Canine distemper virus from diseased large felids: biological properties and phylogenetic relationships

T. C. Harder,[1] M. Kenter,[1] H. Vos,[2] K. Siebelink,[1] W. Huisman,[1] G. van Amerongen,[3] C. Örvell,[4] T. Barrett,[5] M. J. G. Appel[6] and A. D. M. E. Osterhaus[1]*

[1] *Department of Virology, Erasmus University, PO Box 1738, 3000 DR Rotterdam, The Netherlands,* [2] *Seal Rehabilitation Centre, Hoofdstraat 94A, 9968 AG Pieterburen, The Netherlands,* [3] *National Institute for Public Health and Environmental Protection, Antonie van Leeuwenhoeklaan 9, 3720 BA Bilthoven, The Netherlands,* [4] *Laboratory of Clinical Virology, Huddinge Hospital, Huddinge, Sweden,* [5] *Institute for Animal Health, Pirbright Laboratories, Ash Road, Woking, Surrey GU24 0NF, UK and* [6] *J. A. Baker Institute of Animal Health, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853, USA*

Specific pathogen free (SPF) domestic cats were inoculated with tissue homogenate obtained from a Chinese leopard (*Panthera pardus japonensis*) that had died in a North American zoo from a natural infection with canine distemper virus (CDV). The cats developed a transient cell-associated CDV viraemia along with pronounced lymphopenia but did not show any clinical symptoms. Plasma neutralizing-antibody titres against the homologous CDV (A92-27/4, isolated from the Chinese leopard) were consistently higher than against the CDV vaccine strain 'Bussell'. The Chinese leopard CDV isolate showed *in vitro* biological properties reminiscent of virulent, wild-type CDV strains. Sequence analysis of the H gene of two large felid CDV isolates from the USA (A92-27/4 and A92-6) revealed up to 10% amino acid changes including up to four additional potential *N*-linked glycosylation sites in the extracytoplasmic domain as compared to CDV vaccine strains. Phylogenetic analysis was performed using the entire coding region of the H gene and a 388 bp fragment of the P gene of several morbillivirus species. Evidence was obtained that recent CDV isolates from different species in the United States (including isolates from large felids), Europe and Africa are significantly distinct from CDV vaccine strains. All wild-type CDV isolates analysed clustered according to geographical distribution rather than to host species origin. By sequence analysis a CDV epizootic among large felids in a Californian safari park was linked to a virus which most likely originated from feral non-felid carnivores.

Introduction

Within the genus *Morbillivirus* of the family *Paramyxoviridae*, seven virus species have been identified to date which infect humans (measles virus, MV), ruminants (rinderpest virus, RPV and peste-des-petits ruminants virus, PPRV), terrestrial carnivores (canine distemper virus, CDV), marine carnivores (phocine distemper virus, PDV) or cetaceans (porpoise and dolphin morbilliviruses, PMV and DMV) (Pringle, 1992; Visser *et al.*, 1993). Although most of these viruses may infect more than one species, susceptible animals are usually restricted to a single order (Appel & Gillespie, 1972). CDV has been shown to infect terrestrial and aquatic carnivores under natural circumstances (Mamaev *et al.*, 1995, 1996). Sporadic suspect cases of natural CDV infection in large felids were first reported in 1981 (Cook & Wilcox, 1981). Apparently unrelated enzootic outbreaks of CDV infections amongst Old World large felids (tigers, lions and leopards) have been noted in different zoos in the United States in recent years (Appel *et al.*, 1994). Virus isolates from these outbreaks were identified as CDV on the basis of cross-reactivity in virus neutralization assay and typing with a limited set of mouse monoclonal antibodies (MAbs) (Appel *et al.*, 1994). Recently, an epizootic associated with considerable mortality among lions in the Tanzanian Serengeti National Park has also been attributed to CDV infection (Morell, 1994; Harder *et al.*, 1995).

In the large felid populations of North American zoos, infection probably spread from cat-to-cat, although

* Author for correspondence. Fax +31 10 436 5145.
e-mail osterhaus@viro.fgg.eur.nl

The sequences of the H gene of CDV A92-27/4 and CDV A92-6 have been assigned GenBank accession numbers Z54156 and Z54166, respectively.

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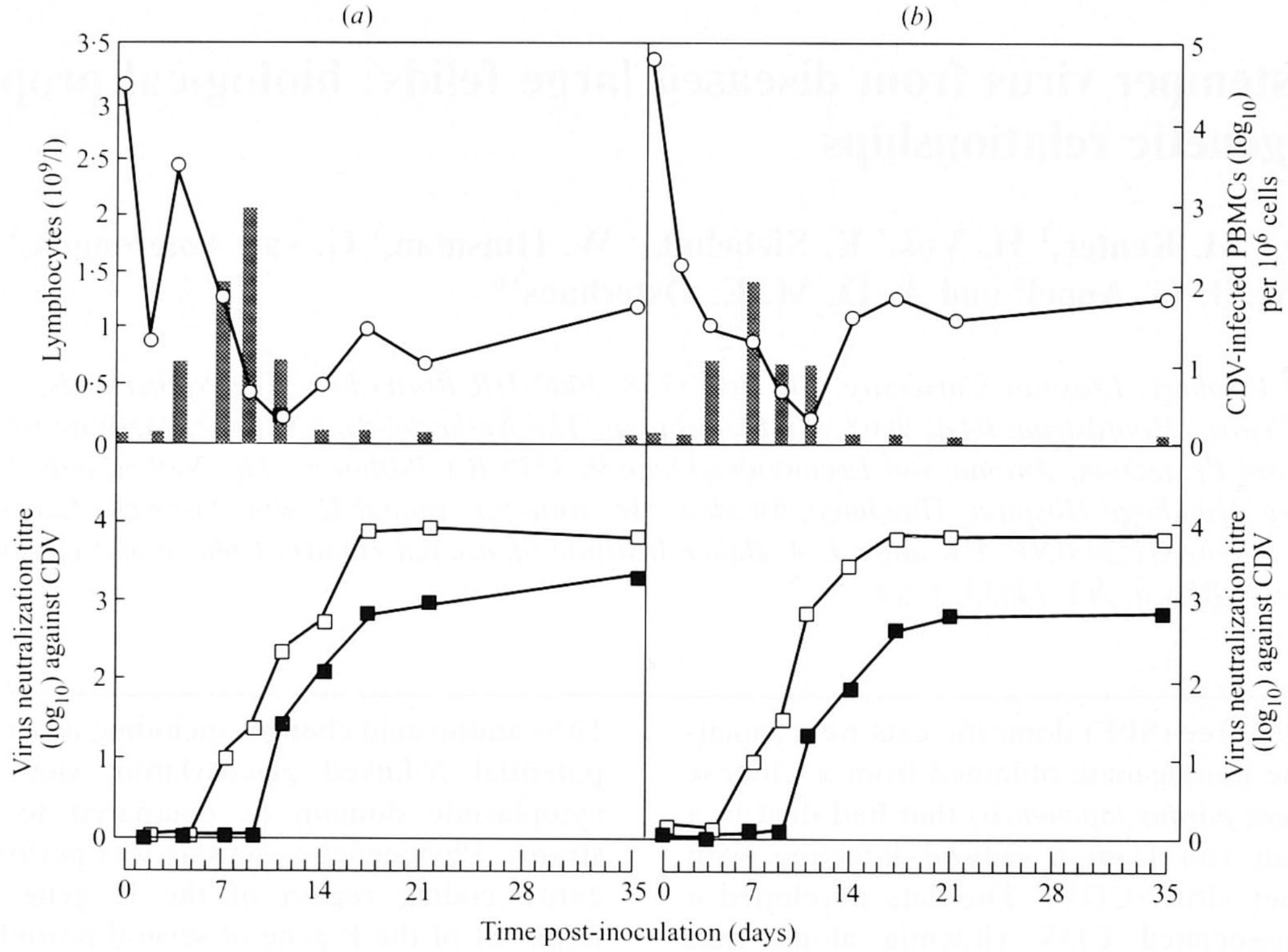

Fig. 1. Experimental inoculation of two domestic SPF cats (*a*, *b*) with tissue homogenate from a Chinese leopard that had died from a CDV infection. Upper panel: peripheral blood lymphocyte counts (○) and numbers of CDV infected PBMC per 10^6 PBMC (▨). Lower panel: kinetics of neutralizing plasma antibody titres ($\log_{10}$ ND_{50}) against the Chinese leopard CDV isolate A92-27/4 (□) and the CDV vaccine strain Bussell (■).

recurrent introduction from a common source of infection (e.g. free-ranging *Procyonidae* species like racoons) was not excluded. It remained elusive whether each outbreak was due to separate introductions of CDV or whether a cat-adapted strain of CDV circulated in these populations (Appel *et al.*, 1994).

To date, there are no indications of naturally occurring morbillivirus infections in domestic cats. Experimental infection of cats with the highly virulent Snyder–Hill strain of CDV resulted in an asymptomatic infection and virus shedding could not be demonstrated (Appel *et al.*, 1974).

Here we describe the results of experimental infection of specific pathogen free (SPF) domestic cats with tissue homogenate from a Chinese leopard that had died recently from a CDV infection in a zoo in the United States (Appel *et al.*, 1994) and the phylogenetic comparison of CDV isolates from felid and non-felid origins.

Methods

Viruses and cells. Canine peripheral blood mononuclear cells (PBMC) obtained from a healthy SPF dog were prepared from heparinized blood samples as previously described (Visser *et al.*, 1993), stimulated overnight in the presence of 20 μg/ml of PHA-M and maintained in Iscove's medium supplemented with 20% fetal calf serum (FCS) and 100 IU/ml of recombinant human interleukin 2 (rh IL-2). Feline PBMC and thymocytes or lion (*Panthera leo*) PBMC were stimulated for 3 days using Concanavalin A (Con A, 5 μg/ml) and expanded in RPMI 1640 containing 10% FCS and 100 IU/ml rh IL-2 (Siebelink *et al.*, 1990). Vero cells as well as CRFK cells (clone ID 10) were kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4% or 10% FCS, respectively.

The CDV strain 'Bussell' a Vero cell-adapted clone of the Onderstepoort vaccine strain (Bussell & Karzon, 1965) was used as a reference virus. The CDV isolate A 92-27/4 was obtained from thymus material of a fatally diseased Chinese leopard (*Panthera pardus japonensis*) from the Shambala Wildlife Waystation, Angeles National Forest, California, USA, using stimulated canine PBMC. CDV A92-27/4 was passaged four times in stimulated canine PBMC and adapted to replication in Vero cells by cocultivation with infected canine PBMC. Stock virus was prepared from material from the third passage in Vero cells. CDV A92-6 originated from a black leopard (*Panthera pardus*) that died of the infection in a zoo located in Illinois (USA). CDV isolate A92-27/14b was obtained from a racoon (*Procyon lotor*) which had been found in close vicinity to the Shambala location. These isolates were passaged three to five times in Vero cells following primary isolation in dog lymphocytes.

Infection and sampling of cats. Two SPF domestic European short hair cats were inoculated intratracheally with 3 ml of 10% (w/v) tissue homogenates (lung, thymus, brain) from a Chinese leopard that had died from a morbillivirus infection and from which CDV A92-27/4 had been isolated (Appel *et al.*, 1994).

Blood samples and tonsillar swabs were collected at days 0, 2, 4, 7, 9, 11, 14, 17, 21 and 36 post-inoculation, and rectal temperature was recorded. PBMC were recovered for virus isolation from heparinized

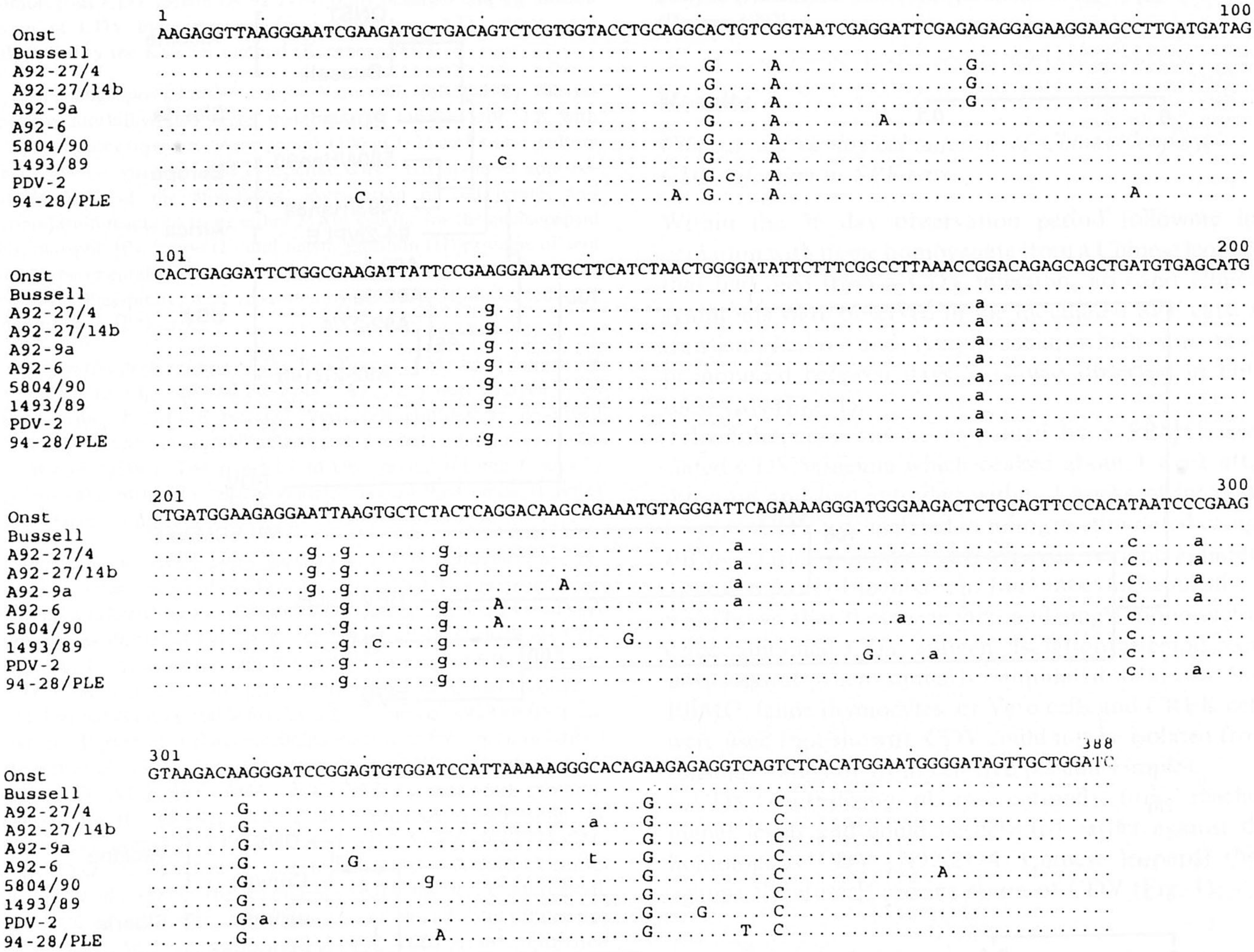

Fig. 2. Alignment of the nucleotide sequences of a P gene fragment (mRNA sense) of CDV strains and isolates. Sequences of primers used for PCR amplification are excluded. Line 1 shows the nucleotide sequence of the Onderstepoort vaccine strain of CDV (GenBank accession no. X51869). Non-synonymous mutations to this sequence are shown in upper case. Bussell, CDV vaccine strain; A92-27/4, Chinese leopard isolate (California, USA, 1992); A92-24/14b, racoon isolate (California, USA, 1992). Other sequences were taken from Harder *et al.* (1995) [A92-9a, grey fox isolate (California, USA, 1992); A92-6, black leopard isolate (Illinois, USA, 1992); 94-28/PLE, Lion (Serengeti, Tanzania, 1994] and from Mamaev *et al.* (1996) [1493/Han89, ferret isolate (Germany, 1989); 5804/Han90, dog isolate (Germany, 1990); PDV-2, isolate from a Baikal seal (*Phoca sibirica*), Siberia]. Sequences were aligned with 'Pileup' included in the GCG software package.

blood samples by Lymphoprep density centrifugation as previously described (Siebelink *et al.*, 1990). Plasma was used for serological analysis. Routine haematological parameters were measured with EDTA blood samples.

Animals were euthanized on day 36. Direct tissue cultures were initiated from kidneys and lymphatic organs (spleen and mesenterial lymphnodes) of all cats using standard procedures. In addition, IL-2 dependent T cell lines were raised from PBMC of the day 0 blood samples from the cats by serial repeats of Con A stimulation and expansion in IL-2 containing medium as previously described (Siebelink *et al.*, 1990).

Virus isolation and identification. PBMC obtained from the inoculated cats were stimulated for 3 h in the presence of 5 μg Con A and then diluted in duplicate in five $\log_{10}$ steps from 100000 to 1 cell/ml. Stimulated canine PBMC (1×10^5) were added and cocultivated for 7 days. Plasma samples, pharyngeal swabs and tissue homogenates of various organs were directly inoculated into canine PBMC cultures containing approx. 1×10^5 cells/ml. Following an adsorption period of 2 h, the cells were washed twice in Iscove's medium and cultivated for 7 days. After one freeze/thaw cycle a second passage of 7 days was initiated for all isolation attempts.

Morbillivirus antigen was detected with the MAb F 3.5 directed against a highly conserved epitope located on the fusion (F) glycoprotein employing an immune peroxidase monolayer assay (IPMA) described elsewhere (Harder *et al.*, 1993).

Virus isolation from tissue homogenates used for the inoculation of SPF cats was also attempted using mitogen-stimulated PBMC of a dog, a cat and a lion, feline thymocytes, and Vero and CRFK cells.

Serological assays. Plasma samples were assayed for morbillivirus neutralizing antibodies against 50–100 $TCID_{50}$ of the Vero cell-adapted

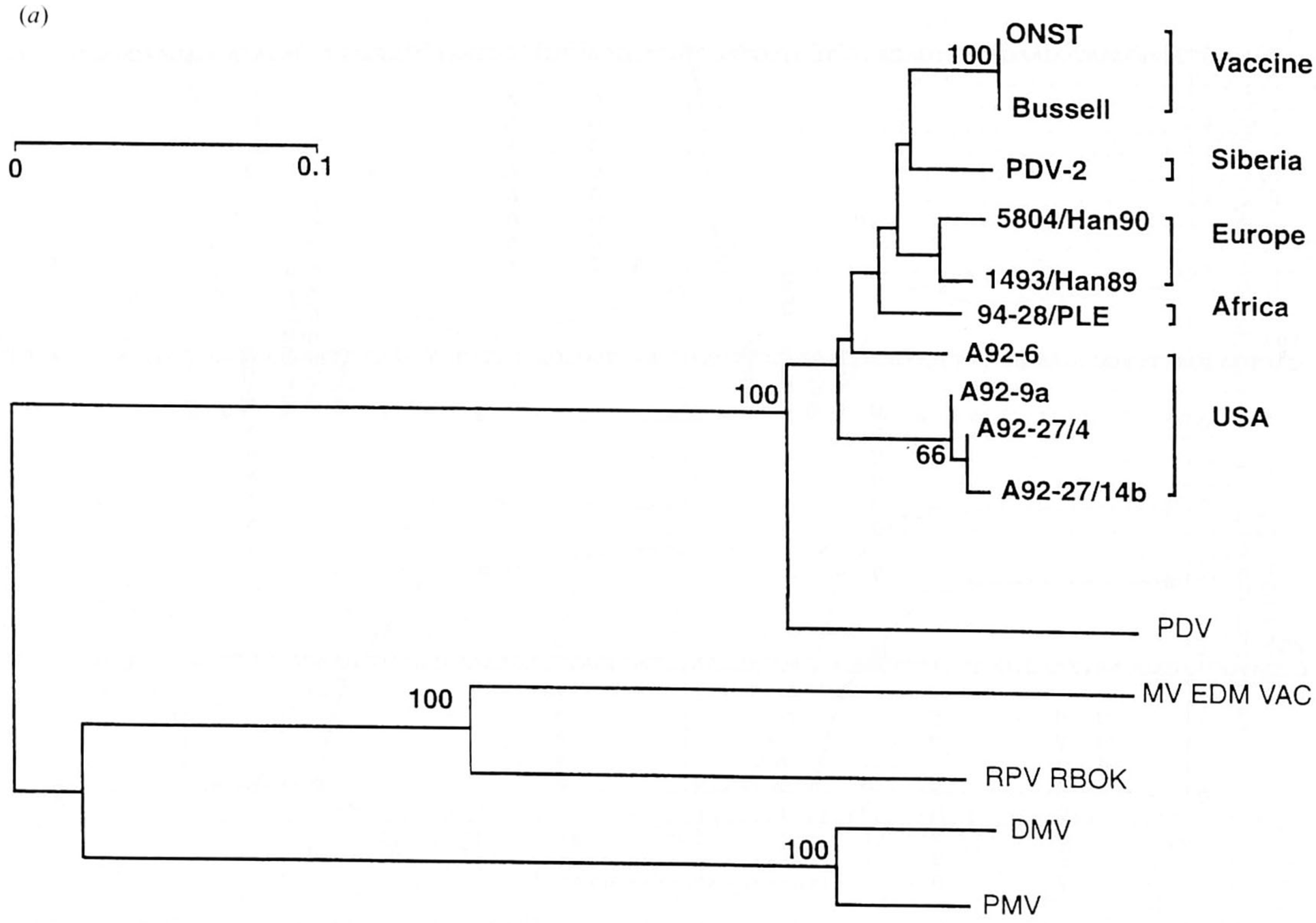

Fig. 3. Phylogenetic analysis of CDV-like virus isolates and other morbillivirus species computed on the basis of sequence analysis of a 388 bp P gene fragment (*a*) and the entire protein coding region of the H gene (*b*). Sequences of CDV Bussell, A92-27/4 (Chinese leopard, California, 1992), A92-6 (black leopard, Illinois, 1992; H gene) and A92-27/14b (raccoon, California, 1992) were generated in this study. Other sequences were extracted from the GenBank database [CDV strain Onderstepoort (P, X51869; H, D00758), CDV strain Convac (H, Z35493), PDV (P, H, D10371), MV vaccine strain Edmonston (P, M89920; H, U03669), RPV strain RBOK (P, X68311; H, M21513), RPV strain L (H, M17434)] or from recent publications [Barrett *et al.*, 1993: dolphin (DMV) and porpoise morbilliviruses (PMV); Mamaev *et al.*, 1995, 1996: 1493/89 (ferret, Germany, 1989), 5804/90 (dog, Germany, 1990), phocid distemper type-2 (PDV-2, Lake Baikal seal); Harder *et al.*, 1995: CDV A92-6 (black leopard, P) and A92-9a (grey fox, California, 1992)]. Multiple sequence alignments were computed by 'Pileup' included in the GCG software package (version 8.0) setting gap creation weights to 3·0 and gap extension weights to 0·1. Distances were calculated by the Kimura 2-parameter method (Kimura, 1980) and used to construct trees according to the neighbour-joining method (Saitou & Nei, 1987). Alignment gaps were ignored in pairwise distance

homologous CDV isolate (A 92-27/4, third passage) and the Bussell strain of CDV by a microneutralization assay. ND_{50} titres were calculated by the Kaerber method (Kaerber, 1931).

Radioimmunoprecipitation assays. Vero cells infected by the respective morbilliviruses were metabolically labelled for 2 h with [^{35}S]methionine/cysteine (Amersham) (100 μCi/25 cm^2 tissue culture flask) when a virus-induced cytopathic effect had involved approximately 50% of the monolayer. Preparation of cell lysates and precipitation reactions using either MAbs specific for the nucleocapsid (N), phospho (P), fusion (F) and haemagglutinin (H) proteins or sera from experimentally infected cats were exactly as described (Barrett *et al.*, 1989). Precipitates were separated by SDS–PAGE under reducing conditions in 10% gels.

Antigenicity profiles using MAbs. Reactivities of MAbs to antigens of the CDV strain Bussell and the isolate A92-27/4 were assayed in an IPMA using heat-fixed infected Vero cell cultures as described previously (Harder *et al.*, 1993). MAbs were tested in dilutions of 1/50, 1/500 and 1/5000. The panel of MAbs specific for the F and H glycoproteins had previously been raised against the Convac strain of CDV (Örvell *et al.*, 1985).

Amplification, cloning and sequencing of a P gene fragment and the complete coding region of the H gene. Total RNA was prepared from Vero cells infected by the different CDV strains and used for cDNA synthesis as described (Visser *et al.*, 1993). All PCR reactions were limited to 25 cycles comprising 30 s at 94 °C, 1 min at 55 °C and 1 min at 72 °C. A final elongation phase of 10 min at 72 °C was allowed.

Universal primers suitable for the amplification of a 429 bp fragment from the P gene of various morbillivirus species have been described (Barrett *et al.*, 1993). The upstream primer (5′ TTA ATT *GAG CTC* ATG TTT ATG ATC ACA GCG GTG 3′) represents positions 400–419 on the measles virus P gene (mRNA sense) while the downstream primer (5′ TTA ATT *ACT AGT* ATT GGG TTG CAC CAC TTG TC 3′) refers to nucleotides P 828–808 (vRNA sense) (Bellini *et al.*, 1985). The amplificate spans the RNA editing site (Cattaneo *et al.*, 1989). Amplificates were cloned into pBluescript SK(+) using *Sac*I (upper primer) and *Spe*I (lower primer) restriction sites (shown as italics in primer sequences).

The H gene was amplified using primers RH-3 (5′ AGG GCT CAG GTA GTC CAG C 3′) representing nucleotides 1–19 of the mRNA and RH-4 (5′ AAT GCT AGA GAT GGT TTA ATT 3′) referring to nucleotides 1937–1917 of the H gene (vRNA sense), thus extending into the 3′ UTR of the H mRNA (sequences taken from the CDV vaccine strain Onderstepoort, GenBank accession no. D00758). Amplificates of approximately 1·9 kb comprising the complete coding region were cloned into a 'sticky' T vector (pCR II, Invitrogen).

Dideoxy sequencing of double stranded plasmid DNA was carried out using M13 universal forward and reverse primers (Murphy & Kavanagh, 1988) as well as sequence-specific 17-mers (H gene). Two clones obtained from a single PCR reaction were sequenced to completion. To correct for possible reading errors of the *Taq* polymerase a third clone was analysed if necessary. Sequences were assembled and analysed using the GCG software package (release 8.0). Computer-assisted phylogenetic analysis (MEGA, version 1.01; Kumar *et al.*, 1994) of the nucleotide sequences was done by the neighbour-joining method (Saitou & Nei, 1987); distances were calculated according to Kimura's 2-parameter method (Kimura, 1980). Bootstrap analysis (Felsenstein, 1985) was performed using 2000 replications (Hedges, 1992).

Results

Clinical and virological outcome of Chinese leopard CDV infection in SPF cats

Within the 36 day observation period following inoculation with tissue homogenate from a Chinese leopard that had died from a CDV infection, no overt clinical symptoms were observed in the inoculated SPF cats. A transient leuko- and lymphopenia, which was most pronounced between days 7–11 was observed in both these cats (Fig. 1).

Lymphopenia was accompanied by a PBMC associated CDV viraemia which peaked about 1 week after infection and had subsided within 2 weeks of infection (Fig. 1). CDV was isolated in mitogen stimulated canine PBMC: large multinucleated syncytia became apparent after 4–6 days of incubation. Immunocytochemically, it was shown that these syncytia, and single mononuclear cells, contained CDV antigen. Isolation attempts were unsuccessful when similarly stimulated feline or lion PBMC, feline thymocytes, or Vero cells and CRFK cells were used (not shown). CDV could not be isolated from tonsillar swabs or from cell-free plasma samples.

CDV neutralizing plasma antibody titres reached higher levels and could be detected earlier against the homologous CDV (A92-27/4, Chinese leopard) than against the Bussell vaccine strain of CDV (Fig. 1).

Patterns of MAb recognition and migration in PAGE of Chinese leopard CDV (A92-27/4) proteins

Using a panel of MAbs specific for seven or three operationally defined non-overlapping epitopes of the CDV H and F proteins respectively, no significant differences in reactivity to A92-27/4 or the CDV Bussell strain were seen in IPMA, except for MAb 3.775 specific for the epitope H-4 which did not react with A92-27/4 (data not shown).

Similarly, migration patterns of CDV A92-27/4 proteins immunoprecipitated by MAbs or convalescent cat sera from infected cell lysates were in the range found for other CDV strains (data not shown). The P protein of CDV A92-27/4, however, migrated at about 85 kDa which is considerably higher than the molecular mass of P proteins of previously examined CDV strains which

estimations. Branches are drawn in proportion to genetic distances as indicated by the bar. Numbers at tree branchings represent bootstrap *P* values after 2000 replications (Felsenstein, 1985; Hedges, 1992). Values ⩾ 95% indicate a statistically significant separation of the respective cluster from the rest of the tree. All calculations were carried out using the 'MEGA' PC program (Kumar *et al.*, 1994).

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          1                                                                                                  100
ONST      MLPYQDKVGAFYKDNARANSTKLSLVTEGHGGRRPPYLLFVLLILLVGILALLAITGVRFHQVSTSNMEFSRLLKEDMEKSEAVHHQVIDVLTPLFKIIG
Convac    --S-----A----------:---:-------E--------------V-----------------------------------------------------
1493      --S----------------:--S:-------EQ-------------------M-----------------------------DR-------------------
5804      --S----------------:--S:-------EQ-------------------MT----------------------------------------------------
A92-27/4  --S----------------:--SR------DQ-------------------M-----------------------------------------------------
A92-6     --S----------------:--S:-------EQ-------------------M-----------------------------------------------------
                             :....:

          101                                                                                                200
ONST      DEIGLRLPQKLNEIKQFILQKTNFFNPNREFDFRDLHWCINPPSTVKVNFTNYCESIGIRKAIASAANPILLSALSGGRGDIFPPHRCSGATTSVGKVFP
Convac    ----S-----------------------------------------K---|---|-----------------------P---S----------------
1493      ----------------------------------------------KI--|---|--DT-----S--------------E--------Y----------R---
5804      --V-------------------------------------------KI--|---|--DT---K-S----------------------Y----------R---
A92-27/4  ----------------------------------------------KI--|---|--DT-----S--------------RS-------Y----------S---
A92-6     --V-------------------------------------------KI--|---|--DT-----S------------HT---------Y----------R---

          201                                                                                                300
ONST      LSVSLSMSLISRTSEVINMLTAISDGVYGKTYLLVPDDIEREFDTREIRVFEIGFIKRWLNDMPLLQTTNYMVLPKNSKAKVCTIAVGELTLASLCVEES
Convac    ---------------I------------------A----------Q------------------S----------E----------------------
1493      ---------------I----------------------Y--G----QK-------------------------E----------------------D--
5804      -----------K---ITS-------------------Y--G----QK--------------N----------E----------------------D--
A92-27/4  ---------------I----------------------YL-G----QK--------------N----------E----------------------D--
A92-6     ---------------I----------------------Y--G----QK-------------------------E----------------------D--

          301                                                                                                400
ONST      TVLLYHDSSGSQDGILVVTLGIFWATPMDHIEEVIPVAHPSMKKIHITNHRGFIKDSIATWMVPALASEKQEEQKGCLESACQRKTYPMCNQASWEPFGG
Convac    ----------------------GT----------------E-------------------------------------------P------|T|-------
1493      -------|N|-----------G-----QV---V----S-VE----------------------V--------N---------S------|T|-------
5804      -------|N|-----------G-----QV----------VE----------------------V--------N----------------|T|-------
A92-27/4  -------|N|--G--------G-----QV------P---VE----------------------V--------N---------S------|T|-------
A92-6     -I-----|N|-----------G-----QV----------VE----------------------V--------S---------S------|T|-------

          401                                                                                                500
ONST      RQLPSYGRLTLPLDASVDLQLNISFTYGPVILNGDGMDYYESPLLNSGWLTIPPKDGTISGLINKAGRGDQFTVLPHVLTFAPRESSGNCYLPIQTSQIR
Convac    ---------------------|---|-------------------------------N|--|L--------------I--------M--------------I
1493      G--------I----P-I-----|---|---------------------D--------RN|--|L-M----S--------T----------------------M
5804      G-------------P-I-----|---|---------------------SD-------RN|--|VL------S--------I----------------------M
A92-27/4  G-------------P-I-----|---|---------------------D--------N|--|VL------S--------I----------------------M
A92-6     G-------------P-I-----|---|----------N----------G--------N|--|VL---------------I----------------------M

          501                                                                                                600
ONST      DRDVLIESNIVVLPTQSIRYVIATYDISRSDHAIVYYVYDPIRTISYTHPFRLTTKGRPDFLRIECFVWDDNLWCHQFYRFEADIANSTTSVENLVRIRF
Convac    ---------L-------F-------------------------------------------------------------------N---|-|----------
1493      -K-------L------NF-----------D-----------------MY--------------------ND-------------T---|S|----------
5804      -K---T---L------NF-----------G-------------------Y----------------------D-------------T---|-|----------
A92-27/4  -K---T---L------NFI----------G-------------------A----------------------D------------ST---|-|----------
A92-6     -K---T---L------NF-----------G-----------------------------------------D----------T-ST---|-|----------

          601  607
ONST      SCNR...
Convac    -|--|SNP
1493      -|---|K-
5804      -|---|K-
A92-27/4  -|---|K-
A92-6     --S--K-
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Fig. 4. Alignment of deduced amino acid sequences of the H proteins of CDV vaccine strain Onderstepoort (D00758), CDV vaccine strain Convac (Z35493), recent CDV field isolates from a dog (5804; Z85000) and a ferret (1493; Z84999), and two CDV isolates obtained from captive large felids (A92-27/4, Chinese leopard; A92-6, black leopard). Selected sequences were aligned using 'Pileup' and secondary structure predictions were computed by 'PeptideStructure' and 'Motifs' included in the GCG software package (version 8.0). A stretch of predominantly hydrophobic character representing the putative membrane anchor is overlined. Potential Asn-glycosylation sites are boxed; the site at position 19 (dotted box) is probably not used as it is located in the intracellular domain.

migrate at about 72–75 kDa (Örvell, 1980; Rima *et al.*, 1991; Shapshak *et al.*, 1982). Also, differences between the molecular masses of the H proteins of the Bussell strain (72 kDa) and A92-27/4 (78/82 kDa) were noticed (not shown).

Sequence comparison and phylogenetic analysis of CDV isolates using a P gene fragment

A 388 bp P gene fragment had previously been shown to be informative for inferring the phylogenetic relationships of morbilliviruses (Barrett *et al.*, 1993). Alignments of the respective fragments from the large felid morbilliviruses A92-27/4 and A92-6, recent American and European CDV field isolates (Mamaev *et al.*, 1995) from non-felid species, a P gene sequence obtained from a PCR amplificate of central nervous system tissues of a Serengeti lion (Harder *et al.*, 1995) and two CDV vaccine strains (Onderstepoort and Bussell) are presented in Fig. 2. The field isolates harboured up to 16 mutations when compared to the sequences of the two vaccine strains, which proved to be identical.

Phylogenetic analysis of these sequences revealed a clustering within the group of canine distemper-like viruses (Fig. 3*a*). The vaccine strains are clearly separated from recent CDV isolates as indicated by the high bootstrap values. Wild-type CDV isolates can be subdivided into clusters reflecting their geographical origin (European, American or African). The two CDV isolates obtained from large cats in Illinois (A92-6) and California (A92-27/4) were quite distinct whereas a grey fox isolate (A92-9a) and a racoon isolate (A92-27/14b) from California were most closely related to the Chinese leopard CDV isolate A92-27/4. It is interesting to note that the racoon was found in close vicinity to the safari park where the Chinese leopard was kept.

Sequence comparison and phylogenetic analysis of the H gene of two CDV isolates from large felids

A 1·9 kb product representing almost the entire H mRNA was amplified by PCR from the CDV isolates A92-27/4 (Chinese leopard, California) and A92-6 (black leopard, Illinois). Sequence analysis revealed unique sequences of both isolates which differed from each other by 3·0% at the nucleotide level. A single large open reading frame (ORF) was identified, and its amino acid composition was deduced and aligned to those of CDV vaccine strains (Fig. 4); this showed scattered predicted amino acid changes of up to 10%. While the Onderstepoort H gene harbours an ORF capable of encoding 604 amino acids (Curran *et al.*, 1991), those of the large felid isolates and the Convac vaccine strain (Kövamees *et al.*, 1991) code for 607 amino acids. Secondary structure analysis revealed that the H protein of the large cat CDV isolates, like all other morbillivirus attachment proteins (Morrison, 1988), represents a class II transmembrane glycoprotein with a putative membrane anchor sequence near its N terminus (positions aa 35–55, overlined in Fig. 4). All 12 cysteine residues were completely conserved between the CDV isolates whereas 94% of the proline residues of the Onderstepoort H protein were retained in the large cat isolates. The glycosylation pattern, however, differed considerably (Fig. 4). While four potential *N*-linked glycosylation sites were shared between all CDV isolates, three (Convac and A92-6) or four (A92-27/4) additional sites, all located in the extracytoplasmic domain, proved to be present in the other sequences.

Phylogenetic analysis of the entire protein-encoding region of the H gene (Fig. 3*b*) confirmed the clustering of the large cat isolates as CDV-like. Bootstrap *P* values indicate that they are significantly separated from CDV vaccine strains and also from current European and Asian CDV wild-type isolates.

Discussion

In the present study we have shown that SPF cats can be infected by CDV from a Chinese leopard without developing overt clinical symptoms and that the CDV isolates which have recently caused disease outbreaks in large felids do not constitute a separate lineage within CDV. Tissue homogenate instead of a CDV isolate was used for inoculation as it has been shown that adaptation of morbilliviruses to replication in cell culture systems may affect their virulence (Friedländer *et al.*, 1985; Appel *et al.*, 1992).

The apparent lack of clinical symptoms in the SPF cats should be interpreted with caution, since we have also shown, for example, that infection with the usually highly virulent Snyder–Hill strain of CDV results only in mild symptoms in SPF dogs (De Vries *et al.*, 1988). Although domestic cats, in contrast to large felids, may have a high intrinsic resistance towards CDV infections, it cannot be excluded that conventionally raised domestic cats may develop clinical symptoms upon CDV infection. So far, however, clinical and epidemiological indications for this assumption are lacking. In contrast, CDV-neutralizing antibodies have been detected in sera of healthy domestic cats from the Netherlands (A. D. M. E. Osterhaus & H. Vos, unpublished).

For the isolation and amplification of large cat CDV isolates mitogen stimulated canine PBMC proved to be the most suitable cells. Replication of CDV A92-27/4 in Vero cells, in contrast, required an adaptation of three passages of 14 days each. In accordance with previously published biological data (Appel *et al.*, 1994) these results confirm that CDV A92-27/4 represents a non-

attenuated strain of CDV. Replication of CDV A92-27/4 in feline lymphoid cells, including IL-2-dependent T cell lines established from each of the SPF cats, could scarcely be demonstrated (data not shown) and during further passaging infectivity rates also declined gradually. This is also in line with results of an earlier study (Appel *et al.*, 1974), in which serial passage of the Snyder–Hill strain of CDV could not be accomplished in feline lung macrophages, while the virus replicated fulminantly in canine lung macrophages. This is yet another indication that the morbillivirus isolates from large felids are not members of a separate *feline* lineage of CDV.

Comparison of the deduced amino acid sequences of the H protein of the CDV isolates A92-27/4 and A92-6 to other CDV-like viruses revealed up to 10% scattered differences, which is within the range reported for other morbillivirus species like MV and RPV. The number of potential *N*-linked glycosylation sites in the H protein of the large cat isolates resembles the situation in recent wild-type CDV isolates (5804, 1493) of non-felid origin (Mamaev *et al.*, 1995), but is quite distinct from that in the vaccine strains. In the measles virus H protein, *N*-linked carbohydrates were found to be involved in the formation of conformational epitopes which appeared to be critical in eliciting neutralizing antibodies (Hu *et al.*, 1994). The differences found in potential *N*-linked glycosylation sites between wild-type and vaccine strains of CDV may explain the differences in neutralization efficacy of sera obtained from the experimentally inoculated domestic cats towards the homologous isolate (A92-27/4) and the Bussell vaccine strain. Similar differences in cross-neutralization efficacies were also described for recent CDV field isolates from a dog (5804) and a ferret (1493) using sera raised against the Rockborn vaccine strain of CDV in a kinetic neutralization assay (Harder *et al.*, 1993). An explicit clustering of predicted amino acid changes around potential Asn-glycosylation sites, as has been described for recent MV field isolates (Rota *et al.*, 1992), however, was found neither in the large felid CDV isolates nor in wild-type CDV isolates from other species (Fig. 4).

Phylogenetic analysis of the H gene and P gene fragment sequences revealed that current wild-type CDV isolates and vaccine strains are clearly distinguishable. Within the group of recent field isolates a tendency to cluster according to geographical rather than to host species origin was noticed. Geographical lineages have also been described for other morbillivirus species (Chamberlain *et al.*, 1993; Rota *et al.*, 1992; Taylor *et al.*, 1991). On the basis of our data we can exclude the possibility that virus isolates from large cats in the United States have recently evolved from CDV vaccine strains. Rather, they seem to be representatives of CDV strains presently circulating in North America, which is also emphasized by the close phylogenetic relationship between the Chinese leopard CDV and a racoon isolate from the same area.

Taken together, the biological and sequence data we have obtained so far do not indicate the existence of a CDV lineage adapted to feline species; rather, they suggest that the outbreaks of distemper-like disease among captive and feral large felids are most likely initiated by interspecies transmission of CDV from local feral (non-felid) carnivores.

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