Partial DNA cloning and sequencing of a canine parvovirus vaccine strain: application of nucleic acid hybridization to the diagnosis of canine parvovirus disease

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Summary. The cloning and sequencing of an *Eco* RI-*Pst* I fragment derived from the replicative form of a canine parvovirus (CPV) vaccine strain are reported. The variability of the 5′ end of NS 1 protein gene in the genome is confirmed by comparison with previously determined DNA sequences. A 15 nucleotide deletion was also observed in this vaccine strain. In order to improve CPV diagnosis, radioactively labelled RNA or DNA and biotin labelled DNA obtained by random priming of the recombinant plasmid were used as probes mainly on gut or stool samples from naturally infected dogs. Results of filter hybridization correlated well with histopathological diagnosis of parvovirus infection and with hemagglutination tests performed on dog faeces. We propose that nucleic acid hybridization may be an alternative diagnostic method to ascertain the presence of CPV, especially in frozen samples.

Introduction

Canine parvovirus (CPV) like other parvoviruses contains a linear 5 kilobase (kb) single stranded DNA (ss DNA). The viral genome encodes two nonoverlapping transcription units [30]. When replication occurs, DNA is converted into double stranded replicative forms (RF) [37]. Restriction sites have been mapped [22] on the genome and the nucleotide sequence has been determined [30, 32].

Canine parvovirus, in spite of vaccination, remains an important cause of disease and is often implicated in fatal disease in young puppies. CPV diagnosis is best achieved with hemagglutination test on faeces or by the detection of histological changes in gut mucosa [7, 24, 28]. Alternatively, virus can be isolated in cell culture from various organs, but this method is reported to be

much less sensitive because of the high lytic properties of the intestinal content. So, even if tests are available CPV diagnosis may be difficult in some cases.

A sensitive CPV diagnosis test based on viral nucleic acid hybridization has been developed. The Eco RI-Pst I restriction fragment of the CPV replicative form DNA has been cloned into the multiple cloning site of the pT7T3 18 U plasmid. Viral nucleic acid hybridization was realized using radioactively labelled DNA or RNA probes and also a biotin labelled DNA probe. The biotin labelled probe was found to be 10 fold less sensitive. The cloned DNA was sequenced and compared to the previously described CPV sequences. Short deletion and point mutations were observed, which emphasised the high variability of C terminal region of the non structural (NS 1) protein gene.

Materials and methods

Cells and virus strain

Cells used for virus propagation were freshly seeded Crandell feline kidney cells maintained with Eagles's minimum essential medium supplemented with 10% foetal bovine serum. The CPV strain used in the present study was derived from the Carmichael strain (CPV-b) partially sequenced at passage 88 [8, 32]. It was obtained from a commercial vaccine at passage 108 and six additional passages in cell culture were performed before DNA cloning. Passage 108 and passage 114 were further designated as CPV-b 108 and CPV-b 114. Feline panleukopenia virus (FPLV), porcine parvovirus, mink aleutian disease virus (Gorham strain) and Derzsy goose virus (kindly supplied by V. Marius, Laboratoire Central de Recherches Avicole et Porcine, Ploufragan) were used to test the probe specificity.

Clinical specimens

Organs and faeces were collected from diseased puppies with parvovirus-related symptoms. Some of them were kindly supplied by A. Moraillon, Veterinary School, Maisons-Alfort. Organs from 3 kittens with panleukopenia-like symptoms were also collected and included in this study. Histological analysis or hemagglutination test on faeces were performed on an aliquot of each sample. Samples were then stored at $-20\,°C$ before being processed.

DNA isolation

The replicative form of CPV-b 114 DNA was extracted by a modified Hirt procedure as described by McMaster et al. [14, 21]. Viral single stranded DNA was either prepared from purified viral particles [26] or directly from infected cell supernatants. Virus was then treated with 0.2% SDS and proteinase K (50 µg/ml) for 2 h at 37 °C, followed by a phenol chloroform extraction and ethanol precipitation. Viral DNA was extracted from organs and faeces of diseased animals as described by Orth [25]. Gut, spleen and faeces were minced and left in lysis buffer (10 mM Tris pH 8, 100 mM NaCl, 50 mM EDTA, SDS 0.5%) for 1 h at room temperature. Proteinase K was added (100 µg/ml) and samples were incubated for 2 h at 37 °C. After clarification, the NaCl concentration of the supernatant was adjusted to 1 M and mixtures were kept on ice overnight. The supernatant was centrifuged for 1 h at 10,000 rpm and treated with an equal volume of phenol for 1 h at room temperature. It was then treated by chloroform and precipitated by ethanol. The DNA pellet was suspended in TE with RNase (20 µg/ml) for 30 min at 37 °C, and 50 µg of this treated DNA were spotted onto a nitrocellulose filter after denaturation by sodium hydroxide.

DNA cloning

DNA was digested with Eco RI and Pst I (Boehringer) and ligated into the pT7T3 18 U plasmid (Pharmacia). *Escherichia coli* NM 522 was transformed with the recombined plasmid as described by Hanahan [13]. Recombinants were identified by in situ hybridization of white colony replicas with a radioactive labelled viral probe obtained from purified virions. The hybridization procedure was performed as described by Maniatis et al. [19]. Recombinant plasmid DNA was also further analyzed by agar gel electrophoresis after digestion with Eco RI and Pst I or with Hind III.

DNA sequencing

M 13 dideoxynucleotide sequencing was carried out as already described [5]. For direct sequencing of denatured plasmid DNA, we used synthetic primers (Biosearch 8600 apparatus) [17] with the Sequenase Kit (USB). Sequence data were analyzed by using the Microgenie (Beckman, 1988) and PC Gene (Intellegenetics, 1990) computer programs.

DNA amplification by polymerase chain reaction (PCR)

CPV-b 108 viral DNA was submitted to 40 amplification cycles in a programmable heating block (MS Research) under standard conditions [35]. Briefly, reaction mixtures consist of template DNA, a mixture of four $dNTP_S$ (final concentration: 0.2 mM each), 100 pmoles of each primer and amplification buffer (50 mM KCl, 10 mM Tris pH 8.3, 1.5 mM $MgCl_2$) in a final volume of 100 µl. 1.25 IU of *Taq* polymerase (Boehringer) was added after the first denaturation step. The temperature and time regime used were as follows: 1 min at 94 °C, 2 min at 48 °C, 2 min at 72 °C.

Probe labelling and hybridization

DNA probe

Whole recombinant plasmid and viral single stranded DNA were labelled in vitro with [^{32}P]dCTP (110 mBq/mmol; Amersham) or with [^{14}C]dATP biotin (BRL) using random oligonucleotides primers (Pharmacia) and Klenow polymerase (Boehringer) as described by Feinberg and Vogelstein [11].

RNA probe

Recombinant plasmid was linearized downstream of the insert with Pst I. After phenol extraction, the DNA was ethanol precipitated and resuspended in water. RNA was synthetized and labelled by incubating 1 µg of linearized plasmid DNA for 30 min at 37 °C in a mixture containing 25 IU ribonuclease inhibitor (Pharmacia), 20 units T 7 RNA polymerase (Pharmacia), reagent buffer (Transprobe-TKit; Pharmacia) with 5 µl [α ^{32}P]UTP (> 15 TB q/mmol; Amersham).

Hybridization

Hybridization were carried out overnight at 42 °C in 50% formamide, 5 × SSC (1 × SSC: 150 mM sodium chloride, 15 mM sodium citrate), 1 × Denhardt's solution (Denhardt's solution: 1% polyvinyl pyrrolidone, 1% Ficoll, 1% bovine serum albumin), 0.5% SDS and 10 µg/ml of sonicated calf thymus DNA (Sigma). 10% dextran sulfate was also added for biotinylated probe. Filters were washed twice in 0.1% SSC, 0.1% SDS for 1 h at 42 °C. Radioactive filters were exposed to X-ray film (Kodak XAR) at −70 °C for 12 h. Biotin labelled probe was detected with streptavidin-alkaline phosphatase conjugate (BRL). After

incubation with a luminescent substrate, PPD (4 methoxy 4-3-phosphate phenyl spiro 1-2-dioxetane 3.2' adamantan), as recommended by the supplier (Photogene-BRL), light emission was detected by autoradiography for 5 min on X-ray film.

Results

CPV-DNA cloning and sequencing

Eight recombinant clones containing CPV sequences were selected by colony hybridization. Six clones contained an insert of the 2 kb; as expected the other two contained 1.5 kb and 1.3 kb inserts. All clones hybridized with purified viral

Table 1. Nucleotide and amino acid differences between one feline and four canine parvovirus strains

Section of genome	Nucleotide position on FPLV-193 genome[a]	FPLV-193 (Martyn)	CPV-N (Reed)	CPV (Parrish-1990)	CPV-b (Carmichael) passage 88	CPV-b 114
Eco RI NS$_1$	1092/1097					
	1124	A[K]	A[K]	A[K]	nd	G[K]
	1490	T[I]	C[I]	C[I]	nd	T[I]
	1593	A[I]	A[I]	A[I]	nd	G[V]
	1724	T[T]	T[T]	T[T]	G[T]	T^b[T]
	1730	A[E]	A[E]	A[E]	A[E]	G^c[E]
	1745	A[V]	A[V]	A[V]	G[V]	A^c[V]
Hind III	1821/1826					
	1875	G[A]	G[A]	G[A]	G[A]	T^c[S]
	1899	C[C]	G[G]	G[G]	G[G]	G[G]
	1944	G[E]	G[E]	G[E]	G[E]	C^c[R]
	2036	T[P]	T[P]	T[P]	T[P]	A^c[P]
	2037/2048	CAG[Q]	CAG[Q]	CAG[Q]	CAG[Q]	del
		AGT[S]	AGT[S]	AGT[S]	AGT[S]	del
		CAA[Q]	CAA[Q]	CAA[Q]	CAA[Q]	del
		GAC[D]	GAC[D]	GAC[D]	GAC[D]	del
	2049/2051	CAC[H]	CAA[Q]	CAA[Q]	CAA[Q]	del
	2159	C[G]	C[G]	C[G]	T[G]	C^b[G]
	2174	C[N]	C[N]	C[N]	G[E]	C^b[N]
	2192	A[T]	G^d[T]	A[T]	A[T]	A[T]
	2225	T[I]	C[I]	C[I]	C[I]	C[I]
	2247/48	GA[E]	GA[E]	GA[E]	AG[R]	GA^b[E]
	2250	G[D]	G[D]	G[D]	A[N]	G^b[D]
	2260/61	AC[D]	AC[D]	AC[D]	CA[A]	CA[A]
VP$_1$ small intron	2375/2376	TC[nc]	TT	TT	TT	C^{c}T
VP$_1$	2423	C[N]	C[N]	C[N]	G[K]	C^b[N]
Hind III	2482/2487					
	2621	A[K]	G[K]	G[K]	G[K]	A^c[K]

Table 1 (continued)

Section of genome	Nucleotide position on FPLV-193 genome[a]	FPLV-193 (Martyn)	CPV-N (Reed)	CPV (Parrish-1990)	CPV-b (Carmichael) passage 88	CPV-b 114
VP$_2$	2910	A[T]	A[T]	A[T]	A[T]	G[c][A]
	3014	T[N]	T[N]	T[N]	G[K]	T[b][N]
	3015	T[Y]	T[Y]	T[Y]	G[D]	T[b][Y]
	3019	A[K]	G[R]	G[R]	G[R]	G[R]
	3026	A[V]	G[V]	G[V]	G[V]	G[V]
Pst I	3049/3054					

It is noteworthy that sequence analysis of *Hind* III fragment allowed differentiation between the four CPV strains

[a] See [20]
[b] Reversions
[c] Consistent differences between CPV-b 114 and the other CPV strains
[d] Unique difference between CPV-N and CPV Parrish
del Deletion
nc Non coding
nd Not done
In brackets, amino acids

probe after restriction enzyme digestion and Southern transfer. As predicted from previously sequenced CPV [30], digestion of the six 2 kb recombinant plasmids with Hind III yielded the predicted 660 bp fragment (Fig. 1). The sequence of both strands of cloned DNA was determined for three different plasmids after subcloning into the M 13 phage. The resulting 1947 nucleotide sequence represented about 37% of the CPV genome. As expected, it included parts of two major open reading frames (ORFs) which are in the same phase corresponding to the 3' end of NSI gene and the first 785 nucleotides of the VP 1/VP 2 gene (Fig. 1) [30].

Comparison of this sequence with that of three other CPV strains [27, 30, 32] and one FPLV strain [20] revealed point mutations between the different isolates (Table 1). Furthermore, a 15 nucleotides deletion, located in the NS 1 gene, and extending from nucleotide 2040 to nucleotide 2056 of the Norden CPVs strain (CPV-N) sequence [30] was observed in the cloned DNA. To elucidate whether the deletion observed was originally in the vaccine or was generated by cell culture passages in our laboratory, DNA sequence was determined on CPV-b 108 after PCR amplification of a 1000 bp fragment including the deleted region and the two *Hind* III restriction sites (Fig. 1). PCR amplification products were then cleaved with Hind III, cloned in pT7T 318U and sequenced. The presence of the deletion in the original vaccine was thus confirmed. Consequently, the modified NS 1 gene did not alter viral replication in cell culture as CPV-b 108 could be multiplied without loss of infectivity.
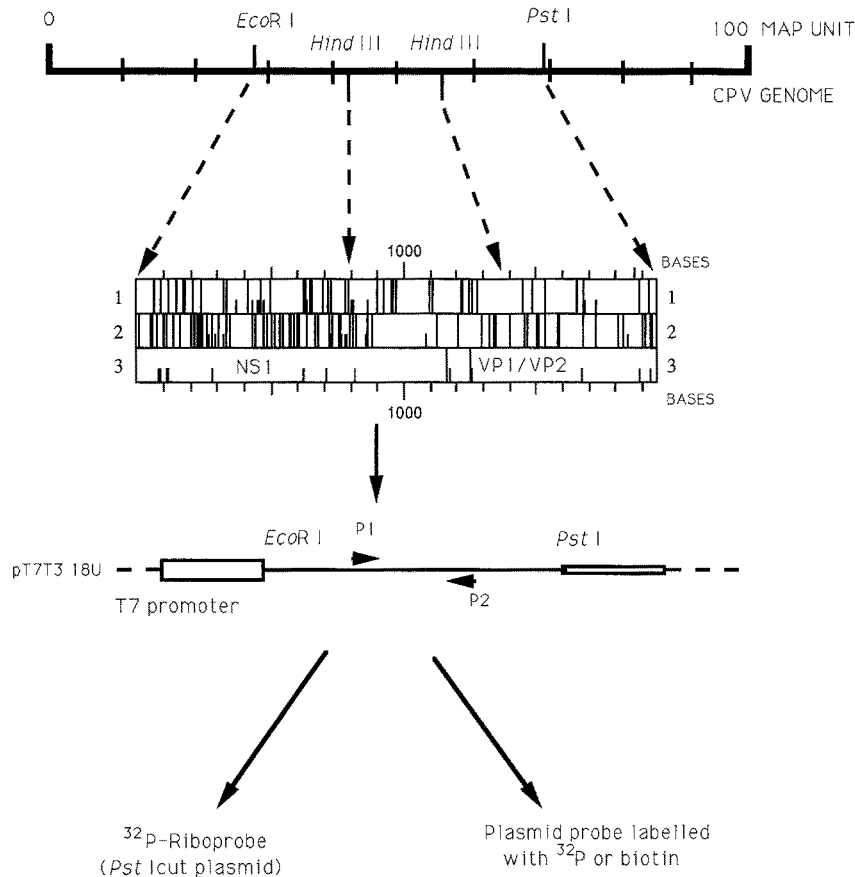
Fig. 1. Location and translation of *Eco* RI-*Pst* I insert of CPV-b genome. Strategy of cloning and preparation of labelled probes. The 5 kb long viral CPV genome is shown in 3' to 5' orientation and is divided into 100 map units. The block diagram is deduced from our sequence data and extends between the conserved *Eco* RI and *Pst* I sites of CPV. The three ORF in the complementary strand are shown with initiation codons (short bars) and termination codons (long bars). ^{32}P riboprobes were obtained by using T_7 polymerase in the Pst I cleaved recombinant plasmid. Probes were alternatively prepared by random priming with whole recombinant plasmid. Primers for PCR: P_1 ATGTAAGCTTCCAG-
GAGACTTTGG, P_2 GCCTCCAGACCCGTTCCCAGATCC

Evaluation of the specificity and sensitivity of ^{32}P labelled DNA and RNA probes

Parvoviruses from various animal species were spotted onto nitrocellulose and assayed either with whole ^{32}P labelled recombinant plasmid (Fig. 2) or with a ^{32}P labelled RNA probe synthetized from the T_7 promoter of the plasmid opened at the *Pst* I site. 0.3 ng of DNA from purified canine parvovirus or $10^{4.8}$ CCID$_{50}$/ ml could be detected. $10^{6.8}$ CCID$_{50}$ of FPLV and $10^{2.6}$ HA unit of porcine parvovirus were also detected. The same sensitivity and specificity were observed with the RNA probe (data not shown).
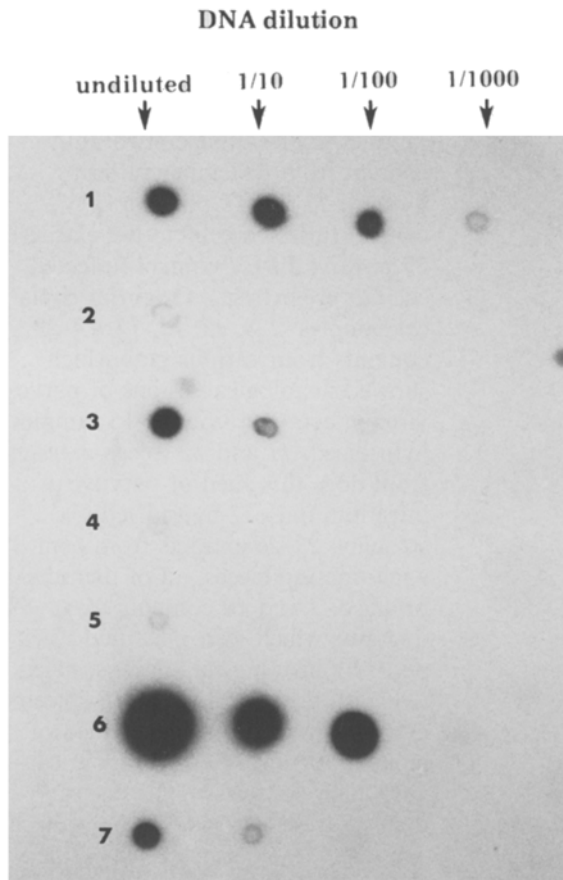
DNA dilution

Fig. 2. Parvovirus DNA from different animal species, diluted from 1 to 1/1000 were spotted onto nitrocellulose sheet and probed with whole ^{32}P-labelled recombinant plasmid. *1* Canine parvovirus ($10^{6.8}$ CCID$_{50}$, cell culture infective dose); *2* non infected cell culture; *3* feline panleukopenia ($10^{7.8}$ CCID$_{50}$); *4* Derszyparvovirus of goose ($10^{5.5}$ CCID$_{50}$); *5* Aleutian mink disease parvovirus (10^{5} CCID$_{50}$); *6* purified DNA of canine parvovirus (30 ng); *7* porcine parvovirus ($10^{4.6}$ HA, hemagglutination unit)

Application of ^{32}P labelled DNA or RNA probe for routine diagnosis

DNA from various organs of dogs and cats was extracted, spotted onto nitrocellulose and assayed with probes. Samples came from animals which died of parvovirus infection as could be deduced from histological examinations, expected for three of them which were classified as "suspect"and one gut sample taken from a puppy suffering from distemper which was chosen as a negative control. Out of 15 gut samples from diseased dogs, 13 were positive with DNA or RNA probes. Two spleen extracts were tested: only one hybridized. Four faecal extracts which showed specific hemagglutinating properties hybridized strongly and the fifth one from a suspect dog was negative. Two samples for panleukopenia diagnosis, one from the gut and the other, a mixture of spleen and kidney, did not hybridize. Results presented with the DNA probe in Fig. 3 were identical with the RNA probe (data not shown).

Biotinylated probe

Twofold dilutions of purified CPV-DNA were spotted in duplicate and assayed respectively with ^{32}P labelled DNA probe and biotin labelled probe in order to determine the sensitivity of the non-radioactive probe. Results are shown in
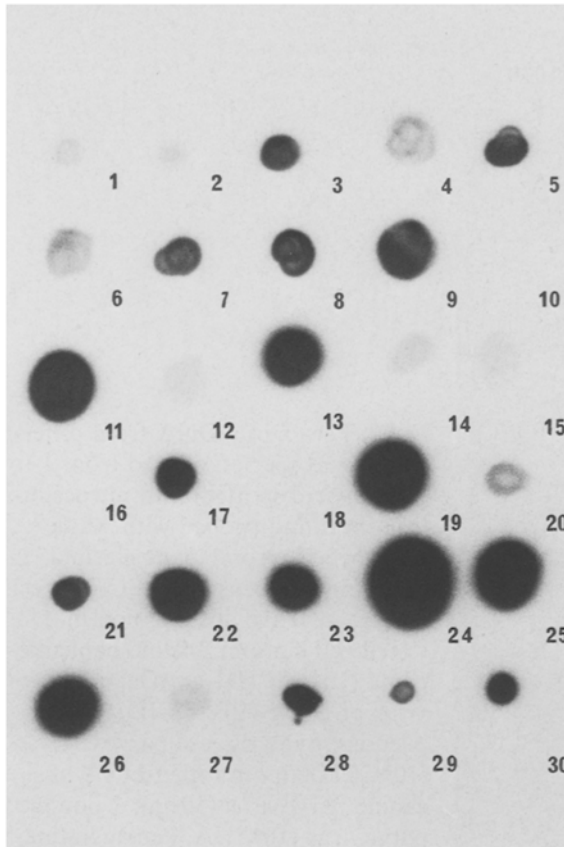
Fig. 3. DNA extracted from clinical samples were spotted onto nitrocellulose and probed with ^{32}P-labelled recombinant plasmid. *1–26* Clinical samples, *27* negative control (gut content from distemper suffering puppy), *28* and *30* positive CPV control (infected cell culture extract), *29* positive FPLV control (infected cell culture extract). Origin of clinical samples: *2–8, 13, 15, 17, 19–22* contents from various guts which showed histological lesions of parvovirus infection: 13 out of 15 samples hybridized; *11* and *12* spleen extracts from dogs that died of parvovirus infection; only *11* hybridized strongly; *23–26* extracts from hemagglutinating faeces, all of them hybridized; *1* and *10* contents from dog guts which were classified "suspect" by histological analysis, negative with probe; *16* gut or *18* spleen extracts from two cats that died of panleukopenia; *14* extract from cat gut which was classified "suspect"; none of these cat samples hybridized
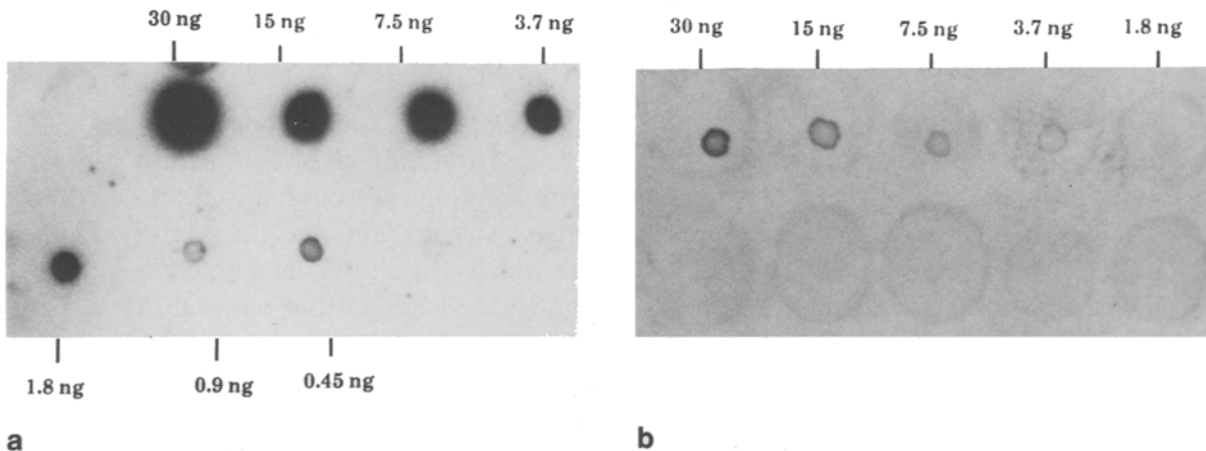

Fig. 4. Comparative sensitivity of radioactive and biotinylated probes on different dilutions of purified CPV DNA spotted onto nitrocellulose. **a** ^{32}P labelled plasmid probe; **b** biotinylated plasmid probe

Fig. 4. Non-radioactive probes allowed the detection of 3.7 ng of purified CPV-DNA. This non-radioactive hybridization test was then performed on the same clinical samples as previously described: the faeces samples were strongly positive
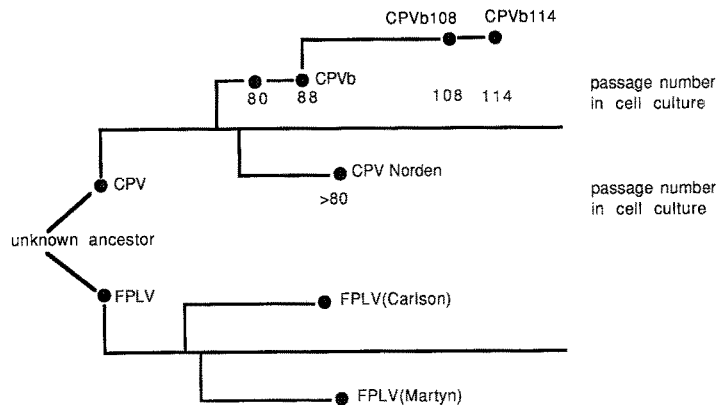
Fig. 5. Origin and passage history of feline (FPLV) and canine (CPV) parvovirus

and 10 out of 15 extracts from the gut of diseased dogs gave a positive signal (data not shown).

Discussion

The vaccine strain used in this study was derived from the Carmichael strain (Fig. 5). It underwent 26 additional passages in cell culture (CPV-b 114) before being cloned and sequenced. Eco RI and Pst I sites were chosen because they are perfectly conserved among different isolates of CPV and FPLV [20].

Moreover, the Eco RI-Pst I fragment covered partially non-structural protein genes which present a strong homology between some members of the family Parvoviridae, allowing its use for diagnosis of related diseases [37].

Comparison with the previously published sequence of CPV-b strain [32] showed a high rate of point mutations and one deletion which could have been generated by cell culture passages.

This result emphasises the high variability of the CPV genome and sequence comparison was further investigated on three other CPV strains [27, 30, 32] and one FPLV strain [20]. 22 point mutations were observed between the different CPV sequences and 15 between CPV-b 108 and FPLV (Table 1).

10 reversions appeared in CPV-b 108 strain; the 10 nucleotidic changes were only observed in CPV-b at passage 88, compared to FPLV and no longer existed in the partially sequenced DNA of CPV-b 108. By contrast, no changes were observed in regulatory regions (polyadenylation sites, P_{38} and P_{45} promoters, potential splicing sites).

Most of the coding mutations appeared in the C terminal part of NS 1 (Table 1) and this is in contrast with the results published for Minute virus of mice [2]. The short deletion observed in CPV-b 108 and CPV-b 114 was also located in this part of the NS 1 gene. Short deletions often appeared in the non-coding part of the genome but were never described in the parvovirus NS 1 gene. This deletion, located 45 bases downstream of the P 38 promoter did not

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FPLV NS1                     PEDWMMLQPDSYIEMMAQPGGENLLKNTLEICTLTLARTKTAFELILEKA    349
CPV Parrish                  PEDWMMLQPDSYIEMMAQPGGENLLKNTLEICTLTLARTKTAFELILEKA    349
Murine minute virus (Astell) PEDWMMMQPDSYIEMMAQPGGENLLKNTLEICTLTLARTKTAFDLILEKA    348
Murine minute virus (Sahli)  PEDWMMMQPDSYIEMMAQPGGENLLKNTLEICTLTLARTKTAFDLILEKA    397
H. parvovirus                PEDWMMMQPDSYIEMMAQPGGENLLKNTLEICTLTLARTKTAFDLILEKA    348
Porcine parvovirus           IEDWMMTDPDSYIEMMAQTGGENLIKNTLEITTLTLARTKTAYDLILEKA    347
CPV-b 114                    PEDWMMLQPDSYIEMMAQPGGENLLKNTLEICTLTLARTKTAFELILEKA     73
B19 parvovirus               EDKWKLVDFNQYTLLSSSHSGSFQIQSALKLAIYKATNLVPTSTFLLHTD    280
Bovine parvovirus            EGMPGGSKTAE-QLLTMIH-----IKLCAKYNAYEFMLMKTPATQNMNPG    263
                                .     .  .   .           ..    .        ..   . .. .

FPLV NS1                     DNTKLTNFDLANSRTCQIFRMHGWNWIKVCHAIACVLNRQGGKRNTVLFH    399
CPV Parrish                  DNTKLTNFDLANSRTCQIFRMHGWNWIKVCHAIACVLNRQGGKRNTVLFH    399
Murine minute virus (Astell) ETSKLTNFSLPDTRTCRIFAFHGWNYVKVCHAICCVLNRQGGKRNTVLFH    398
Murine minute virus (Sahli)  ETSKLTNFSLPDTRTCKIFAFHGWNYVKVCHAICCVLNRQGGKRNTVLFH    447
H1 parvovirus                ETSKLANFSMASTRTCRIFAEHGWNYIKVCHAICCVLNRQGGKRNTVLFH    398
Porcine parvovirus           KPSMLPTFNISNTRTCKIFSMHNWNYIKCCHAITCVLNRQGGKRNTILFH    397
CPV-b 114                    DNTKLTNFDLANSRTCQIFRMHGWNWIKVCHAIACVLNRQGGKRNTVLFH    123
B19 parvovirus               FEQVM---CIKDNKIVKLLLCQNYDPLLVGQHVLKWIDKKCGKKNTLWFY    327
Bovine parvovirus            APHYD---C-QGNLVFKLLNLQGYNPWQVGHWLVMMLSKKTGKRNSTLFY    309
                               .. .  ...   ....     .   ....  **.*.   *.

FPLV NS1                     GPASTGKSIIAQAIAQAVGNVGCYNAANVNFPFNDCTNKNLIWVEEAGNF    449
CPV Parrish                  GPASTGKSIIAQAIAQAVGNVGCYNAANVNFPFNDCTNKNLIWIEEAGNF    449
Murine minute virus (Astell) GPASTGKSIIAQAIAQAVGNVGCYNAANVNFPFNDCTNKNLIWVEEAGNF    448
Murine minute virus (Sahli)  GPASTGKSIIAQAIAQAVGNVGCYNAANVNFPFNDCTNKNLIWVEEAGNF    497
H1 parvovirus                GPASTGKSIIAQAIAQAVGNVGCYNAANVNFPFNDCTNKNLIWVEEAGNF    448
Porcine parvovirus           GPASTGKSIIAQHIANLVGNVGCYNAANVNFPFNDCTNKNLIWIEEAGNF    447
CPV-b 114                    GPASTGKSIIAQAIAQAVGNVGCYNAANVNFPFNDCTNKNLIWVEEAGNF    173
B19 parvovirus               GPPSTGKTNLAMAIAKSVPVYGMVNWNNENFPFNDVAGKSLVVWDEGIIK    377
Bovine parvovirus            GPASTGKTNLAKAICHAVGLYGCVNHHNNKQFPFNDAPNKMILWWEECIMT    359
                             **.****. .* * . *    * * .* .*****..* .. .*

FPLV NS1                     GQQVNQFKAICSGQTIRIDQKGKGSKQIEPTPVIMTTNENITIVRIGCEE    499
CPV Parrish                  GQQVNQFKAICSGQTIRIDQKGKGSKQIEPTPVIMTTNENITIVRIGCEE    499
Murine minute virus (Astell) GQQVNQFKAICSGQTIRIDQKGKGSKQIEPTPVIMTTNENITVVRIGCEE    498
Murine minute virus (Sahli)  GQQVNQFKAICSGQTIRIDQKGKGSKQIEPTPVIMTTNENITVVRIGCEE    547
H1 parvovirus                GQQVNQFKAICSGQTIRIDQKGKGSKQIEPTPVIMTTNENITVVRIGCEE    498
Porcine parvovirus           SNQVNQFKAICSGQTIRIDQKGKGSKQIEPTPVIMTTNEDITKVRIGCEE    497
CPV-b 114                    GQQVNQFKAICSGQTIRIDQKGKGSKQIEPTPVIMTTNENITIVRIGCEE    223
B19 parvovirus               STIVEAAKAILGGQPTRVDQKMRGSVAVPGVPVVITSNGDITFVVSGNTT    427
Bovine parvovirus            TDYVEAAKCVLGGTHVRVDVKHKDSRELPQIPVLLSSNHDVYTVVGGNAT    409
                             . *.. * . .*  .*.* * ..* ..  .**....*  ..  *  * ..

FPLV NS1                     RPEHTQPIRDRMLNIKLVCKLPGDFGLVDK---EEWPLICA---------    537
CPV Parrish                  RPEHTQPIRDRMLNIKLVCKLPGDFGLVDK---EEWPLICA---------    537
Murine minute virus (Astell) RPEHTQPIRDRMLNIHLTHTLPGDFGLVDK---NEWPMICA---------    536
Murine minute virus (Sahli)  RPEHTQPIRDRMLNIHLTHTLPGDFGLVDK---NEWPMICA---------    585
H. parvovirus                RPEHTQPIRDRMLNIHLTRTLPGDFGLVDK---HEWPLICA---------    536
Porcine parvovirus           RPEHTQPIRDRMLNINLTRKLPGDFGLLEE---TEWPLICA---------    535
CPV-b 114                    RPEHTQPIRDRMLNIKLVCKLPGDFGLVDK---EEWPLICS---------    261
B19 parvovirus               TTVHAKALKERMVKLNFTVRCSPDMGLLTEADVQQWLTWCNAQSWDHY--    475
Bovine parvovirus            FGVHAAPLKERITQMNFMKQLPNTFGEITPGMISNWLSHCAHIHQEHLSL    459
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Fig. 6. Highly conserved aminoacid sequences of different parvoviruses NS 1 peptide. Multiple sequence alignment (Clustal-PC gene) of different NS 1 peptides is performed by comparison of amino acid sequence of: FPLV NS 1 [6], CPV Parrish [27], murine minute virus [3], murine minute virus [33], H 1 parvovirus [31], porcine parvovirus [29], CPV-b 114, B 19 parvovirus [39], bovine parvovirus [9]. Amino acid number is indicated, highly conserved sequence are boxed, asterisks indicate exact homology, dots indicate conservative change

alter the reading frame and defined a genetic marker for this vaccine strain. The N terminal part of the NS 1 gene appeared more conserved in the analyzed sequences. Furthermore, the alignment of NS 1 protein sequences from different parvovirus allowed identification of a highly conserved sequence already de-

scribed as a homologous domain of proteins which used purines nucleotides (Fig. 6) [1].

The 2 kb insert has been used as probe on CPV and other parvoviruses available in the laboratory for cross-hybridization in order to measure to what extent the probe could be useful for other parvoviruses. Results were as expected [23, 29]. CPV and FPLV are closely related but the sensitivity of the probe for FPLV is one hundred fold lower as measured by the difference in titer of the infectious particles detected.

The sensitivity of the DNA CPV probe on homologous DNA is comparable to that reported for other viruses: herpesvirus [18], adenovirus [12] and, rotavirus [10]. Probes for viruses in the family Parvoviridae have only been reported for the human B 19 parvovirus [4, 34] with higher sensitivity in terms of quantity of target DNA detected but the number of infectious particles detected is of the same order. In fact, there is a discrepancy between the number of infectious particles detected ($10^{4.8}$ CCID$_{50}$) and the quantity of purified DNA spotted and detected: 0.3 ng DNA correspond to 10^8 viral particles. This may be explained by the production of defective DNA genomes and/or a greater number of RF copies in the supernatants of infected cell culture which cannot be measured in terms of infectivity. RNA probes have been developped to overcome any background due to hybridization of the plasmid with bacteria present in stools and gut contents but we did not encounter such difficulty. Although RNA probes have been shown to offer up to tenfold more sensitivity than DNA probes, at least on RNA viruses such as enterovirus [15] and on DNA viruses such as B 19 parvovirus [34], the results obtained in our study did not demonstrate any difference in the sensitivity and specificity of the two types of probes.

Data presented on clinical samples demonstrated that CPV nucleic acid probes were effective for diagnosis of parvovirus disease even on specimens which had been stored under unappropriate conditions. We plan to simplify DNA extraction and to use this probe in combination with PCR to improve the sensitivity of the test.

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