccine comprises a live attenuated virus, many ways of administration, such as oral, intranasal, I.M. and subcutaneous administration are feasible. A preferred route of administration is the subcutaneous administration.

[0034] Animals susceptible to parvovirus infection such as i.a. cats and dogs are frequently vaccinated against several other

diseases at the same time. Therefore it would be practical to combine a vaccine according to the invention with an additional antigen of a virus or micro-organism pathogenic to dogs and cats or genetic information encoding said antigen.

[0035] Thus, another embodiment of the invention relates to a combination vaccine comprising a vaccine according to the invention and an additional antigen of a virus or micro-organism pathogenic to animals or genetic information encoding an immunogenic polypeptide of said virus or micro-organism.

[0036] The additional antigen of a virus or a micro-organism can be the whole virus or micro-organism (in a live attenuated form or in an inactivated form) or an immunogenic polypeptide or another immunogenic part of that virus or micro-organism such as e.g. a (lipo-)polysaccharide, capable of inducing a protective immune response.

[0037] Preferably, the virus or micro-organism pathogenic to animals is selected from the group of *Ehrlichia canis*, *Babesia gibsoni*, *vogeli*, *rossi*, *Leishmania donovani-complex*, Canine adenovirus, Canine coronavirus, Canine distempervirus, *Leptospira interrogans serovar canicola*, *icterohaemorrhagiae*, *pomona*, *grippotyphosa*, *bratislava*, Canine hepatitisvirus, Canine parainfluenzavirus, rabies virus, *Hepatozoon canis*, *Borrelia burgdorferi*, *Bordetella bronchiseptica*, feline Herpesvirus, feline calicivirus, feline panleucopenia and *Chlamydomphila felis*.

[0038] Vaccines comprising live attenuated viruses must be stored at low temperature, or they have to be in a freeze-dried form. Freeze-dried vaccines can be kept under moderate cooling conditions or even at room temperature. Often, the vaccine is mixed with stabilizers, e.g. to protect degradation-prone proteins from being degraded, to enhance the shelf-life of the vaccine, or to improve freeze-drying efficiency. Useful stabilizers are i.a. SPGA, carbohydrates e.g. sorbitol, mannitol, trehalose, starch, sucrose, dextran or glucose, proteins such as albumin or casein or degradation products thereof, and buffers, such as alkali metal phosphates.

[0039] Therefore, preferably, the (combination) vaccine according to the invention is in a freeze-dried form. In addition, the vaccine may be suspended in a physiologically acceptable diluent. Such buffers can e.g. be sterile water, a buffer and the like.

[0040] It goes without saying, that diluents and compounds for emulsifying or stabilizing viruses are also embodied in the present invention.

[0041] Again another embodiment of the invention relates to methods for the manufacture of a (combination) vaccine according to the invention wherein these methods comprise the mixing of a live attenuated CPV2 parvovirus according to the invention and a pharmaceutically acceptable carrier.

[0042] Still another embodiment of the invention relates to live attenuated CPV2 parvovirus according to the invention for use as a medicament. More specifically, this embodiment relates to live attenuated CPV2 parvovirus according to the invention for use in the treatment of parvovirus infection.

[0043] Finally, another embodiment of the present invention relates to methods for the preparation of a CPV2 parvovirus mutant according to the invention, wherein such methods comprise exchanging in a gene encoding a capsid protein of CPV2 serotype 2a, 2b or 2c of a canine parvovirus that is already attenuated in a part other than the part encoding the capsid a DNA fragment encoding at least part of the parvovirus capsid protein having at amino acid position 219 a codon encoding Isoleucine and/or having at amino acid position 386 a codon encoding Glutamine, by a DNA fragment encoding at least part of the parvovirus capsid protein having at amino acid position 219 a codon encoding an amino acid other than Isoleucine and/or having at amino acid position 386 a codon encoding an amino acid other than Glutamine.

[0044] Such exchanging of DNA can be done using recombinant DNA techniques well-known in the art, such as site-directed mutagenesis, exchange of restriction fragments and the like. There are several ways of making the 219 Isoleucine an X1 or 386 Glutamine an X2 substitution. Such changes could be introduced by chemical synthesis or PCR followed by recombination of the newly synthesized fragment with viral DNA.

Examples

Example 1: Production of Canine parvovirus Clone 630att

Starting Materials

[0045]

Viruses:

Virus	Source	Cell substrate	Other Information
Nobivac parvo	Intervet (Nobivac parvoC vaccine vial)	A72	Virus is a type 2 strain dating from before 1979
Isolate "Jes"	Intervet inc. Millsboro USA	CrFK	Type 2c virus isolated in USA in 2008. Typing performed by DNA sequence analysis

E. coli strains

[0046] The E. Coli strains JC811 obtained from the E. coli genetic stock centre (USA) and strain DL795 (Kramel Biotech UK) were selected for plasmid propagation of complete infectious clones.

DNA synthesis

[0047] Custom DNA synthesis was performed by Eurofins MWG GmbH. The synthesised DNA fragment was supplied in the pBluescript cloning plasmid.

[0048] The construction of the canine parvovirus clone 630att was a multi-step process and is described here in its separate steps.

1) Construction of an infectious molecular clone of Nobivac Parvo C (p154att)

[0049] Replicative form (RF) viral DNA was obtained from A72 cells infected with Nobivac Parvo C infected cells using a modified "Hirt" preparation method (Parrish et al 1988, Virology 166, 293-307). Viral DNA was digested with a number of restriction enzymes and the genome was assembled in pBluescript using routine cloning methodology. The cloning scheme is outlined in figure 1-4.

RF DNA of CPV 154att was first "end filled" using T4 DNA polymerase. The DNA was then digested with EcoRI and SpeI. These enzymes cut once at positions 1099 and 3459 respectively. This digestion results in three fragments labelled A, B & C in order of their size. Fragments were separated by gel electrophoresis and the EcoRI/SpeI fragment (fragment A) was then cloned into plasmid pBluescript which had been prepared by digestion with the same enzymes (see figure 1). The EcoRI terminal fragment (fragment C) from the RF DNA digest was then cloned into pBluescript digested with EcoRI and EcoRV to produce pCPV C (see figure 2).

The canine parvovirus A and C fragments were sub-cloned together into the into the same plasmid. Plasmids pCPV A and PCPV C were digested with SpeI and EcoRI. The CPV insert was purified from pCPV A and the vector portion was taken from pCPV C. Ligation then resulted in a plasmid in which fragments A and C were "re-united" (see figure 3).

The SpeI terminal fragment (fragment B) from the CPV RF DNA digest was then cloned into pCPV AC. Plasmid pCPV AC was digested with Spe I and an enzyme HincII which cuts leaving a blunt ends. The fragments were ligated and cloned. (see figure 4).

[0050] The resultant plasmid was called p154att.

[0051] Confirmation that a complete clone had been assembled was achieved by transfection of the plasmid DNA in to A72 cells resulting in the production of infectious virus.

[0052] DNA sequence analysis of the plasmid clone was performed; this sequence was shown to be identical to that determined from viral DNA extracted from infected cells, as shown further below.

2). Construction of 2/2c Hybrid D9

[0053] Viral DNA was obtained from both Nobivac parvo C infected cells and separately from CPV2c "Jes" infected cells. Viral DNA preparations were each digested with a single restriction enzyme to produce 2 DNA fragments from each. The enzyme digests were done in such a way that the left hand fragment of the Nobivac genome and the right hand fragment of the "Jes" genome shared >200bp of overlapping sequence. The Nobivac left end and "Jes" right end fragments were separated purified and mixed (Figure 7).

[0054] Transfection of A72 and CrFK cells with these overlapping fragments allowed infectious virus to be produced by natural recombination. The resulting virus was cloned by limiting dilution and termed 2/2c hybrid D9.

3). Construction of Clone 630.

[0055] Clone 630 was developed from the infectious plasmid clone p154att and DNA prepared from 2/2c hybrid D9.

[0056] The restriction enzyme *PacI* cuts the CPV genome in two places around positions 561 and 4651; the far left and right ends of the genome. Therefore the plasmid p154att was digested with *PacI* and the ~ 4kbp *PacI* fragment containing over 80% of the genome was separated from the vector and terminal sequences and replaced with that obtained from 2/2c hybrid D9 DNA. The resultant plasmid was termed p630. This is illustrated in Figure 5.

[0057] As predicted, transfection of A72 or CrFK cells with p630 results in the generation of infectious virus, this virus is termed 630.

[0058] Virus 630 like 2/2c hybrid D9 retained a low level of pathogenicity.

4). Construction of Clone 630att

[0059] 630 virus showed some low level clinical signs when injected into dogs.

[0060] A portion of the capsid gene was chemically synthesised incorporating amino acid changes observed in Nobivac parvo C but not found to occur in field strains. This fragment was then substituted for the same region in plasmid p630 to create plasmid p630att; this is illustrated in Figure 6.

[0061] The DNA sequence corresponding to that between positions 3356 and 4029 on the CPV genome was synthesised. The exact DNA sequence, provided here as SEQ ID NO: 2, is shown below

```

1   agatctgaga cattggggtt ttatccatgg aaaccaacca taccaactcc
   atggagatat tattttcaat gggatagaac attagtacca totcatactg
101  gaactagtgg cacaccaaca aatatatacc atggtacaga tccagatgat
   gtccaatttt ataactatga aaattctgtg ccagtagact tactaagaac
201  aggtgatgaa ttgtctacag gaacattttt ttttgattgt aaaccatgta
   gactaacaca tacatggcaz acaaatagag cattgggctt accaccattt
301  ctaaaattctt tgccctcaagc tgaaggaggt actaacctttg gttatatagg
   agttcaacaa gataaaagac gtggtgtaac tcaaatggga aatacaaaact
401  atattactga agctactatt atgagaccag ctgagggttg ttatagtcca
   ccattattatt cttttgaggg gtctacacaa gggccattta aaacacctat
501  tgcagcagga cgggggggag cgcaaacaga tgaaaatcaa gcagcagatg
   gtgatccaag atatgcattt ggtagacaac atggtaaaaa aactaccaca

601  acaggagaaa cacctgagag atttacatat atagcacatc aagatacagg
   aagatatcca gaaggagatt gg

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[0062] The restriction enzyme sites *Bgl* II and *Xcm*I are shown in bold and underlined.

[0063] The sequence was liberated from the plasmid in which it was provided by digestion with *Bgl* II and *Xcm*I. The DNA fragments were separated by agarose gel electrophoresis and the 672 bp fragment was isolated and purified.

[0064] Transfection of A72 or CrFK cells with p630att resulted in the generation of infectious virus (630att) which when

administered to pups gave no clinical signs.

[0065] In a comparative study, a vaccine comprising clone 630 and a vaccine comprising clone 630att were compared.

Five MDA negative dogs were vaccinated subcutaneous $10^{8.0}$ - $10^{8.3}$ TCID₅₀ of Clone 630 in 1 ml. This led to mild to moderate signs in all dogs. Weight change over 5 day period was -6% on average in 5 dogs, as follows from the table below.

		day -1	day+3	day +4	day +5	Change
Clone 630	964	7145	6740	6440	6650	-7
	320	7840	7625	7270	7060	-10
	148	6115	5915	5665	5740	-6
	761	5740	5525	5595	5530	-4
	959	5040	4890	5030	4910	-3
	Mean	6376	6139	6000	5978	-6

[0066] Five MDA negative dogs were vaccinated subcutaneous $10^{8.0}$ - $10^{8.3}$ TCID₅₀ of Clone 630att in 1 ml.

[0067] In this group, no clinical symptoms, no temperature rises, no leukopaenia, no diarrhoea or vomiting was seen. Moreover, there was a substantial weight gain in this group, as follows from the table below.

% Weight Gain			
	Days -4 to 0	Days 0 to +7	Days +7 to +14
Group 1 630att	7	13	11

[0068] It was therefore concluded that vaccines on the basis of clone 630att indeed behave attenuated when compared to clone 630, and have an excellent safety profile.

Example 2: generation of a recombinant virus having an attenuating mutation outside the capsid gene

[0069] Strain 154 att was obtained from a commercially available Nobivac Parvo C (Intervet Schering-Plough Animal Health) and strain Jess was a field isolate of a type 2c virus.

[0070] Viruses were grown on adherent canine or feline kidney cells (e.g. A72 & CrFK) using M6B8 medium containing 5% fetal calf serum. Replicative form (RF) DNA was prepared from infected cell cultures using a modification of the standard "Hirt" method (McMaster et al 1981).

[0071] RF DNA prepared from the 154 att strain was digested with the restriction enzyme PstI and the fragments separated by agarose gel electrophoresis. The 3055 base pair (bp) band (corresponding to the left hand end of CPV) was excised from the gel and purified using Qiagen Qiaquick gel extraction columns. RF DNA isolated from CPV Jess infected cells was digested with the restriction enzyme XmnI. Again the DNA fragments were separated by agarose gel electrophoresis followed by purification of an approximately 2750 bp band (corresponding to the right hand end of CPV including the capsid sequence) using Qiagen Qiaquick gel extraction columns.

[0072] The purified 3055 bp and 2750bp fragments from 154att and Jess were combined and transfected into A72 or CrFK cells in culture. Transfections were performed using Lipofectamine 2000 (Invitrogen) with approximately 3µg of each fragment, following the manufactures instructions.

[0073] Following transfection, cells were passaged and monitored by haemagglutination (HA) assay. Virus was detected by HA at pass 4. DNA sequence determination of hybrid viruses was performed using standard DNA sequencing protocols using either RF DNA or PCR fragment templates. Virus was purified by limiting dilution on adherent susceptible canine or feline cells.

Example 3: Recombinant Virus Constructed from Cloned Viral DNA

[0074] Recombinant virus was generated from cloned fragments. The genome of virus strain 154att was cloned into the standard cloning vector pBluescript (Stratagene inc.). In order to maintain the palindromic terminal sequences intact the plasmid was propagated in the bacterial host DL795 which is defective in a number of recombination systems. Cloning of parvovirus genomes has been described in the literature and the techniques required are known to someone skilled in the art.

The obtained clone of 154att (p154att) was digested with the restriction enzyme Pac I such that the digestion was not allowed to go to completion, i.e. the restriction enzyme digest was only partial. The digested fragments were then subjected to digestion with the restriction enzyme Xmn I. The digested DNA fragments were then separated by agarose gel electrophoresis and the fragment indicated in the diagram below was excised from the gel and purified using Qiagen Qiaquick gel extraction columns. The Xmn I and right hand Pac sites flank the capsid region in the parvovirus genome.

The capsid gene of 154 att was replaced by the capsid gene of a virulent strain of CPV as follows. The Xmn I site and the right hand Pac I indicated in figure 8 lie outside the boundaries of the capsid gene. The approximately 110bp sequence between the Pac I site and the end of the capsid gene differs significantly between the 154att strain and virulent isolates. There are as yet no recorded sequence changes in the short sequence (~55 bp) between the Xmn I site and the start of the capsid gene. Therefore in order to limit the exchange of material just to the capsid sequence; the virulent CPV capsid sequence was chemically synthesized and vaccine specific sequence between the PacI site and the capsid stop signal was retained.

[0075] Below, the chemically synthesized sequence is shown containing the CPV capsid gene. The sequence as shown below is provided herein as SEQ ID NO: 3.

AGAGGCGAGACCTGAGAGCCATCTTTACTTCTGAACAATTGGAAGAAGATTTTCGAGA

Xmn I

CGACTTGGATTAAAGTACGATGGCACCTCCGCEAAAGAGAGCCAGGAGAGGTAAGGGTGT
 GTTAGTAAAGTGGGGGAGAGGAAAGATTCAATAACTTAAGTATGTTGTTTTTTAT
 AGGACTTGTGCTCCAGGTTATAATATCTTGGGCTTGGGAACAGTCTTGACCAAGGAGA
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PacI

[0076] The Xmn I & Pac I sites are indicated and underlined. The stop codon (TAA) of the capsid coding region The capsid (Vp1/Vp2) coding sequence is in bold.

[0077] The synthesized fragment was liberated from the plasmid in which it was provided using the enzymes Xmn I and Pac I, it was then ligated to the fragment shown in Figure 9. Competent E. coli (strain DL795) was transformed with the ligation mix using standard protocols and bacteria harboring the recombinant plasmids isolated and identified. The resultant plasmid p 1542c illustrated below (Figure 10) was then prepared from the cloned E. coli.

Hybrid virus was prepared as follows. Plasmid p 15 42c DNA was transfected into A72 or CrFK cells in culture. Transfections were performed using Lipofectamine 2000 (Invitrogen) with approximately 3 microgram of DNA, following the manufacturer's instructions. Following transfection, cells were passaged and monitored by haemagglutination (HA) assay. Virus was detected by HA at pass 4. DNA sequence determination of hybrid viruses was performed using standard DNA sequencing protocols using either RF DNA or PCR fragment templates. Virus was purified by limiting dilution on adherent susceptible canine or feline cells.

Legend to the figures.

[0078]

Figure 1: Construction of pCPV A

Figure 2: Construction of pCPV C

Figure 3: Construction of pCPV AC

Figure 4: Construction of pCPV 154_{att}

Figure 5: Construction of p630

Figure 6: Construction of p630_{att}

Figure 7: schematic representation of the natural recombination (non-GM) method of obtaining a hybrid 2/2c virus isolate. Two overlapping fragments from the type 2 vaccine and type 2c field virus were transfected into cells and virus isolated following homologous recombination.

Figure 8: schematic representation of the infectious plasmid clone of CPV strain 154att showing the restriction enzyme sites Pac I and Xmn I. The shaded boxes illustrate the terminal palindrome sequences

Figure 9: schematic showing the selected product of the partial Pac I / Xmn I digest that was selected for further manipulation

Figure 10: plasmid containing the 154att vaccine virus DNA in which the capsid gene is substituted by a virulent CPV2c capsid sequence.

SEQUENCE LISTING

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REFERENCES CITED IN THE DESCRIPTION

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LEVENDE, SVÆKKET PARVOVIRUS

PATENTKRAV

1. Levende, svækket CPV2-parvovirus, kendetegnet ved, at CPV2 omfatter et capsidgen af CPV2 serotype 2a, 2b eller 2c, der koder for en anden aminosyre end isoleucin ved aminosyreposition 219 af capsidproteinet og/eller en anden aminosyre end glutamin ved aminosyreposition 386 af capsidproteinet, og kendetegnet ved, at et DNA-fragment af en del af ikke-capsidområdet af CPV2 udskiftes af et homologt DNA-fragment af en del af ikke-capsidområdet afledt af et andet parvovirus, hvor det homologe DNA-fragment af det andet parvovirus bærer en svækkende mutation.
2. Levende, svækket CPV2 ifølge krav 1, kendetegnet ved, at CPV2 omfatter et capsidgen, der koder for en anden aminosyre end isoleucin ved aminosyreposition 219 af capsidproteinet, og en anden aminosyre end glutamin ved aminosyreposition 386 af capsidproteinet.
3. Levende, svækket CPV2 ifølge krav 2, kendetegnet ved, at CPV2 omfatter et capsidgen, der koder for et valin ved aminosyreposition 219 af capsidproteinet og/eller et lysin ved aminosyreposition 386 af capsidproteinet.
4. Levende, svækket CPV2-parvovirus ifølge et hvilket som helst af kravene 1-3, kendetegnet ved, at det homologe DNA-fragment af det andet parvovirus bærer en svækkende mutation i det ikke-strukturelle område, i området fra position 2061 til 2070 ifølge SEQ ID NO: 1.
5. Vaccine til beskyttelse af dyr mod infektion med CPV2-parvovirus, kendetegnet ved, at vaccinen omfatter et levende, svækket CPV2-parvovirus ifølge et hvilket som helst af kravene 1-4 og en farmaceutisk acceptabel bærer.
6. Kombinationsvaccine til beskyttelse af dyr mod patogener, kendetegnet ved, at kombinationsvaccinen omfatter en vaccine ifølge krav 5 og et supplerende antigen af et virus eller en mikroorganisme, der er patogen/patogen for dyr, eller genetiske informationer, der koder for et immunogent protein af viruset eller mikroorganismen.
7. Kombinationsvaccine ifølge krav 6, kendetegnet ved, at viruset eller mikroorganismen, der er patogen/patogen for dyr, er udvalgt fra gruppen bestående af *Ehrlichia canis*, *Babesia gibsoni*, *vogeli*, *rossi*, *Leishmania donovani-complex*, hunde-adenovirus, hunde-coronavirus, hunde-distempervirus, *Leptospira interrogans serovar canicola*, *icterohaemorrhagiae*, *pomona*, *grippotyphosa*, *bratislava*, hunde-hepatitisvirus, hunde-parainfluenzavirus, rabiesvirus, *Hepatozoon canis*, *Borrelia burgdorferi*, *Bordetella bronchiseptica*, katte-herpesvirus, katte-calicivirus, katte-panleucopeni og *Chlamydomydia felis*.
8. Fremgangsmåde til fremstilling af en vaccine ifølge krav 5 eller en kombinationsvaccine ifølge krav 6 eller 7, kendetegnet ved, at fremgangsmåden omfatter blanding af et levende, svækket CPV2-parvovirus ifølge et hvilket som helst af kravene 1-4 og en farmaceutisk acceptabel bærer.
9. Levende, svækket CPV2-parvovirus ifølge et hvilket som helst af kravene 1-4 til anvendelse som et medikament.
10. Levende, svækket CPV2-parvovirus ifølge et hvilket som helst af kravene 1-4 til anvendelse i behandlingen af en parvovirusinfektion.

- 2 -

11. Fremgangsmåde til fremstilling af en CPV2-parvovirusmutant ifølge et hvilket som helst af kravene 1-4, kendetegnet ved, at fremgangsmåden omfatter trinnet med ændring i et gen, der koder for et capsidprotein af CPV2 serotype 2a, 2b eller 2c, af et hunde-parvovirus, der allerede er svækket i en anden del end den del, der koder for capsidet, ved rekombinante DNA-teknikker, et codon ved aminosyreposition
- 5 219, der koder for isoleucin og/eller et codon ved aminosyreposition 386, der koder for glutamin, ind i et codon ved aminosyreposition 219, der koder for en anden aminosyre end isoleucin og/eller et codon, der koder for en anden aminosyre end glutamin ved aminosyreposition 386.
12. Fremgangsmåde til fremstilling af en CPV2-parvovirusmutant ifølge et hvilket som helst af kravene 1-4, kendetegnet ved, at fremgangsmåden omfatter udskiftning i et gen, der koder for et capsidprotein af CPV2 serotype 2a, 2b eller 2c, af et hunde-parvovirus, der allerede er svækket i en anden
- 10 del end den del, der koder for capsidet, et DNA-fragment, der koder for mindst en del af parvoviruscapsidprotein, der ved aminosyreposition 219 har et codon, der koder for isoleucin og/eller ved aminosyreposition 386 har et codon, der koder for glutamin, med et DNA-fragment, der koder for den samme del af parvoviruscapsidproteinet, der nu ved aminosyreposition 219 har et codon, der koder for en
- 15 anden aminosyre end isoleucin og/eller ved aminosyreposition 386 har et codon, der koder for en anden aminosyre end glutamin.

DRAWINGS

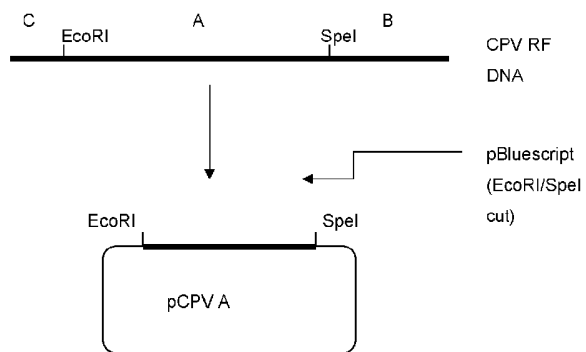


Figure 1

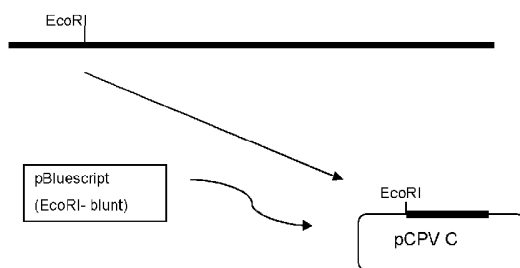


Figure 2

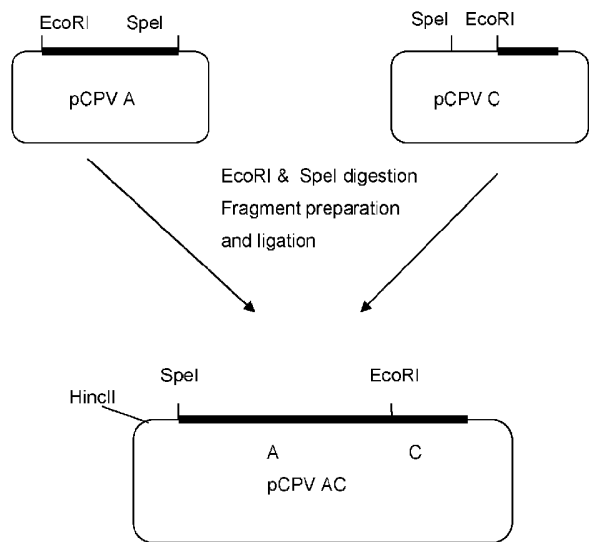


Figure 3

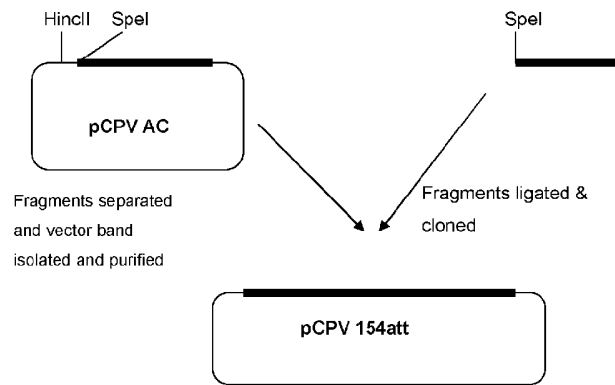


Figure 4

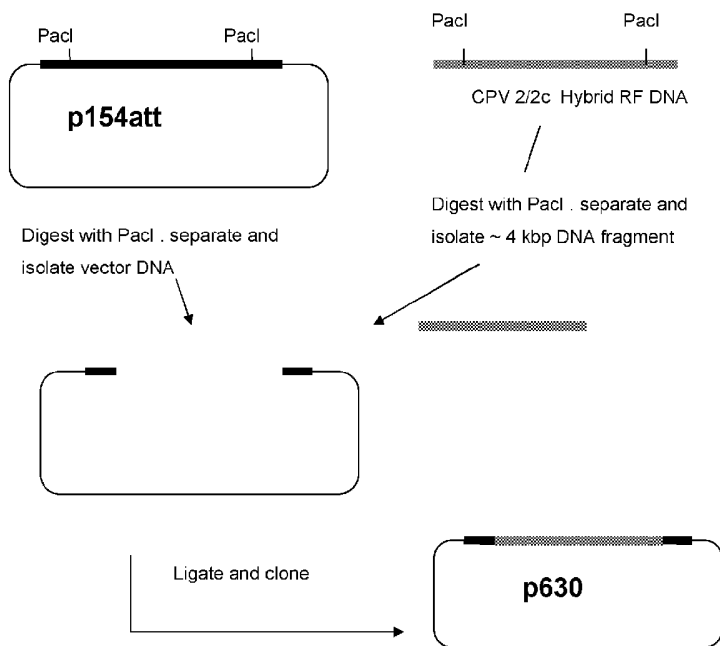


Figure 5

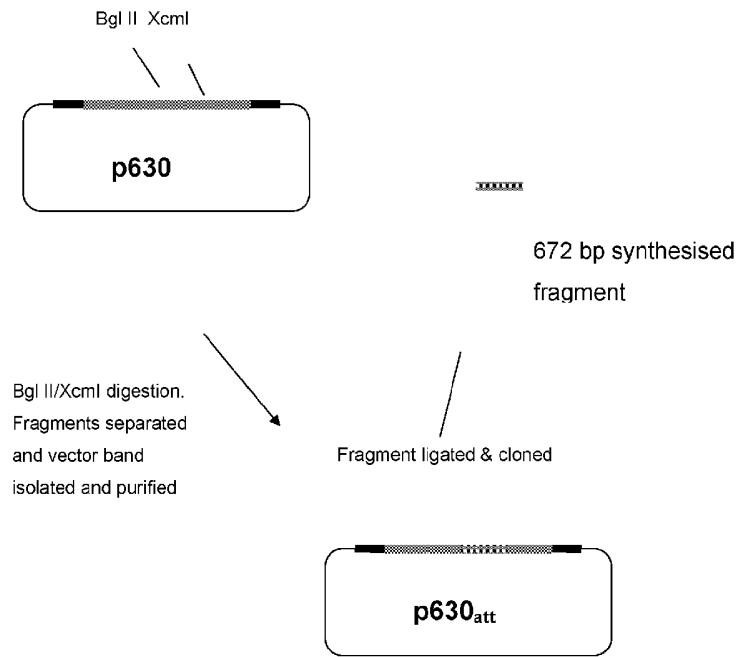


Figure 6

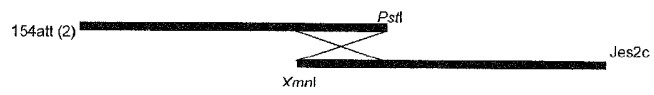


Figure 7

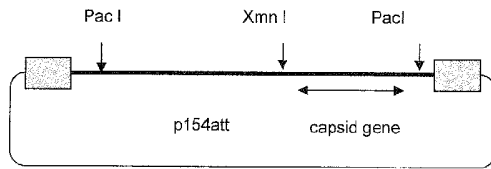


Figure 8

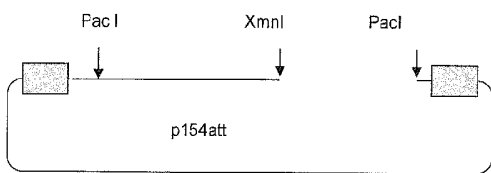


Figure 9

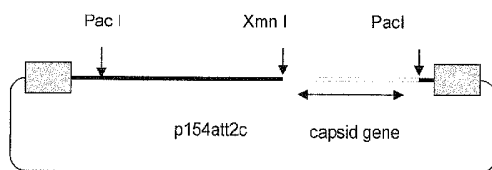


Figure 10